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Applicative and ecological aspects of mycorrhizal symbioses

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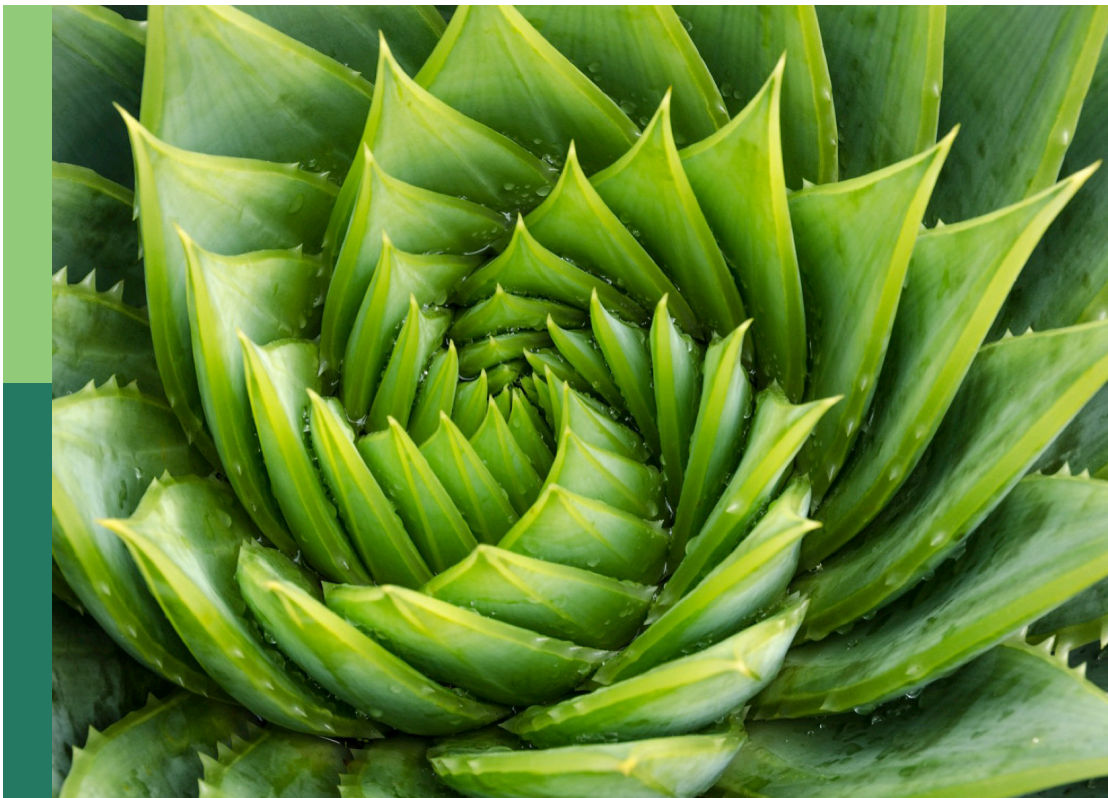

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Applicative and ecological aspects of mycorrhizal symbioses

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Editorial: Applicative and ecological aspects of mycorrhizal symbioses

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Editorial on the Research Topic
Applicative and ecological aspects of mycorrhizal symbioses

Fungal symbioses, particularly mycorrhizal relationships between fungi and plant roots, are critical for ecological dynamics and have significant implications for agriculture and environmental management (Martin and van der Heijden, 2024). Understanding these interactions is essential for recognizing ttheir roles in ecosystems and potential benefits across various biomes (van der Heijden et al., 2008; Mikryukov et al., 2023; Tedersoo et al., 2014; Bahram et al., 2018). Mycorrhizal fungi enhance plants' ability to absorb essential nutrients, particularly phosphorus, nitrogen, and micronutrients (Van Der Heijden, 2004). By forming extensive hyphal networks, these fungi increase the surface area for nutrient absorption, improving plant health and growth (Jastrow et al., 1998).

Mycorrhizal fungi also play a vital role in carbon cycling, storing carbon in the soil, influencing global carbon dynamics and helps mitigate climate change (Hawkins et al., 2023). Additionally, they contribute to soil structure by forming aggregates and enhancing porosity, improving water retention and aeration (Jastrow et al., 1998). This ultimately promotes overall soil health and stability. Furthermore, mycorrhizal networks can influence plant competition and coexistence by transferring nutrients between plants and fostering cooperation by forming common mycorrhizal networks (CMNs) where mycobiont establishes an association with the roots of two or more plant species (Henriksson et al., 2023). These symbiotic relationships support plant diversity, enabling various species to thrive in nutrient-poor soils. Many types of mycorrhizal fungi, such as arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (ECM) fungi, orchid mycorrhizal (ORM) fungi, and ericoid mycorrhiza, work with different plant species. These fungi make ecosystems more resilient and help plants survive in harsh conditions like drought, high salt levels, and heavy metal pollution. AM fungi form symbiotic relationships with the roots of most land plants, including many important crops and plants (Brundrett and Tedersoo, 2018). These fungi enhance nutrient uptake, especially phosphorus, and improve plant growth, yield, and stress tolerance, thereby contributing to sustainable agriculture. In this context, nine studies in this Research Topic highlight various aspects of AM fungi in

agricultural systems, providing valuable insights into their diversity, identification methods, and potential applications. Figure 1 summarizes the different benefits AM fungi provide plants, soil, and ecosystems.

This editorial emphasizes the urgency of understanding mycorrhizal symbioses for ecosystem function and agricultural sustainability. The studies showcase the significant roles of mycorrhizal fungi and beneficial microorganisms in enhancing plant health, growth, and resilience while promoting sustainable agricultural approaches. For example, Liang et al. studied the mycorrhizal fungi communities that are connected to *Bulbophyllum tianguii*. They found that the diversity of the fungi was very different between the roots, rhizomes, and rhizosphere soil, with *Sebacina* and *Exophiala* being the most common species. This knowledge aids in the conservation strategies for orchids.

Wang et al. examined how AM fungi influence the growth and herbivore resistance of *Artemisia ordosica* under varying water and nutrient levels. Contrary to expectations, they observed reduced plant height and biomass under low water and nutrient conditions. They reported that while AM fungi generally support plant growth, their effects can be context-dependent, sometimes inhibiting growth in low-resource conditions. Chen et al. studied how adding AMF fungi to *Catalpa bungei* seedlings affected their growth and nitrogen metabolism at different nitrogen levels. They found that adding AM fungi increased the plants' ability to take in nitrogen and phosphorus and improved photosynthesis at low to medium nitrogen levels. At medium nitrogen levels, AM fungi significantly promoted root growth by altering root hormone levels and improving root architecture and activity. Their findings highlight the potential of AM fungi in tree cultivation strategies. Pokluda et al. investigated the impact of AM fungi and plant growth-promoting microorganisms on onion seedlings. The results showed that the microbial groups worked together to help the plants grow and deal with stress better, especially in organic-rich substrates. This shows how the groups of microbes work together to make things better. Xia et al., investigated the impact of AM fungi inoculation on alfalfa growth and photosynthetic performance under different phosphorus application levels. Using a controlled pot experiment, they demonstrated that inoculation with AM fungi, particularly a mixed inoculation of *Funneliformis mosseae* and *Glomus etunicatum*, significantly improved alfalfa's photosynthetic efficiency, chlorophyll content and dry matter yield, particularly at optimal phosphorus fertilization. Yu et al., conducted a proteomic analysis of *Poncirus trifoliata* (a common citrus

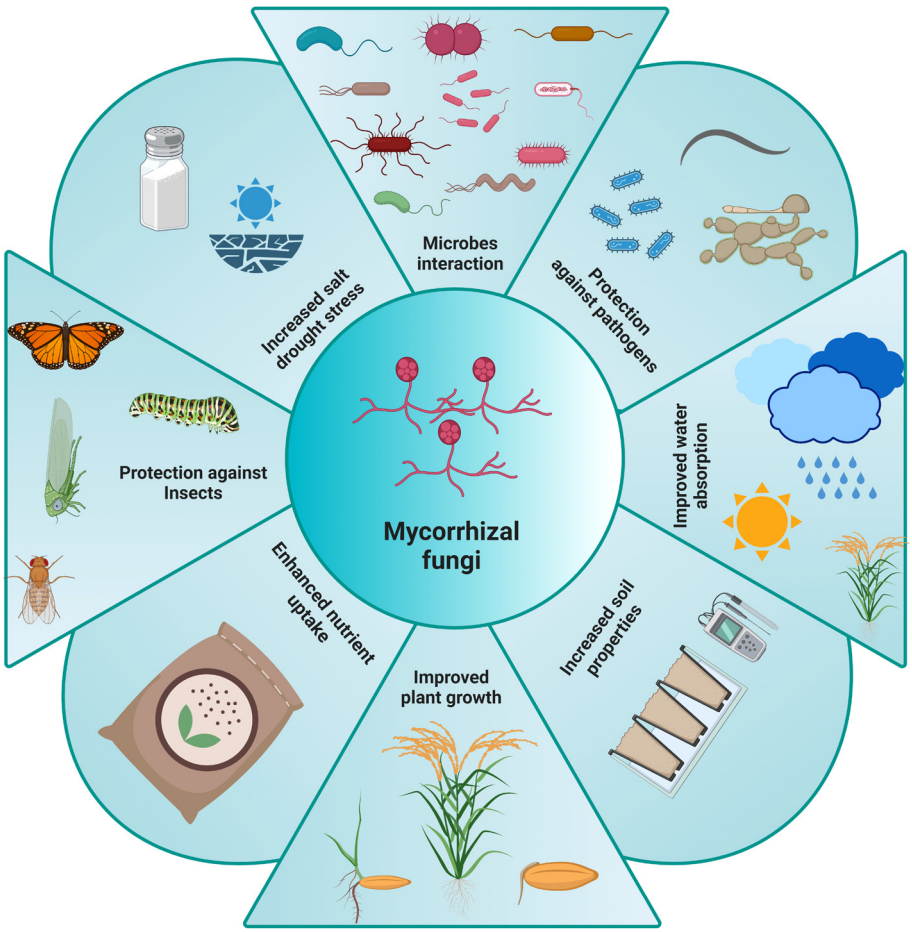

FIGURE 1
The presence of mycorrhizal fungi provides many benefits to the ecosystem, soil and plants. Understanding their role can improve future farming practices.

rootstock) roots that were colonized by *Rhizophagus irregularis* and identified key genes and proteins involved in AM colonization. This study contributes to a deeper understanding of the molecular mechanisms underlying AM symbiosis in woody plants like citrus.

Duan et al., discovered that intercropping sugarcane with *Dictyophora indusiata* and *Bacillus aryabhattai* promotes growth through a unique "white root" phenotype and enhanced flavonoid metabolism, providing new strategies for improving sugarcane yield. Maússe-Sitoe and Dames, focused on characterizing AM fungal species associated with maize using single-spore propagation techniques. Through a combination of morphological and molecular methods, they identified several AM fungal genera, including *Claroideoglomus*, *Funneliformis*, *Gigaspora*, *Paraglomus*, and *Rhizophagus*. Their work highlights the diversity of AM fungi in agricultural soils and provides insights into effective methods for isolating and identifying these important symbionts. They proposed that their isolates might be categorized into effective agents that stimulate maize growth and mycorrhization regardless of where they were found. Their findings can greatly contribute to crop productivity and sustainable management of the agricultural ecosystem. Finally, Hassan et al. showed that inoculating *Eruca sativa* with plant growth-promoting bacteria *Jeotgalicoccus* sp. significantly boosts its nutritional quality and biological value by enhancing amino acid and phenolic metabolism, thereby boosting the health benefits of this leafy vegetable.

Harnessing mycorrhizal associations can enhance soil carbon sequestration, contributing to climate change mitigation efforts and improving agricultural sustainability (Averill et al., 2022; Baldrian et al., 2023). Mycorrhizal inoculants can boost crop yields and reduce chemical fertilizer dependence, particularly in organic farming, where soil health and sustainability are prioritized (Martin and van der Heijden, 2024). Moreover, mycorrhizal fungi are valuable in mining and industrial land reclamation, supporting vegetation establishment and promoting ecological recovery. Mycorrhizal fungi are employed in restoration projects to rehabilitate degraded ecosystems. By promoting plant establishment and growth, they aid in restoring biodiversity and ecosystem functionality (Delgado-Baquerizo et al., 2016). They also play a crucial role in restoring degraded ecosystems and enhancing forest resilience.

Conclusion

Mycorrhizal symbioses provide a complex interplay of ecological benefits and practical applications. Their roles in nutrient cycling, soil health, and plant resilience underscore their importance in natural ecosystems. Furthermore, the potential for enhancing agricultural productivity and supporting ecological restoration positions mycorrhizal fungi as key players in sustainable land management practices. Understanding and harnessing these relationships will be crucial in addressing global challenges in agriculture, conservation, and climate change adaptation.

Author contributions

SM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. MM: Conceptualization, Formal analysis, Investigation, Project administration, Writing – review & editing.

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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

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Structure and diversity of mycorrhizal fungi communities of different part of *Bulbophyllum tianguii* in three terrestrial environments

Jiayu Liang[1,2†], Rong Zou[3†], Yang Huang[4†], Huizhen Qin[3], Jianmin Tang[3], Xiao Wei[3*], Yu Liang[1,2*] and Shengfeng Chai[3*]

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Mycorrhizal fungi plays important roles in the seed germination and subsequent growth of orchids. The research of fungi in orchid roots, especially dominant mycorrhizal fungi is critical for orchids protection. In this study, the fungal community and composition of mycorrhizal fungi in roots, rhizomes and rhizosphere soil of *Bulbophyllum tianguii* grown in three terrestrial environments were analyzed by the second generation sequencing technology. The results of OTU clustering and α and β diversity analysis showed that there were significant differences in fungal communities in roots, rhizomes and rhizosphere soil of *B. tianguii*. The total number of OTUs in rhizomes was much less than that in roots and rhizosphere soil. The number of OTUs in rhizosphere soil and the diversity of mycorrhizal fungi were the highest. Meanwhile, the species and abundance of mycorrhizal fungi in roots and rhizomes of *B. tianguii* were different from those in rhizosphere soil. For different elevations, compared with *B. tianguii* that grow in middle of Tiankeng and top of Tiankeng, the OTUs number of *B. tianguii* in orchid garden is richest, and the diversity of mycorrhizal fungi in orchid garden was significantly higher than other locations. Among the three different habitats of *B. tianguii*, the number of OTUs in humus soil and stone habitats was notably higher than tree habitats, and the diversity of mycorrhizal fungi in humus soil was the highest. The analysis of mycorrhizal fungi in different habitats and altitudes of *B. tianguii* showed that Sebacina and Exophiala were the dominant mycorrhizal fungi in *B. tianguii*. The results of species annotation, phylogenetic

tree and co-occurrence network analysis showed the dominant mycorrhizal fungi of *B. tianguii* mainly included Sebacina, Cladosporium, Exophiala, Fusarium. This study reveals the symbiotic relationship between Sebacina, Exophiala, Cladosporium and the *B. Tianguii*. It will provide a theoretical basis for the protection and biological function study of *B. Tianguii*.

KEYWORDS

orchidaceae, mycorrhizal fungi, *bulbophyllum tianguii*, microbial community, orchids

Introduction

Orchidaceae, commonly known as Orchids, is one of the most abundant and diverse groups in the plant kingdom, widely distributed in various terrestrial ecosystems with a long evolutionary history (Li T. et al., 2021; Wang et al., 2021). Due to the important ornamental value, medicinal value and cultural value of orchids, the phenomenon of excessive excavation of orchids is hardly avoided, resulting in almost all wild orchids in different degrees of endangerment (Pandey et al., 2013). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) includes all wild orchids in appendices I and II, and more than 90% of the protected plants covered by CITES are orchids.

Bulbophyllum tianguii is a new species of orchidaceae which was found in Guangxi Yachang orchidaceae National Nature Reserve in 2007 (Lang and Luo, 2007). It is mainly distributed on the surface of karst stone mountains or on the stems of humus trees. This species was only found in four distribution sites in Yachang Reserve, and was subsequently found in Guangxi Mulun National Nature Reserve, Guizhou Wangmo Sutie Nature Reserve (Luo et al., 2020). The associated plants include trees, shrubs, vines, herbs, and orchids: *Eriacoronaria*, *Cymbidiumfloribundum*. As one of the star orchids in Yachang Reserve, the related researches about *B. tianguii* is little. Only one biological study on the pollination mechanism of *B. tianguii* in Laowuji Tiankeng, Yachang Reserve is reported. This study showed that the seed setting rate of *B. tianguii* was very low under the natural state, and the seed setting rate of *B. tianguii* could be significantly increased to 100% by pollinating by crushing pollen (Jiang et al., 2020). Therefore, the further study of mycorrhizal fungi in roots and rhizosphere soil can provide effective measures for the conservation of *B. tianguii*. Orchids growing at different altitudes will be affected by the gradual decrease of temperature, humidity, nitrogen (N), phosphorus (P) and potassium (K) contents in soil, and orchids will improve their adaptability to the environment, which will in turn affect the number and species of mycorrhizal fungi to a certain extent. At the same time, mycorrhizal fungi could promote the absorption of N, P, K, Ca, Mg and other elements (Chen et al., 2017; Ren et al., 2021).

The survival and growth of orchids are highly depend on the symbiotic fungi in their roots, among which mycorrhizal fungi is important in the seed germination and subsequent growth of orchids (Hou et al., 2010; Tian et al., 2022). Research on mycorrhizal fungi in orchid roots has significance in the protection of orchids. Mycorrhizal fungi, also known as symbiotic fungi, are a group of fungi that can form a specific symbiotic structure with the roots of higher plants or other root-like organs in contact with the substrate. They live on the surface, in the cortex, or around the epidermal cells of roots (root-like organs) and are beneficial to both plants and fungi (Wang et al., 2021). The study of mycorrhizal fungal community of *Spiranthes sinensis* found that there were significant differences in the composition of the mycorrhizal diversity of the 6 samples of *Spiranthes sinensis*, and there was no obvious correlation between the differences and geographical distance, suggesting that the composition of the mycorrhizal community was more affected by habitat factors (Li J. et al., 2021). Studies about *Cypripedium* species at different altitudes found that the community structure of mycorrhizal fungi was significantly different among three *Cypripedium* species, indicating that the preference of *Cypripedium* to mycorrhizal fungi significantly affected the community structure of mycorrhizal fungi in the same habitat (Xu et al., 2019).

The root system of plants is critical in the whole life activities of plants, mainly absorbing water and elements in the soil. Plant roots can provide sufficient energy and nutrition for various microorganisms in the rhizosphere by secreting rich organic substances into the soil, and regulating the types and numbers of these microorganisms (Wu et al., 2014). At the same time, the detection of mycorrhizal fungi in the soil can determine whether the site is suitable for the orchids growth (Li T. et al., 2021). Therefore, studying the distribution of mycorrhizal fungi in the roots of orchids in the surrounding soil is important in the population restoration of orchids. Some studies about mycorrhizal fungi in orchid roots and the surrounding soil are reported. The results show that most of the mycorrhizal fungi

found in roots are also widely distributed in soil (Waud et al., 2016). At the same time, other studies found that the mycorrhizal fungi communities in orchid roots and rhizosphere soils are significantly different (Liu et al., 2015; Han et al., 2016). The second generation sequencing technology was used to analyze fungal communities and mycorrhizal fungi composition in roots, rhizosphere soil and rhizosphere soil of nine orchids belonging to minimal populations in Liaoning Province, China. It was also found that the OTUs in roots of orchids were less than that in rhizosphere soil and the bulk soil. The species and abundance of mycorrhizal fungi in orchid roots were not significantly correlated with that in the rhizosphere soil and the bulk soil, indicating that the fungal communities in orchid roots and soil were independent to some extent (Jiang et al., 2019).

The relationship between the community structure and habitat of symbiotic mycorrhizal fungi in the same orchids is one of the important contents in the study of both orchids and mycorrhizal fungi. Some studies have proved that the mycorrhizal fungi communities of the same orchids have great variability, which may vary under different habitats. Environmental conditions can strongly affect the mycorrhizal fungi communities of orchids (Esposito et al., 2016; Li T. et al., 2021). On the other hand, microbial communities in soil are influenced not only by rhizosphere secretions but also by many other factors, such as climate and the physical and chemical properties of the soil (Kaushik et al., 2021). The changes of mycorrhizal fungi communities in the same orchids in different habitats may be related to the physical and chemical properties of local soil. By analyzing the diversity of endophytic fungi in *Cremastra appendiculata* roots and exploring the effects of altitude and rhizosphere soil physical and chemical properties on fungal diversity, it was found that the diversity of fungi varied in different elevations. Nitrate nitrogen and available phosphorus in rhizosphere soil decreased with elevation. The higher the altitude, the less endophytic fungi. And the more uneven the community distribution leads to the more prominent the dominant fungi (Ren et al., 2021). In the study of mycorrhizal fungi frequency on the volcano Mount Koma in northern Japan, it was found that ammonium nitrogen content at the lowest altitude was more than twice as high as that at the highest altitude. The nitrate content also fluctuated with altitude, but the difference was not significant except the lowest at 750 m altitude. With the increase of altitude, nitrogen and phosphorus levels decreased, indicating that higher altitude may have a certain impact on the survival of mycorrhizal fungi and their host environmental adaptation (Tsuyuzaki et al., 2005). While comparing the fungal community composition of *Gymnadenia conopsea* in southern Tibet (3600 m) and northern Heilongjiang Province (496 m), it was found that Ambisporaceae, Archaeosporaceae, Acaulosporaceae, Gigasporaceae, Glomeraceae and Paraglomeraceae were significantly enriched in the soil of southern Tibet (3600 m), while these fungi were almost not found in northern Heilongjiang Province (496 m). In addition, Clavariaceae, Russulaceae, Leotiaceae and Dictyosporiaceae are dominant in the roots of southern Tibet (3600 m), while these families are rare in the roots of northern Heilongjiang Province (496 m). The low pressure, low oxygen and strong radiation in high altitude areas will have adverse effects on the growth of plants. The above research results show that these fungal groups may play a role in *G. conopsea*'s adaptation to the high altitude environment in southern Tibet (Lin et al., 2020). It can be seen from the above studies that the increase of altitude will be accompanied by the decrease of air temperature and pressure, and the increase of photosynthetic radiation. Whether orchid mycorrhizal fungi or their hosts, they will have corresponding adaptation to environmental changes, as well as the interaction between microorganisms and plants, which may cause differences in fungal community composition. Other studies showed that there was specificity between mycorrhizal fungi and orchids. It was found that there were commonness and difference in the dominant population of wild orchid mycorrhizal fungi with different geographical distribution. The mycorrhizal fungi of *C. goeringii* in Dongbu Mountain were more diverse and distributed more evenly. Meanwhile, due to the influence of regional habitat, the diversity, good conditions and rich mycorrhizal fungi are conducive to the growth of *Cymbidium goeringiensis* (Xu and Liu, 2020). These results suggest that the changes of orchidaceae mycorrhizal fungi in different habitats may be related to the characteristics of plants themselves, but the effect of habitats on orchidaceae mycorrhizal fungi needs further research.

B. tianguii is an epiphytic plant of the orchid family. Its roots can attach to humus, rocks and tree trunks. The root mycorrhizal fungi and the root and rhizosphere soil mycorrhizal fungi may be different in the three habitats. Different altitudes affect the adaptability of *B. tianguii*, and the species and quantity of mycorrhizal fungi may change. The present study analyze the similarities and differences of mycorrhizal fungi communities in roots and rhizosphere soil of *B. tianguii* in different habitats, and find out the most important mycorrhizal fungi species, which can provide significant basis for the conservation and biological research of orchid plants.

Materials and methods

Plant material and soil sampling

The roots, rhizomes and rhizosphere soil (bark from trees and moss from rocks) of *B. tianguii* were collected from three populations at different elevations in Yachang Orchidaceae National Nature Reserve, Guangxi province. The three main habitats of *B. tianguii* are humus soil, stones and trees. The different samples from all these three habitats were collected. In order to investigate the influence of altitudes for *B. tianguii*, the

samples from three different altitudes (middle of Tiankeng, top of Tiankeng and orchid garden) were also collected. The fungal species of each part were determined with 3 biological replicates and a total of 81 samples were collected. The distribution habitat (Figure 1) and population situation and details of *B. tianguii* samples are shown in Table 1. The collected root segment and environmental samples were stored in dry ice and brought back to the laboratory for subsequent nucleic acid extraction experiments.

DNA extraction and sequencing

E.Z.N.A.® Soil DNA Kit was used to extract total microbiome DNA from all samples, and 1% agarose gel electrophoresis was used to detect extracted genomic DNA. Specific primers with barcode were synthesized according to the designated sequencing area. PCR amplification primers corresponding to the region of ITS1F_ITS2R (Lemons et al., 2017) the upstream primer ITS1F: CTTGGTCATTTAGAGGAAGTAA, and the downstream primer ITS2R: GCTGCGTTCTTCATCGATGC. PCR amplification was performed using TransGen AP221-02: TransStart Fastpfu DNA Polymerase and PCR instrument: ABI GeneAmp® 9700. Reaction PCR reaction system includes: 4 μL5×FastPfu Buffer; 2 μL2.5 mM dNTPs; 0.8 μL upstream primer (5 μM); 0.8 μL downstream primer (5 μM); 0.4 μL FastPfu DNA Polymerase; 0.2 μL BSA; 10 ng genome DNA; supply ddH_2O to 20 μL. The PCR amplification cycle in this study was pre-denaturation at 95°C for 3 min, 30 cycles: denaturation at

FIGURE 1
Growth situation of *B tianguii*. [**(A)**: *B tianguii*; **(B)**: Roots attached to stone walls; **(C)**: Roots attached to humus soil; **(D)**: Roots attached to the trunk.].

TABLE 1 Habitat and population profile of *Bulbophyllum tianguii*.

Plot	Site	Altitude (m)	Slope (°)	Aspect	Slope position	Distribution area (m^2)	Number of pseudobul	Number of stems	Density of dense distribution area (Numberof stems/m^2)
P1	Orchid Garden	950	30	Northwest	The upper part of Shishan Mountain	12	1270	125	35
P2	Middle of Tiankeng	1198	80	Southwest	The top of Tiankeng	35	2039	200	32
P3	Top of Tiankeng	1281	15	West	Near the mountain top	15	4360	427	82

95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 45 s, and finally stable extension at 72°C for 10 min (Xu et al., 2019). Each sample had three replicates. The PCR products of the same sample were mixed and detected by 2% agarose gel electrophoresis. The products were eluted with Tris_HCl reagent, and the PCR products were recovered by gel cutting with AxyPrepDNA gel recovery kit (AXYGEN Company). The PCR products were detected and quantified using the QuantiFluor™ -ST blue fluorescence quantification system (Promega Company) based on the preliminary quantitative results of electrophoresis, and the products were mixed in corresponding proportions according to the sequencing requirements of each sample. Purified amplified fragments were used to construct Miseq library using NEXTFLEX Rapid DNA-Seq Kit and sequenced using Illumina Miseq PE300 platform (Shanghai Meiji Biomedical Technology Co., Ltd.). FASTP (Chen et al., 2018) (https://github.com/OpenGene/fastp,version 0.20.0) software was used to control the quality of the original sequencing sequences of all samples. According to the overlap relationship between PE reads, pairs of reads were spliced into a sequence. The minimum overlap length was 10 bp, and the maximum mismatch ratio allowed in the overlap region of the spliced sequence was 0.2, which was used to screen sequences that did not meet the requirements of this experiment. Use FLASH (Magoc and Salzberg, 2011)(http://www.cbcb.umd.edu/software/flash,version 1.2.7) software for quality control sequence stitching.

Data analysis

OTU analysis

In order to facilitate the analysis of the species and number of fungi in the sequencing results, UPARSE software (Version 7.1) (Edgar, 2013) was used to cluster the optimized sequences and they were divided into groups according to the similarity between sequences (97%). A group is an Operational Taxonomic unit (OTUs) (He et al., 2015). The representative sequences of OTUs were taxonomic analyzed and summarized by RDP classifier (similarity > 97%) to obtain the classification information related to each OTUs (Wang et al., 2007), and the microbial community composition of each sample was counted at each classification level. In this study, Unite8.0/ITS_Fungi classification database was used for taxonomic analysis of OTUs.

Rarefaction curve analysis

The Rarefaction curve (Bacaro et al., 2012) is constructed by randomly selecting the result sequence from sequencing and combining the sequence number with the corresponding species number or diversity index. Mothur (Schloss et al., 2009) was used to analyze various Alpha diversity indexes, and R language was used to make dilution curves of each sample, so as to analyze whether the sample sequencing depth was sufficient. Dilution curves can also be used to compare species richness, uniformity or diversity among samples with different data volumes.

Community composition analysis

Through the amount of sample data in overlapping and separated areas in the Venn diagram, the number of common and unique OTUs in each sample was shown, so as to directly observe the similarity and overlap of OTUs number composition among each sample, as well as the number of unique groups in each sample's microbial community. And through the community Bar diagram to analyze the fungal community at all levels of classification groups and dominant species.

Alpha-diversity analysis

Alpha diversity is used to explore the diversity of microbial communities within the sample and the richness and evenness between communities by means of a variety of statistical indices (Baczkowski et al., 1998). Good's Coverage index is mainly used to reflect community coverage, while sobs, chao1 and Ace (Adaptive coherence estimator) can reflect the community richness of microbial communities. Shannon and Simpson index can show the community diversity of microbial community. The samples were divided into groups, and the Wilxocon rank sum test was used to analyze the differences between groups of Alpha diversity, and to detect whether the index values between the two groups had significant differences.

Beta-diversity analysis: NMDS analysis

NMDS (non-metric multidimensional scaling) analysis is used to simplify the research objects (samples or variables) of multidimensional space to low-dimensional space for positioning, analysis and classification. At the same time, it can retain the original relationship between objects data analysis method. According to the species information contained in the samples, it is reflected in the multidimensional space in the form of points, and the degree of difference between different samples is reflected by the distance between points, and finally the spatial location map of the samples is obtained. Using Qiime to calculate beta diversity distance matrix, R language (Version 3.3.1) vegan software package for NMDS analysis and mapping.

Phylogenetic tree analysis

Phylogenetic trees were constructed according to the evolutionary relationships among microbial species in *B. tianguii*, and the phylogenetic relationships among species in the samples were revealed from the perspective of molecular evolution. Through the software: FastTree (version 2.1.3 http://www.microbesonline.org/fasttree/). By selecting the sequence corresponding to the classification information at the OTU level, the evolutionary tree is constructed according to

Maximum Likelihood (ML), and the evolutionary tree is plotted using R language (Version 3.3.1).

Co-occurrence network analysis

Co-occurrence network analysis was used to display samples and species distribution. By analyzing the species abundance information between different environmental samples, the co-existence relationship of species in environmental samples can be obtained to highlight the similarities and differences between samples. Co-occurrence network analysis was performed by Networkx software.

Results and analysis

Fungal composition in roots and rhizosphere soil of *B. tianguii*

In the present study, a total of 81 samples were collected from 27 root samples, 27 rhizome samples and 27 rhizosphere soil samples of *B. tianguii*, which grow in Guangxi Yachang orchidaceae National Nature Reserve (Table 1). These 81 samples of *B. tianguii* were collected from three different growth environments of humus soil, stone and tree in three different altitude sites: middle of Tiankeng, top of Tiankeng and orchid garden (Figure 1). The different habitats and altitudes may affect the number and species of mycorrhizal fungi in orchid roots and rhizosphere soil. At different altitudes, the number of possible mycorrhizal fungi could be different; similarly, in different habitats, the *B. tianguii* grown in humus soil habitat may have more mycorrhizal fungi than stones and trees, speculated that humus soil may be moist and rich in nutrients suitable for fungal growth. After Illumina MiSeq high-throughput sequencing, quality control splicing of double-end sequences and OTU clustering, a total of 4447492 sequences were obtained, including 1667918 for roots, 1379856 for rhizomes and 1399718 for rhizosphere soil. A total of 6051 OTUs were obtained from root samples, 3482 OTUs from rhizome, and 5560 OTUs from rhizosphere soil. The common and unique fungal OTUs of *B. tianguii* samples were expressed as the Venn diagram to evaluate the relationship between fungal communities in roots, rhizomes and rhizosphere soils of *B. tianguii* at different habitats and altitudes. There are 2514 OTUs were shared by root, rhizome and rhizosphere soil; 293 OTUs were shared by root and rhizome; 331 OTUs were shared by rhizome and rhizosphere soil; and 2266 OTUs were shared by root and rhizosphere soil. The numbers of OTUs unique to each species were as follows: 978 for root, 344 for rhizome, and 1643 for rhizosphere soil. Except for non-annotated fungi, the distribution of fungi in the root, rhizome and rhizosphere soil of *B. tianguii* at different classification levels among domain to species is shown in Table 2. The number of OTUs of mycorrhizal fungi in B.tianguii samples varied in different habitats and altitudes. Overall, the number of OTUs of mycorrhizal fungi in the roots, rhizomes and rhizosphere soil of B.tianguii in humus soil habitat and orchid garden was more abundant. On phylum classification level, Ascomycota, Basidiomycota, Rozellomycota, Mortierellomycota, Chytridiomycota, Glomeromycota, Mucoromycota, unclassified_k:Fungi are the common phylums of the fungi detected in roots, rhizomes and rhizosphere soil samples of *B. tianguii*. In addition, the fungi in root also include Kickxellomycota, and the fungi in rhizosphere soil also includes Kickxellomycota, Basidiobolomycota, Neocallimastigomycota, Olpidiomycota.

It can be seen from the above results that the number of fungi in the root and rhizosphere soil of *B. tianguii* was apparently higher than that in the rhizome, and the species of fungi in the rhizosphere soil was the richest. The number of effective sequences in 81 samples was randomly selected by Mothur analysis, and the dilution curve (Supplementary Figure S1) was drawn by sobs index. It can be seen that the curve began to flatten after exceeding 25000, indicating that the sequencing has reached saturation and the most OTUs have been obtained.

Mycorrhizal fungi composition of *B. tianguii*

According to the published list of common orchid mycorrhizal fungi summarized by Wang et al. (2021), mycorrhizal fungi were selected from all the fungi detected in 81 samples of *B. tianguii*. A total of 209, 162 and 215 fungi were detected from root, rhizome and rhizosphere soil, respectively (Table 3), all belonging to the following four phylums: Ascomycota, Basidiomycota, Mucoromycota and Mortierellomycota. It can be seen that the OTUs in rhizosphere soil of *B. tianguii* are more than those in root, but the number at each classification level is basically the same. And the OTUs of rhizome are far less than those of root and rhizosphere soil. There are 187 common OTUs in root,

TABLE 2 OTU statistics of each classification level (Fungi).

Sample name	Domain	Kingdom	Phylum	Class	Order	Family	Genus	Species
Root	1	1	9	40	126	344	880	1511
Rhizome	1	1	8	32	104	286	663	1053
Rhizosphere soil	1	1	12	53	141	354	860	1429

TABLE 3 OTU statistics of each classification level (Mycorrhizal fungi).

Sample name	OTUs	Domain	Kingdom	Phylum	Class	Order	Family	Genus	Species
Root	431	1	1	4	9	21	35	52	209
Rhizome	286	1	1	4	9	21	33	49	162
Rhizosphere soil	478	1	1	4	8	20	34	52	215

rhizome and rhizosphere soil; 31 OTUs were shared by root and rhizome; 30 OTUs were shared by rhizome and rhizosphere soil; and 150 OTUs were shared by root and rhizosphere soil. The numbers of OTUs unique to root, rhizome and rhizosphere soil were 63, 38 and 111, respectively (Figure 2A). The results show that the number of mycorrhizal fungi in rhizosphere soil of *B. tianguii* was the highest, which was much higher than that of the rhizome. It is known that the roots of *B. tianguii* are closer to soil while the rhizomes are farther. The number of mycorrhizal fungi in the roots of is close to that in the rhizosphere soil, and both are significantly higher than rhizomes. There were 234 species of mycorrhizal fungi in the samples collected from humus soil, stone and tree habitats. The number of mycorrhizal fungi in humus soil habitats was 87, and 96 in stone habitats, and 25 in tree habitats. The results showed that the number of mycorrhizal fungi in the root endophyte and rhizosphere soil of *B. tianguii* grown on the tree was significantly lower than that in the humus soil and stone habitats. There are 168 OTUs were shared by the middle of Tiankeng, the top of Tiankeng and the orchid garden; 31 OTUs were shared by the middle of Tiankeng and the top of Tiankeng; 71 OTUs were shared by the top of Tiankeng and the orchid garden; and 59 OTUs were shared by root and rhizosphere soil. The numbers of OTUs unique to the middle of Tiankeng, the top of Tiankeng and the orchid gardenwere as follows: 45 for the middle of Tiankeng, 59 for the top of Tiankeng, 117 for orchid garden (Figures 2B, C). The number of mycorrhizal fungi in the orchid garden population was significantly higher than that in the two places, which was consistent with the results of fungal statistics.

In order to identify the fungal taxa related to *B. tianguii* and analyze their similarities and differences in root and rhizosphere soil of *B. tianguii* under different habitats, especially the distribution of mycorrhizal fungi. Two taxonomic levels (phylum and genus) were selected for further analysis (Figures 3A, B). Among the fungal taxa of *B. tianguii*, Ascomycota and Basidiomycota were the most representative phylum. In most samples, Ascomycota dominated (79.16%-50.96%), and Ascomycota was particularly abundant in root samples (79.16%). The mycorrhizal fungi belonging to Ascomycota were dominant in the roots and rhizomes of *B. tianguii* (71.75% and 59.52%), and the dominant group in rhizosphere soil was Basidiomycota (68.56%). The results showed that Ascomycota fungi were the main endophytic fungi of *B. tianguii*, and Basidiomycota fungi were the main exogenous fungi, indicating that there were significant differences between endophytic fungi and exogenous fungi in *B. tianguii*. The fungal classification at different sites showed great differences in the phylum classification level of fungi and mycorrhizal fungi in the sites with different elevation. At the same time, the main dominant groups of *B. tianguii* fungal community in each taxonomic unit (phylum to genus) were also sorted out (Supplementary Table 4). Notably, Ascomycota (87.16%) was the dominant phylum in the fungal community of root samples collected in the middle of Tiankeng. And Basidiomycota (51.34%/61.86%) was the dominant phylum in rhizomes and rhizosphere soil (Figure 3C). In the mycorrhizal fungal community of root samples collected in the middle of Tiankeng, Ascomycota (71.02%) was the dominant phylum, and

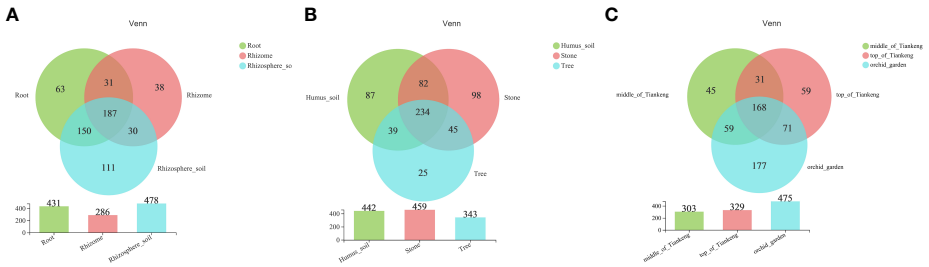

FIGURE 2
Venn chart. Different colors represent different groups, the number of overlapping parts represents the number of species shared by multiple groups, and the number of non-overlapping parts represents the number of species unique to the corresponding group. [**(A)**: root, rhizome and rhizosphere soil groups; **(B)**: different habitat groups; **(C)**: different altitude groups].

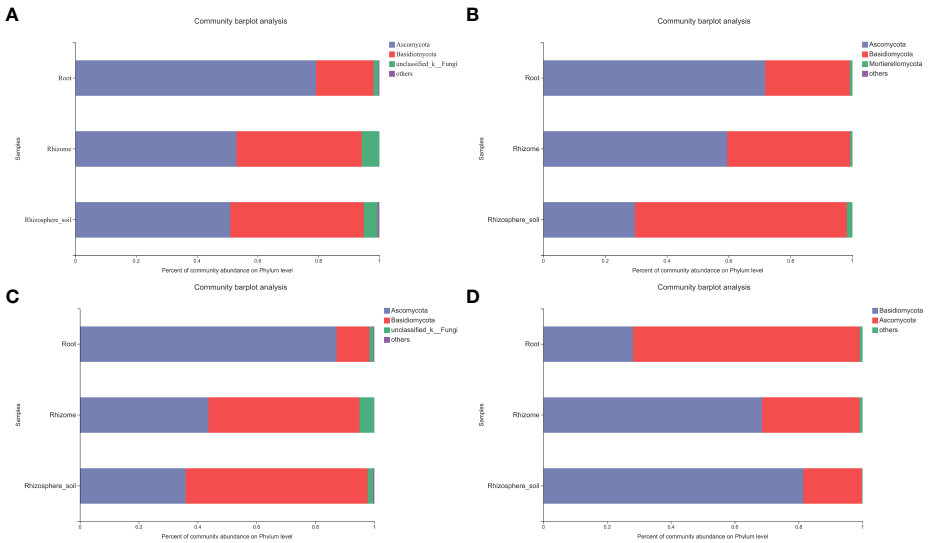

FIGURE 3
Bar diagram of fungal community composition. The ordinate is the sample name, and the abscissa is the proportion of species in the sample. Columns with different colors represent different species, and the length of the column represents the proportion of the species. **(A)**: fungi composition at Phylum level; **(B)**: mycorrhizal fungi composition at Phylum level; **(C)**: fungal composition of the middle of Tiankeng (Phylum); **(D)**: mycorrhizal fungi composition of the middle of Tiankeng (Phylum).

Basidiomycota (68.54%/81.45%) was the dominant phylum in rhizome and rhizosphere soil (Figure 3D). The dominant groups of *B. tianguii* fungi and mycorrhizal fungi in the middle of Tiankeng are different from the overall situation, which maybe related to the environment in the middle of Tiankeng and the adaptability of orchids to this altitude.

According to the genera of some common orchid mycorrhizal fungi published by Wang et al. (2021), mycorrhizal fungi were screened from all fungi detected in all 81 samples. In addition to unclassified _o:Sebacinales, combined with the main dominant groups of mycorrhizal fungi in each taxonomic unit (Supplementary Table 5), it can be seen that the species of endophytic mycorrhizal fungi, rhizome mycorrhizal fungi and rhizosphere soil mycorrhizal fungi in *B. tianguii* are basically similar to those of endophytic mycorrhizal fungi at the phylum to genus levels, but there are great differences in the species and abundance of rhizosphere soil mycorrhizal fungi (Figure 4). And the main dominant groups also have significant differences. The dominant genus of endophytic fungi in *B. tianguii* is Exophiala (39.43%), followed by Fusarium (14.74%). The dominant genus of endophytic fungi in rhizomes of *B. tianguii* is Fusarium (20.13%), and the dominant species is Fusarium_concentricum (18.85%). It can be seen that the dominant mycorrhizal fungi in the roots and rhizomes were Exophiala and Fusarium, while the dominant mycorrhizal fungi in the rhizosphere soil of *B. tianguii* were Sebacina (39.87%), and the dominant species were Sebacina_ sp. (39.85%). The results showed that *B. tianguii* chose Exophiala and Fusarium as its endogenous dominant mycorrhizal fungi. And Sebacina that function as its exogenous dominant mycorrhizal fungi was more conducive to the growth and reproduction of orchids in rhizosphere soil. Among the *B. tianguii* samples in different habitats (humus soil, stone and tree), mycorrhizal fungi belonging to Ascomycota were dominant in stone habitat (69.27%), and the dominant groups in humus soil and tree habitat were Basidiomycota (54.19% and 58.30%). At genus level, the dominant mycorrhizal fungi in humus habitat are Sebacina (29.48%), Exophiala (34.79%) in stone habitat, and unclassified in tree habitat_o:Sebacinales(36.36%). Among the *B. tianguii* samples from different altitudes (middle of Tiankeng, top of Tiankeng and orchid garden), mycorrhizal fungi belonging to Ascomycota were dominant in the orchid garden population (70.18%), and the dominant groups of middle of Tiankeng and top of Tiankeng populations were Basidiomycota (66.38% and 51.14%). At genus level, the dominant mycorrhizal fungi in the middle population of Tiankeng are Sebacina (64.30%), and the dominant mycorrhizal fungi in the top population of Tiankeng are unclassified_ o: Sebacinales (37.79%), and Exophiala (24.23%) was the dominant mycorrhizal fungus in Orchid Garden population.

Alpha-diversity analysis

Based on the results of t-test of the difference between groups of alpha diversity index, this paper selects sobs, Shannon and coverage

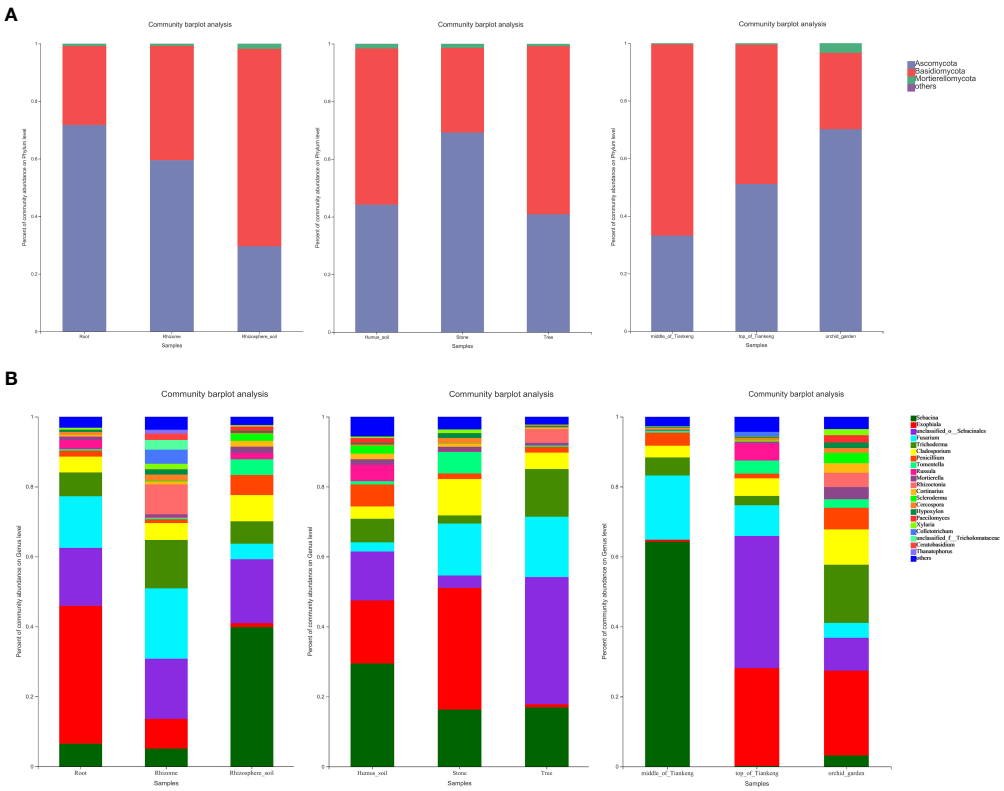

FIGURE 4
Bar diagram of mycorrhizal fungal community composition. The abscissa is the sample name, and the ordinate is the proportion of species in the sample. Columns with different colors represent different species, and the length of the column represents the proportion of the species. Composition of mycorrhizal fungi is at Phylum **(A)** and Genus levels **(B)**.

to analyze the diversity of *B. tianguii* fungal community. According to the grouping analysis of the sample type (root, rhizome and rhizosphere soil) of *B. tianguii*, the diversity index coverage results are higher than 0.95, and the difference between groups is not significant, indicating that the sequencing results represent the real situation of microorganisms in each group of samples (Figures 5A, D, G). The t-test of inter group difference of sobs index showed that the number of mycorrhizal fungi OTUs observed in rhizome was significantly lower than that in root and rhizosphere soil samples ($P \leq 0.001$, marked as * * *), the difference of OTUs observed in root and rhizosphere soil samples was not significant, and the number of mycorrhizal fungi in rhizosphere soil was slightly higher than that in root (Figure 5B). The t-test results of the inter group difference of Shannon index show that there is no significant difference in the community diversity of mycorrhizal fungi among roots, rhizomes and rhizosphere soil (Figure 5C), which is consistent with the species annotation results in the previous classifications. The order of diversity is rhizosphere soil > root > rhizome. The results showed that the mycorrhizal fungi in *B. tianguii* rhizosphere soil were the most abundant. According to different altitude populations of *B. tianguii* (middle of Tiankeng, top of the Tiankeng, orchid garden), T test of Sobs index showed that the number of fungal OTUs observed in samples collected at orchid garden was higher than that at middle of Tiankeng and top of Tiankeng, and the difference was extremely significant ($P \leq 0.001$ marked as * * *). There is no significant difference between the middle of Tiankeng and the top of Tiankeng (Figure 5E). The T-test results of the inter-group difference of the shannon index (Figure 5F) indicated that the mycorrhizal fungal community diversity of the samples from the orchid garden was significantly higher than that of the top of Tiankeng ($0.001 < P \leq 0.01$ marked as * *). This indicated that the mycorrhizal fungi in the roots and rhizosphere soil of *B. tianguii* grown in orchid gardens were the most abundant. According to the grouping analysis of different habitats (humus, stone, and tree) of *B. tianguii*, the T test results of the difference between groups of Sobs index (Figure 5H) indicated that the number of mycorrhizal fungi OTUs observed in the samples collected in the tree habitat was significantly lower than that in the humus and stone habitats ($0.01 < P \leq 0.05$ marked as *), while there was no significant difference in the number of mycorrhizal fungi OTUs between the humus and

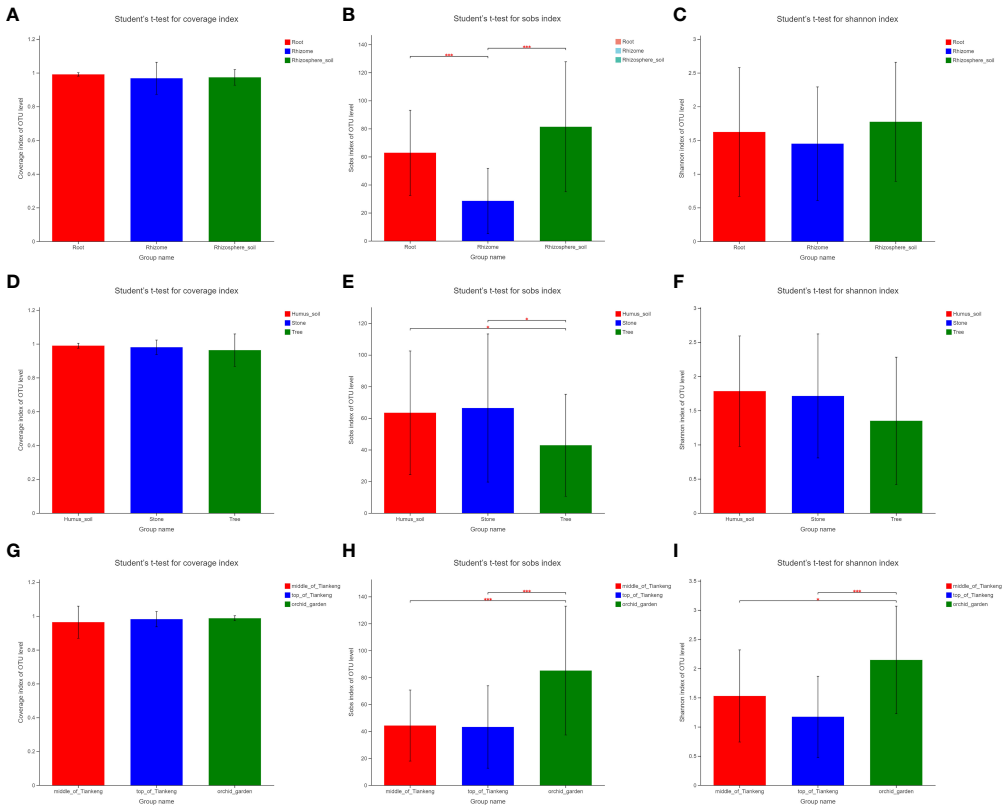

FIGURE 5
Wilxocon rank sum test of Alpha index difference between groups. The significance of the difference between the two groups is expressed as: 0.01 <p ≤ 0.05 marked for *, P ≤ 0.001 marked for ***. The abscissa is the grouping name, and the ordinate is the index average of each group. **(A-C)**: root, rhizome and rhizosphere soil groups; **(D–F)**: different habitat groups; **(G–I)**: different altitude groups.

stone habitats. The shannon index of each group of *B. tianguii* on humus soil, stone and tree ranked as humus soil > stone > tree (Figure 5I). The number and species of mycorrhizal fungi of *Bulbophyllum tianguii* growing in humus soil and stone habitat are more abundant, indicating that the mycorrhiza fungi of *B. tianguii* are more abundant in humus soil, which may be more suitable for *B. tianguii* habitat.

Beta-diversity analysis: NMDS analysis

NMDS analysis (Figure 6) showed that the order of sample points in root and rhizosphere soil was basically concentrated, while the order of sample points in rhizome was relatively dispersed, and the order of sample points in humus soil, stone and tree was also relatively dispersed. The order of sample points

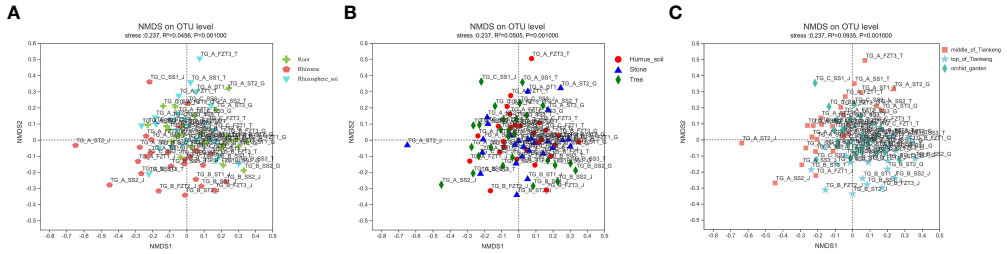

FIGURE 6
NMDS analysis. Points of different colors or shapes represent samples of different groups. The closer the two sample points are, the more similar the species composition is. **(A)**: root, rhizome and rhizosphere soil groups; **(B)**: different habitat groups; **(C)**: different altitude groups.

in the middle and top of the Tiankeng was relatively concentrated, but the order of sample points in orchid garden was relatively dispersed. However, there was no obvious separation among the groups of samples, indicating that there were some differences in mycorrhizal fungi groups in samples from different parts of *B. tianguii* and the samples from different habitats or from different altitude populations, but there was no significant difference in mycorrhizal fungi groups.

Phylogenetic tree analysis

We analyzed the top 50 species of total abundance at the classification level, and the genus of all fungi detected was classified from the OTU classification level. It can be seen from the figure that the Reads proportion of OTU3318 (unclassified_o:Sebacinales) and OTU3273 (Exophiala) was the highest, which are close to 50k, followed by OTU1190 (Sebacina), OTU1933 (Sebacina), OTU4448 (Cladosporium), OTU2455 (Fusarium) and OTU4001 (unclassified_g: Trichoderma), which is also exceeded 20k. In addition, there are 35 OTUs whose reads proportion is less than 5k, and 8 OTUs whose reads proportion is at 5k-10k (Figure 7). The above results showed that Sebacina and Exophiala accounted for the largest proportion of mycorrhizal fungi in *B. tianguii*, which indicated that Sebacina and Exophiala are the predominance mycorrhizal fungi of *B. tianguii*.

Co-occurrence network analysis of environmental samples of *B. tianguii*

The co-occurrence network analysis was performed on the environmental samples of *B. tianguii* (rhizosphere soil samples) to explore the relationship of mycorrhizal fungi in different habitats or at different altitudes (Figure 8). According to the different habitats of *B. tianguii*, OTUs in humus soil, stone, and tree are OTU4448 (unclassified_g:Cladosporium) and OTU1132 (unclassified_g:Trichoderma). According to the different altitude populations of *B. tianguii*, OTUs in the middle of Tiankeng, the top of Tiankeng and orchid garden is OTU4448 (unclassified_g: Cladosporium). The above results showed that the common

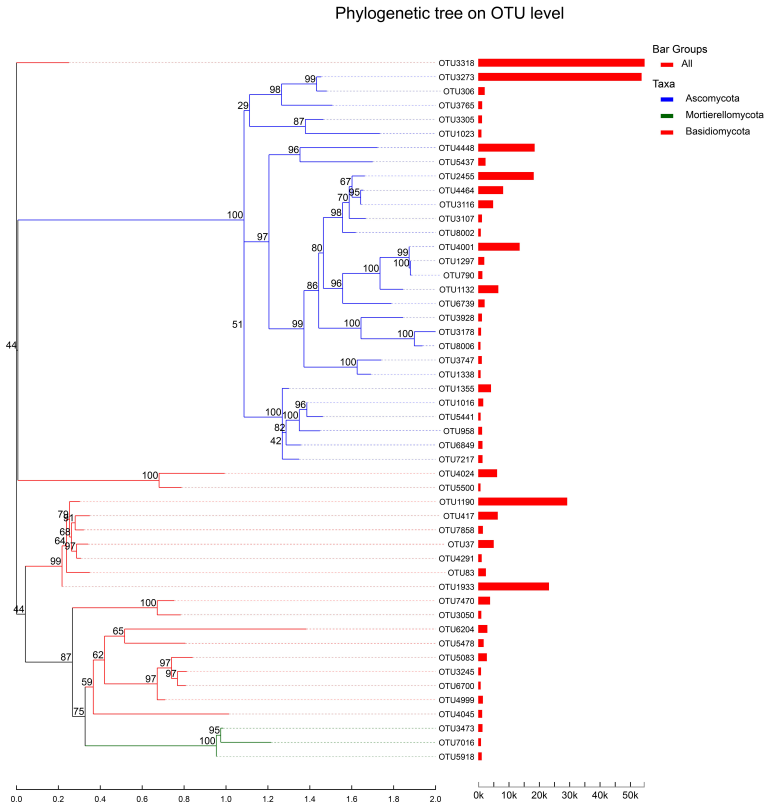

FIGURE 7
System evolution tree. Each branch represents each OTU, and the branch is colored according to the advanced Phylum level of the species. The length of the branch is the evolutionary distance between the two species, namely the degree of species difference. The column shows the proportion of Reads in different groups.

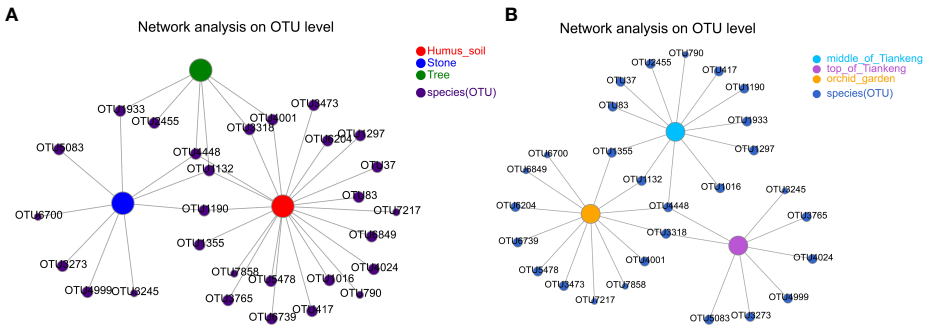

FIGURE 8
Co-occurrence network diagram. Co-occurrence relationship of species in different samples is visually displayed. Nodes in the network represent sample nodes or species nodes, and the connection between sample nodes and species nodes represents the sample containing the species. **(A)**: different habitat groups; **(B)**: different altitude groups.

species in different habitats and different altitudes were unclassified_g:Cladosporium, indicating that the mycorrhizal fungi of Cladosporium may be the specific symbiotic fungi of *B. tianguii*.

Discussion

Mycorrhizal fungi have vital impacts on orchid seed germination, nutrient absorption of adult plants and population dynamics. Therefore, exploring the internal mechanism of the interaction between mycorrhizal fungi and orchids is a hot field of orchid mycorrhizal research at present (Zhao et al., 2021). However, the research on mycorrhizal fungi of *B. tianguii* is still very scarce. Studies on mycorrhizal fungi of orchids are being gradually improved, and more and more fungi are confirmed to be mycorrhizal fungi with some connection to orchids. In order to study the species of orchid mycorrhizal fungi growing in Minas Gerais state, Brazil, Nogueira et al. (Nogueira et al., 2005) isolated Ceratorhiza from the roots of *Bulbophyllum weddelii*, which is the first time that Rhizobium like fungi were isolated from orchid roots in Minas Gerais state. To identify and store important mycorrhizal fungi that can protect orchid species through symbiotic seed germination. Three mycorrhizal Rhizoctonia-like fungi were isolated from the roots of three neotropical orchid species *Gomesa crispa*, *Campylocentrum organense* and *Bulbophyllum* sp. from three different Atlantic rain forest fragments in Brazil. It was found that the isolates belonged to the genus Ceratorhiza (Pereira et al., 2005). *B. tianguii* is known to grow not only in soil and stone habitats, but also attached to the trunks of tall trees. Other researchers analyzed the fungal community composition of two orchids, *Panisea unifora* and *Bulbophyllum odoratissimum*, which grow together in forests in southern China. *P. unifora* was found to grow *Quercus yiwuensis* and *B. odoratissimum* was grown on *Pistacia weinmannifolia* trees. It was observed that Serendipitaceae (71%) and Nectriaceae (24%) were the most abundant families related to *B. odoratissimum*. Fusarium,which belongs to Nectriaceae, was found in the barks of two orchid epiphytic trees, so it cannot be ruled out that Fusarium may be important for the early stage of *B. odoratissimum* development (Pecoraro et al., 2021). The above studies have found that Fusarium and Rhizoctonia maybe a mycorrhizal fungus closely related to *B. odoratissimum* and *B.* sp., which is consistent with our findings. In addition, Ceratorhiza had been confirmed to be related to neotropical orchids *B. weddelii*, not the same to our results. This may be related to the species, distribution and adaptability of the orchid to the growing environment, and mutual selection between dominant mycorrhizal fungi and host plants (Luo et al., 2020), which needs further research and verification.

Previous studies have also confirmed that orchid mycorrhizal fungi can promote plant growth, suggesting that the mycorrhizal fungi isolated from *B. tianguii* may also have the potential function to promote plant growth and impact the host. Several different orchid mycorrhizal fungi were inoculated into *Dendrobium candidum*. It was found that Epulorhiza sp. and Rhizoctonia sp. had a good effect on the growth of *D. candidum* (Li et al., 2022). Four orchid mycorrhizal fungi isolated from the roots of wild *Paphiopedilum* (Cladosporium perangustum, Kirschsteinothelia tectonae, Phialophora sp. and Cypellophora sp.) were used to carry out bacteria seedling symbiosis test with test tube seedlings and nutrient pot seedlings of *Paphiopedilum* with leaves. The changes of growth and physiological indexes were observed. It was found that Cladosporium perangustum and Phialophora sp. increased the fresh quality of test tube seedlings. The activities of three protective enzymes and the total amount of chlorophyll were significantly affected. Kirschsteinothelia tectonae and physiophora sp. significantly improved the fresh quality increment of nutrient bowl seedlings (Chen et al., 2022). Although these observations

suggest that orchid mycorrhizal fungi (OMF) is crucial in orchid microflora in orchid microbial community, little is known about the mycorrhizal fungi associated with *B. tianguii*, their interaction with host and their distribution in plants.

In the present study, the similarities and differences of mycorrhizal fungi communities in roots and rhizosphere soil of *B. tianguii* in different habitats were analyzed in order to find out possible important mycorrhizal fungi species. The analysis focused on different mycorrhizal fungi in the roots and rhizosphere soil of *B. tianguii*. OTU cluster analysis and mycorrhizal fungal community composition analysis showed that mycorrhizal fungi in roots and rhizosphere soil of *B. tianguii* mainly composed of Ascomycota and Basidiomycota. The dominant mycorrhizal fungi in roots were Ascomycota, and the dominant mycorrhizal fungi in rhizosphere soil were Basidiomycota. The dominant mycorrhizal fungi in roots and rhizomes were unclassified_g_Exophiala (37.94%) and Fusarium_concentricum (18.85%), while Sebacina_sp. (39.85%) was the dominant mycorrhizal fungi in rhizosphere soil of *B. tianguii*, indicating that Exophiala and Fusarium were the dominant genus of endophytic mycorrhizal fungi in *B. tianguii*, Sebacina_sp. is a dominant ectomycorrhizal fungus. This may be caused by the effects of different fungi on orchids or the selection of endophytic and exogenous fungi in orchids. This is consistent with other studies, which have also confirmed that Exophiala and Fusarium are common genera of orchid endophytic fungi (Tan et al., 2012).

Furthermore, the dominant mycorrhizal fungi in different habitats and altitudes of *B. tianguii* analyzed in this study are as follows: Sebacina, Exopiala in humus and stone habitat, unclassified_o:Sebacinale in tree habitat; In the middle of the Tiankeng is Sebacina, and at the top of the Tiankeng is unclassified_o: Sebacinale, the orchid garden is Exophiala. The results showed that the dominant mycorrhizal fungi of *B. tianguii* in different habitat were different, indicating that *B. tianguii* would like to choose mycorrhizal fungi that were more conducive to its survival or affected by other microorganisms. The dominant mycorrhizal fungi of *B. tianguii* at different altitudes are different, which indicates that these different mycorrhizal fungi are beneficial for *B. tianguii* to adapt to climate and soil physical and chemical properties at different altitudes. The results of phylogenetic tree and co-occurrence network analysis also indicated that the fungi belonging to Sebacina, Cladosporium, Exophiala and Fusarium might be the dominant mycorrhizal fungi symbiotic with *B. tianguii*.

Former studies have proved that orchids have a close symbiotic relationship with mycorrhizal fungi, and the dominant mycorrhizal fungi have an impact on the growth and seed germination of orchids (Qin et al., 2021; Gao et al., 2022; Li et al., 2022). Some other studies have shown that Sebacina, Exophiala and Fusarium fungi are common species in orchid mycorrhizal fungi (Tan et al., 2012; Wei et al., 2016; Fu et al., 2019), and most of them belong to Ascomycota and Basidiomycota, which is consistent with previous studies (Li T. et al., 2021; Pecoraro et al., 2017). Sebacina, Exophiala, Fusarium and Cladosporium may be related to the germination of plant seeds, plant biomass or influenced the stress resistance of plants. At present, the mycorrhizal fungi associated with *B. Tianguii* have not been reported, but some studies have found that the above mycorrhizal fungi have a certain impact on the growth of orchids. The symbiotic germination test was carried out between mycorrhizal fungi isolated from Orchidaceae and their seeds, the results showed that mycorrhizal fungi *Sebacina* sp. significantly promoted the seed germination of *Bletilla ochracea*, and the germination rate was 18.43% higher than those in the control group. This indicated that *Sebacina* sp. had a good promoting effect on seed germination of *B. striata*, which was beneficial to the protection and reproduction of *B. striata* (Chen et al., 2012). Moreover, it was also found that Sebacina vermifera can improve plant productivity and stress tolerance (Ray and Craven, 2016), Sebacina can promote *Dendrobium ofcinale* seed germination and seedling growth (Zhang et al., 2020). Fusarium is proved not only to promote the synthesis of GAs causing orchid disease, but also may form symbiotic mycelium with orchids, which is conducive to the growth of orchids (Tsavkelova, 2016; Chen et al., 2022). Attention should be paid to the interaction between Sebacina, Exophiala, Fusarium and orchids.

In the case of Cladosporium, it has positive effects on orchids as well as the possibility of causing orchid diseases. Inoculation of Cladosporium did not cause disease to the tissue culture seedlings of *Dendrobium* and *Bletilla*, and had significant growth promoting effects. Cladosporium can infect the roots of tissue culture seedlings of *D. officinale* and *B. striata*, and form a typical structure of orchid mycorrhizal fungi in root cortex cells-mycelium group. The mycorrhizal seedlings grow well and establish a mutually beneficial symbiotic system (Tang et al., 2018). The symbiotic test between *Cladosporium perangustum* isolated from the roots of wild Paphiopedilum and the test-tube seedlings of Paphiopedilum with leaf was conducted to study the changes of growth and physiological indexes. It was found that *C. perangustum* had a good inoculation effect on the test-tube seedlings. The fresh weight of the plant inoculated with *C. perangustum* increased by about 300%, and the leaf area increased by 1.11 cm^2, which greatly improved the absorption of water in the medium. The activities of peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) and total chlorophyll content in leaves were 130.3%, 1016.9%, 196.1% and 62.2% higher than those in the control, respectively, indicating that inoculation of strains was conducive to improving the protective enzyme activity and chlorophyll content in plant leaves (Chen et al., 2022). However, some studies have found that Cladosporium is related to black spot and blossom blight of *Dendrobium*, which may be an important factor leading to the wilt of precious orchids (Xiao et al., 2018; Ismail and Pauzei, 2022). Some studies have shown that Exophiala is a symbiotic mycorrhizal fungus of *Cephalanthera*

damasonium and *Orchis pauciflora*, but no study has clearly pointed out that Exophiala can promote or inhibit the seed germination and growth of orchids (Pecoraro et al., 2012; Pecoraro et al., 2017). Therefore, it will be necessary for the next step to study the symbiotic relationship between the fungi of Cladosporium and *B. tianguii*.

α and β diversity analysis showed that there were significant differences in mycorrhizal fungi species in roots and rhizosphere soil of *B. tianguii*, and there were also significant differences in mycorrhizal fungi species and relative abundance of *B. tianguii* in different habitats. However, no matter from the root, rhizome and rhizosphere soil to classify or from three different habitats (humus soil, stone and tree) to classify and analyze, mycorrhizal fungi found in the root will be widely distributed in the rhizosphere soil. Species of mycorrhiza fungi of the same plant in different habitats will be similar and not completely independent of each other, which is consistent with previous research results (Waud et al., 2016).

The diversity analysis of mycorrhizal fungi in roots, rhizomes and rhizosphere soil of *B. tianguii* showed that there were significant differences in the number of mycorrhizal fungi in roots (roots, rhizomes) and rhizosphere soil, and the dominant mycorrhizal fungi were different. This difference has been confirmed in other studies that there are differences between fungal communities in plant roots and rhizosphere soil. The reason for this difference is that plant roots secrete some substances, which may regulate the types and quantities of microorganisms in soil (Wu et al., 2014; Han et al., 2021).

The diversity of mycorrhizal fungi in humus soil, stone and tree of *B. tianguii* was analyzed to verify the effects of different habitats on the number and species of mycorrhizal fungi in roots and rhizosphere soil of *B. tianguii*. The results showed that the number of mycorrhizal fungi in humus soil and stone was significantly higher than that in tree, but the number of mycorrhizal fungi in humus soil was almost equal to that in stone. The shannon index (fungal community diversity) among mycorrhizal fungi in different growth environments (humus, stone, tree) ranked as humus > stone > tree, and this difference was consistent with results found in other orchid studies (Jiang et al., 2018). The reason for these differences may be the composition of mycorrhizal fungi in orchid roots is not completely controlled by plants themselves, but the main mycorrhizal fungi still depend on the selection of orchid plants (Chen et al., 2019).

The diversity analysis of mycorrhizal fungi in the samples of different altitude populations (middle of Tiankeng, top of Tiankeng and orchid garden) of *B. tianguii* was performed to disgust the structure and diversity of mycorrhizal fungi in *B. tianguii* root and rhizosphere soil at different altitudes. The results showed that the number of mycorrhizal fungi in the middle and top of the Tiankeng was significantly lower than that in the orchid garden, but the diversity of mycorrhizal fungi in the top of the Tiankeng was significantly higher than that in the orchid garden. This was the first report on the diversity of mycorrhizal fungi of *B. tianguii* growing at different altitudes. The dominant mycorrhizal fungi in these three locations were different. This difference is consistent with previous findings, one study analyzed the diversity of root fungi of *Cremastra appendiculata* at different altitudes in Taibai Mountain Nature Reserve and Sichuan Huanglonggou. It was found that the diversity of fungi was different at different altitudes. With the increase of altitude, the number of endophytic fungi gradually decreased (Ren et al., 2021). To explore the relationship between the changes of species richness and distribution of ecorrhizal fungi (ECM) fungal community along the altitude gradient and the host distribution, the ECM fungal community in the roots of *Pinus sylvestris* at 300m and 550 - 600 m altitude in Scotland was analyzed. The results showed that the OTU richness was not related to altitude, while the ectomycorrhizal fungus *Pilderma sphaerosporum* was the most common in low altitude areas, and *Russula sardonia* was the most common in high altitude areas, indicating that with the change of altitude, the ECM fungal diversity of *P. sylvestris* did not change, but the composition of its fungal community changed, which may be closely related to the changes of soil humidity and soil temperature (Jarvis et al., 2015). The community composition of arbuscular mycorrhiza fungi (AMF) in rhizosphere soil and roots of *Spiraea pubescens* at 1515 m, 1410 m and 1305 m altitudes in Daqing Mountain, Inner Mongolia was studied. It was found that the number of OTUs of AMF was the largest at 1515 m altitude, and the highest AMF richness and diversity also appeared at the highest altitude. Altitude did not affect the species diversity of AMF, but it would affect the community structure. The reason for this phenomenon may be that these AMFs can help plants survive in a more arid and barren environment (Liu et al., 2017). And the more uneven the community distribution, and the more prominent the dominant fungi (Xu et al., 2019). The reason for this difference is that with the increase of altitude, affected by climate and environmental factors, along with changes in soil physical and chemical properties and nutrient elements, the environment is more and more unsuitable for microbial growth.

Conclusion

In this study, high-throughput sequencing technology was used to study mycorrhizal fungal communities in roots, rhizomes and rhizosphere soil of *B. tianguii*. Species diversity analysis showed that mycorrhizal fungal communities in *B. tianguii* roots were significantly different from those in rhizomes and rhizosphere soils. The number of OTUs in rhizosphere soil and the diversity of mycorrhizal fungal

communities were the highest. *B. tianguii* in different habitats and different altitudes of mycorrhizal fungi in the species and abundance are also different, the number and diversity of mycorrhizal fungi in the orchid garden and humus soil habitat are the most abundant. Species annotation, phylogenetic tree and co-occurrence network analysis indicated that the dominant mycorrhizal fungi of *B. tianguii* mainly included Sebacina, Cladosporium, Exophiala, Fusarium, belonging to Ascomycota and Basidiomycota. The results of this study showed that the symbiotic relationship between Sebacina and Cladosporium and *B. tianguii* plants needs further study. At present, there are very few studies on mycorrhizal fungi related to *B. tianguii*, and there is little knowledge about the fungal species that are closely related to *B. tianguii*. The results of this study can provide some reference for the subsequent studies on dominant mycorrhizal fungi in orchids and the protection of *B. tianguii*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA857524.

Author contributions

SC and XW designed the study, RZ, HQ, JT performed the experiment, JL and YL analyzed the data and drafted the manuscript, HQ, JT, RZ and SC helped in sample collection, YH helped in data analysis. All authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.992184/full#supplementary-material

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Effects of arbuscular mycorrhizal fungi on plant growth and herbivore infestation depend on availability of soil water and nutrients

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Introduction: Fitness of plants is affected by their symbiotic interactions with arbuscular mycorrhizal fungi (AMF), and such effects are highly dependent on the environmental context.

Methods: In the current study, we inoculated the nursery shrub species *Artemisia ordosica* with AMF species *Funneliformis mosseae* under contrasting levels of soil water and nutrients (diammonium phosphate fertilization), to assess their effects on plant growth, physiology and natural infestation by herbivores.

Results: Overall, plant biomass was synergistically enhanced by increasing soil water and soil nutrient levels. However, plant height was surprisingly repressed by AMF inoculation, but only under low water conditions. Similarly, plant biomass was also reduced by AMF but only under low water and nutrient conditions. Furthermore, AMF significantly reduced leaf phosphorus levels, that were strongly enhanced under high nutrient conditions, but had only minor effects on leaf chlorophyll and proline levels. Under low water and nutrient conditions, specific root length was enhanced, but average root diameter was decreased by AMF inoculation. The negative effects of AMF on plant growth at low water and nutrient levels may indicate that under these conditions AMF inoculation does not strongly contribute to nutrient and water acquisition. On the contrary, the AMF might have suppressed the direct pathway of water and nutrient absorption by the plant roots themselves despite low levels of mycorrhizal colonization. AMF inoculation reduced the abundance of the foliar herbivore *Chrysolina aeruginosa* on plants that had been grown on the low nutrient soil, but not on high nutrient soil. Fertilization enhanced the abundance of this herbivore but only in plants that had received the high water treatment. The lower abundance of the herbivore on AMF plants could be related to their decreased leaf P content. In conclusion, our results indicate that AMF negatively affect the growth of *Artemisia ordosica* but makes them less attractive to a dominant herbivore.

Discussion: Our study highlights that plant responses to AMF depend not only on the environmental context, but that the direction of the responses can differ for different components of plant performance (growth vs. defense).

KEYWORDS

arbuscular mycorrhizal fungi, P fertilization, water addition, *Funneliformis mosseae*, nursery shrub species

Introduction

Nearly 90% of terrestrial plants are able to form a symbiosis with arbuscular mycorrhizal fungi (AMF), which is considered evolutionarily important for plants to cope with many environmental challenges (Begum et al., 2019). In exchange for providing photosynthetic carbon in the form of lipids and sugars to AMF, plants obtain water and nutrients such as phosphorus, nitrogen and micronutrients from the fungus, that can utilize its complex hyphal network to forage for these limiting resources beyond the root zone (Smith and Read, 2008). Symbiotic associations between plants and AMF have been intensively studied, and a wide range of benefits from the symbiosis in terms of plant growth have been reported. These benefits include enhancement of plant uptake of water and nutrients, as well as the promotion of tolerance to environmental stresses like drought, salinity, heavy metal contamination, shade and extreme temperature (Mathur et al., 2018; Evelin et al., 2019; Pasbani et al., 2020; Adeyemi et al., 2021; Begum et al., 2021; Saha et al., 2022). In addition to enhancing plant tolerance to abiotic challenges, AMF are also identified to play important roles in protecting plants against many types of biotic stresses, e.g. pathogen infection and herbivore feeding (Koricheva et al., 2009; Bonfante and Genre, 2010). The mitigation of effects of biotic stress by AMF operates *via* a large set of induced or primed morphological, physiological and biochemical changes in response to AMF colonization, including alterations in plant size, phenology, nutrition, palatability, digestibility and toxicity (Qu et al., 2021).

Drought is among the most frequent and devastating stresses plants experience globally (IPCC 2018), and its associated soil desiccation often causes strong plant growth depression by inducing closure of stomata and decreases in CO_2 flux for photosynthesis (Osakabe et al., 2014; Chitarra et al., 2016). Many studies have shown that mycorrhizal plants may utilize AMF hyphal networks as extension of their root systems to scavenge water beyond the root depletion zone, allowing these plants to overcome the drought-induced depression (Ruth et al., 2011; Zou et al., 2017; Mickan et al., 2021). For example, AMF inoculation to strawberry plants under drought was shown to successfully restore plant growth to similar or higher levels compared to well-watered non-mycorrhizal plants (Boyer et al., 2015). Similar results were found in tobacco seedlings where AMF inoculation significantly decreased negative effects of drought stress and accordingly increased plant growth (Liu et al., 2021). Such AMF-mediated enhancement of plant drought tolerance can be either attributed to higher water uptake efficiency as a result of AMF extra-radical mycelia, or due to AMF-activated plant adaptation to drought in the form of multiple functional traits (Nath et al., 2016; Ruiz-Lozano et al., 2016; Liu et al., 2018; Wu et al., 2019; Zou et al., 2021).

In addition to providing drought tolerance, AMF are well known to assist the plants in the uptake of nutrients, in particular phosphorus, nitrogen and specific micronutrients (reviewed in Bucking and Kafle, 2015; Xie et al., 2022). Inorganic phosphorus (Pi) is a key nutrient that is essential for various plant functions but not easily accessible to plants due to its low solubility and mobility in soil. Many plants cannot absorb sufficient Pi for structural or metabolic use *via* their own root system (direct pathway) and partly rely on uptake of Pi through the AMF hyphal network (the mycorrhizal pathway) (Ferrol et al., 2019). Although AMF symbiosis has traditionally been considered as a mutualistic interaction, the outcome of the association for the plant can range from mutualism to antagonism, depending on environmental conditions (Smith et al., 2010). AMF-induced plant growth depressions have been reported in environments where costs of the association outweigh the benefits for plants, e.g. under low light conditions (Konvalinková and Jansa, 2016) and in P rich systems (Johnson et al., 2015). For instance, Schroeder and Janos (2005) showed that root colonization and growth benefit of red pepper and acorn squash from AMF was strongly reduced with increasing amounts of soil P. Similar results were found in studies of Johnson et al. (2015) who showed that mutualism between *Andropogon gerardii* and AMF only occurred in P-deficient soil. The mechanisms underlying antagonistic plant-AMF associations are still in debate (Johnson, 2010; Jin et al., 2017; Řezáčová et al., 2017; Zhao et al., 2019; Kaur et al., 2022). Traditionally, failures to observe positive mycorrhizal growth responses have been explained as cases in which the net costs of the symbiosis in terms of fungal carbon use outweigh the net benefits in terms of P delivery by the fungus to the plant *via* the AM pathway (imbalanced C-P trade). However, observations of negative mycorrhizal growth responses even when P-transfer was highly functional, have shifted this paradigm. AMF symbiosis commonly results in a repression of the plant's own (direct) P-uptake pathway. Negative mycorrhizal growth responses are currently thought to arise from a reduction in P delivery *via* the plant's direct pathway that is insufficiently compensated by P-uptake *via* the AM pathway (Smith et al., 2009).

Moreover, recent studies have shown that not only plant growth benefits but also plant defense benefits from AMF partners depend on environmental conditions (Bernaola and Stout, 2019; Diaz et al., 2021, Qu et al., 2021). AMF can prime plants, i.e., sensitize their immune system for stronger or faster responses to upcoming herbivores

(Pozo and Azcon-Aguilar, 2007; Jung et al., 2012; Song et al., 2013), or induce higher levels of plant defensive metabolites that reduce the damage or population size of concurrent or later arriving herbivores (Vannette et al., 2013; Wang et al., 2015; Sharma et al., 2017; Meier and Hunter, 2018; Kaur and Suseela, 2020). Other studies have shown that mycorrhizal plants tend to have enhanced tolerance to herbivory (Dowarah et al., 2022) or are better at recruiting beneficial organisms (Ujvari et al., 2021). Regardless of the underlying mechanisms, the occurrence and strength of AMF effects on plant defense is often affected by the environment in which the host plants and AMF interact, leading to difficulties in application of mycorrhizal inoculation to consistently control pests in field (Delavaux et al., 2017; Bernaola and Stout, 2019). The availability of soil phosphorus and water are two major environmental factors that have individually been shown to determine the outcome of AMF-induced defense (Irankhah et al., 2020; Qu et al., 2021). However, their interactive effects on growth and defense, i.e., whether the effect of one factor depends on the presence of the other, are largely unknown.

In the current study, we manipulated soil water and nutrient content to examine how they individually and interactively affect the effects of AMF on growth and herbivore abundance of a dominant nursery shrub species *Artemisia ordosica* that dominates in semiarid region NW China. We hypothesize that (1) plants have overall enhanced growth and reduced herbivore abundance when roots are colonized by AMF, and these effects are stronger at relatively low levels of soil nutrients and water; (2) the impact of soil nutrient level on AMF benefits in terms of growth and lower herbivore abundance depends on soil water content, and *vice versa*.

Materials and methods

Plant, soil and AMF species

Artemisia ordosica Krosch. (Asteraceae) is a woody species that is widely distributed in the fixed and semi-fixed sand dunes of northwestern China. This species is a deciduous, multi-stemmed, dwarf shrub that has plumose, linearly lobate leaves and a branch system that consists of old brown branches and purple current-year twigs near the soil surface (She et al., 2017). *A. ordosica* has a deep taproot system that can reach a depth of 1–3 m, but its lateral roots are mainly distributed in the upper soil layer of 0–30 cm, and limited to a range of 0.4 m in diameter from the trunk (Zhang et al., 2008). *A. ordosica* is a typical shrub species in Mu Us desert, China and is widely used as a nursery plant species for soil restoration in this region (Li et al., 2011). The leaves of this species often start to expand in early April and the shoot biomass reaches its maximum in July, followed by a flowering season from August to late September and leaf abscission in mid-November. The species is wind dispersed by tiny light seeds over distances up to several miles. In our study, seeds of *A. ordosica* were collected from wild individuals at Shapotou Desert Experimental Station of the Chinese Academy Sciences, Ningxia Province China (104°43'8"E, 37°26'28"N) in October 2016, where the annual mean precipitation is 191 mm and the annual average temperature is 10.0°C. The collected seeds were kept dry in the dark at room temperature until use.

Soils were collected from a semi-arid restoration field near the Shapotou research station, where *A. ordosica* is the dominant species. On average, soils from this field have a water content of 1.1%, a C/N ratio of 17.0, and contain 0.28 g/kg total N and P, and 5.31 mg/kg available N and 1.95 mg/kg available P. A soil core borer of 5 cm in diameter was used to collect a total of ca. 600 kg of soil from the 0-15 cm layer. After being collected, soils were fully mixed and immediately sieved through a 5 mm mesh and sterilized in an autoclave. A thorough sterilization of soil often requires that the standard mode of autoclaving (121°C and 100 Kpa for 1 hour) is repeated two or three times. To minimize the time for sterilization yet ensuring thorough sterilization, we instead used a slightly stronger mode of autoclaving at 130°C and 200 Kpa for 15 min. Heat sterilization of soil can change soil physico-chemical properties including increases in pH, electrical conductivity, release of macronutrients, and changes in soil organic matter such as increases in the levels of humic acids. These changes may have affected plant interactions with AMF. For instance, increased release of phosphorus may reduce AMF colonization whereas higher levels of humic acids can often be well combined with successful establishment and growth promoting effects of AMF (Nobre et al., 2013; Cozzolino et al., 2016). Sterilized soils were stored at room temperature until use. *Funneliformis mosseae* was used as the source of AM fungal inoculum in this study. This species forms symbioses with a wide range of plant species, including *A. ordosica* and was identified as one of the dominant AMF species in the rhizosphere of *A. ordosica* (Qian and He, 2009). *F. mosseae*-BGC NM04A was purchased from the Institute of Plant Nutrition and Resource in Beijing Academy of Agriculture and Forestry Sciences, Beijing, China. The strain was originally collected from another semi-arid field site at Ejin Horo Banner, Inner Mongonia, China, ca. 400 km away from the experimental field, with a mean annual precipitation of 346 mm and average annual temperature of 6.3°C.

Experimental design

In total, 128 pots were prepared and each pot was filled with 4 kg sterilized soil. The pots were assigned to eight treatments representing a full-factorial combination of two mycorrhizal treatments (M+/-), two water treatments (W+/-) and two fertilization treatments (F+/-). Each treatment had 16 biological replicates (2M × 2W × 2F × 16 replicates = 128 pots). To create the fertilization treatment (F+), half of randomly selected pots were individually fertilized with 0.0966 g granules of $(NH4)_2HPO_4$ and thus ca. 23 mg phosphate and 21 mg nitrogen was added to each pot. The other half of the pots did not receive the fertilizer and was used as the non-fertilized treatment (F-). Therefore, following the fertilization treatment there was 10.56 mg/kg available N and 7.70 mg/kg available P in F+ soils, and 5.31 mg/kg available N and 1.95 mg/kg available P in F- soils. Tap water was used to create water treatments: half of the fertilized and non-fertilized pots individually received tap water every other day, adjusting the soil water content to 4.5% (W+). The water content of the other half of the pots was adjusted to 1.5% (W-). The water contents (4.5% vs. 1.5%) were calculated from the estimated highest and lowest percentage of water retention capacity of the soil in our experimental region within a typical growing season. The amount of added water was calculated by weighing the

experimental pot and estimating plant weight from a modeled plant growth curve. The mycorrhizal treatment (M+) was created by adding fifteen grams of vital *Funneliformis mosseae* inoculum contained in granules consisting of hyphae, spores and substrate to half pots of each above-mentioned water or phosphate treatment prior to seedling transplantation. The other half of the pots received sterilized inocula to create the non-mycorrhizal treatment (M-).

Greenhouse bioassays

Seeds of *A. ordosica* were surface-sterilized using 0.1% $KMnO_4$ for 30 min and rinsed twice in distilled water. Sterilized seeds were air-dried and germinated in commercial culture soils (Green Energy Inc., Wuzhong, China) at 20°C and a 16 h photoperiod for 6 weeks. Similar-sized seedlings were selected and individually transplanted into plastic pots (diameter 25cm, 16.5 cm height). All pots were grouped into 16 blocks and each block consists of one replicate from each of the eight treatment combinations. All the pots were fully watered with distilled water in the early 11 days after transplantation to ensure survival of seedlings, and dead plants were immediately replaced. After this early period when all seedlings successfully survived, plants started to receive the different watering regimes to establish the watering treatments (W+/-). Following the watering treatments, height of all plants was measured every two weeks for the next 90 days. Plants were grown in a greenhouse with a 16 h photoperiod. Natural daylight was automatically supplemented with light from 400-W metal halide lamps to keep the light intensity at ca. 2000 μmol $m^{-1}s^{-1}$ photon flux density. After these measurements, pots from block 1-8 were transplanted to the field to assess herbivore abundance. Those from the other blocks (block 9-16) were harvested to measure plant traits (see below).

Field bioassay

The pots from block 1-8 were transferred to an undisturbed field site where *A. ordosica* populations were naturally distributed. Pots were dug into the soil with their tops leveling the soil surface and arranged in blocks at least 20 m apart according to their original block identity, with a distance of approximately 80 cm between two pots. Plants received no further fertilization or watering treatment and were naturally exposed to herbivorous insects for 4 days. After this period, plants were individually investigated to examine their colonization by herbivorous insects. Adults of the chrysomelid beetle *Chrysolina aeruginosa* Fald (Coleoptera: Chrysomelidae) showed high colonization whereas no other herbivorous insect species were found. Therefore, the numbers of *C. aeruginosa* were recorded as a measure of herbivore abundance.

Measurements of plant functional traits

Plant shoot biomass - During the harvest of the greenhouse plants, each plant was separated into roots, stems and leaves. Plant leaves and stems were separately collected and oven dried at 70°C for 72 h to determine their dry weights. Plant roots were gently washed to remove soil particles and treated as described below.

Root architecture - Intact fresh roots of each plant were individually scanned using an Epson Perfection 4990 Photo scanner. The obtained photos were analyzed with WinRHIZO software (Regent Instruments Inc., Quebec, Canada) to estimate specific root length (SRL), root surface area (RSA), total root volume (TRV) and average root diameter (ARD). Specific root length (SRL) was calculated as total root length divided by total root biomass of an individual plant.

Root biomass - A subset of lateral roots was randomly selected from each plant. After recording their fresh weight, they were stored in 70% alcohol to determine root colonization by *F. mosseae* (see below). For the remaining roots of each plant, we measured both fresh weight and dry weight after oven drying at 70°C for 72 h. The dry-to-fresh weight ratio of these remaining roots was used to calculate the dry weight of the corresponding root subsample that was used for determining AMF colonization of the corresponding plant.

Mycorrhizal infection - Root colonization by *F. mosseae* was quantified using a gridline intersection method (Bierman and Linderman, 1981). Briefly, each stored fresh root subsample was cut into at least 100 segments of 0.5-1 cm in length. These root segments were cleared in 10% KOH for 10 min at 95°C, and stained using vinegar (5% acetic acid) and 5% black ink (Hero 440, Shanghai, China) for 8 min at 80–90°C. Stained roots were mounted on slides and checked for mycorrhizal structures (arbuscules, vesicles, spores and intercellular hyphae) under a compound microscope (BH-2; Olympus, Tokyo, Japan) at ×40 magnification. Presence of any of these structures was scored at 100 grid intersections, and mycorrhizal colonization rate of an individual plant was quantified as the percentage of intersections with mycorrhizal structures present (Wang et al., 2015).

Element analyses - Dried plant tissues, viz. leaves, stems and roots, were separately ground to a powder and 1 mg of the powder was weighed into tin capsules. The total nitrogen (N) content was measured using an elemental analyzer (Vario EL III, Elementar, Germany). Tissue samples were acid digested using a mixed solution of H_2SO_4 and H_2O_2 in a microwave oven, which continued until the samples were fully dissolved in the solution. The phosphorus (P) content in plant tissues was determined by ICP-OES (Optima 8300, PerkinElmer, USA).

Photosynthetic pigment - Leaf chlorophyll a (Chl-a), chlorophyll b (Chl-b) and carotenoid (Car) contents were determined according to the method described in Lichtenthaler and Welburn (1983) using 80% acetone as extraction solution. The Chl-a, Chl-b and Car contents were measured by spectrophotometry at wavelengths of 663, 645 and 480 nm, respectively (T60U, PG Instruments Ltd, Japan). Leaf proline content was measured using the acidninhydrine method described in Bates (1973). In brief, 0.25 g fresh leaf sample was weighed and homogenized in 5 ml 3% sulfosalicylic acid at 100°C for 10 min before filtration. L-Proline was used as a standard for the colorimetric determination of the filtrated solution at a wavelength of 520 nm.

Statistical analyses

Plant functional traits, including plant biomass, root traits, and leaf N and P concentrations were analyzed using a general linear

mixed model (GLMM) with a maximum-likelihood (ML) iterative algorithm in R version 3.6.1. In this model, AMF inoculation (M+/-), fertilization (F+/-) and water (W+/-) additions as well as their interactions were added as fixed factors, and the identity of the block to which the plants were assigned was added as a random factor. The model was run using the *lmer* function in the "lme4" package (Bates et al., 2015), and the significance of the test was estimated using the *anova* function in the "lmerTest" package (Kuznetsova et al., 2017). In the above models, data on leaf biomass and leaf and stem P content were log-transformed, and data on N content, root P content and SRL were square root-transformed prior to the analyses to meet assumptions of normality of the residual distribution and homogeneity of variances in data.

A repeated measures ANOVA (Zar, 1999) was used to analyze data on plant height, in which the treatments M, F and W were included as fixed factors and plant identity as a repeated subject. Data on insect abundance were analyzed using a generalized linear mixed model, following a "poisson" distribution, where M, F and W as well as their interactions were included as fixed factors, and block and plant height at the time of exposure to insect as random factors. A similar model was run without plant height as a random factor. The models were run using the *glm* function and the significance of the tests was estimated using the *Anova* function in the "car" package (Fox and Weisberg, 2019). All analyses were performed using R version 4.2.1 (R Core Development Team, 2022).

Results

AMF colonization

Overall, AMF colonization was relatively low in mycorrhizal plants (ca. 5% on average). Surprisingly, roots of plants from the non-mycorrhizal treatments were also colonized, but their colonization rate was significantly lower than that of plants from the mycorrhizal treatments ($F = 4.85$, $p = 0.036$, Figure S1). Root colonization by AMF was not affected by fertilization and water supply, nor by their interaction (all $p > 0.20$).

Plant growth

Plant height – Plant height at the harvest was significantly enhanced by both additional water supply (+10.0%) and by additional fertilizer supply (+4.5%), and their effects acted synergistically (+20.8%) (Table 1, Figure 1, W × P interaction, $p < 0.01$). Surprisingly, plant height was unaffected by AMF inoculation when plants received additional water, but was reduced by AMF when additional water was not supplied (Table 1, Figure 1, M × W interaction, $p < 0.001$). The reduction in plant height by AMF under low water conditions tended to be stronger in plants that also did not receive fertilization (-33.5%) than in plants that received the fertilizer treatment (-10.6%).

Plant biomass – Like plant height, total plant biomass was significantly enhanced by additional water (+38.0%), and fertilizer (9.0%) supply, and their joint effects were strongly synergistic (+234%, Figure 2D, Table 1, W × F interaction, $p < 0.001$). The same was observed for individual leaf, stem and root biomass (Table 1, Figures 2A–C). Effects of AMF depended on water conditions. Overall, inoculation with AMF reduced leaf, stem and total biomass, but this effect was only observed in plants that did not receive additional water supply (Table 1, M × W, $p < 0.05$, Figures 2A, B, D). Notably, when mycorrhizal effects were tested separately under each of the four environmental conditions, biomass reductions were only observed for plant stems under low fertilizer and soil phosphorus conditions (paired t-test, $t=5.32$, $p < 0.05$). Contrary to expectation, the benefits of AMF inoculation were thus not more pronounced at lower soil water and nutrient conditions. Root biomass was not influenced by inoculation with AMF (Table 1, Figure 2C).

Plant functional traits

Photosynthetic and physiological traits – Leaf chlorophyll b, carotenoid, and proline contents were predominantly affected by water treatment. On average, proline content was higher but chlorophyll b and carotenoid contents were lower in leaves of plants that received additional water, whereas leaf chlorophyll a

TABLE 1 Effects of fertilization (F), water addition (W) and inoculation with arbuscular mycorrhizal fungi (M) on plant height (repeated measures ANOVA) and on plant biomass, root traits and photosynthetic traits (General Linear Mixed Models, GLMM) of *Artemisia ordosica* plants grown in the greenhouse.

Source	*df*	Plant growth traits					Photosynthesis traits				Root traits			
		Height	Leaf	Stem	Root	Total	Chl-a	Chl-b	Car	Proline	SRL	ARD	RSA	TRV
M	1	**5.60***	**15.0*****	**10.0****	1.11	**4.48***	0.06	0.12	0.05	3.38	**4.47***	2.72	0.05	0.43
F	1	**54.4*****	**146.3*****	**119.0*****	**14.0****	**60.0*****	0.26	0.88	0.00	3.36	**60.0*****	**21.2*****	0.86	2.94
W	1	**117.1*****	**157.8*****	**237.0*****	**94.6*****	**162.7*****	2.08	**15.8*****	**8.84****	**8.84****	**162.7*****	**40.3*****	**40.2*****	**53.1*****
M×F	1	1.95	3.95	**8.79****	2.03	**4.56***	0.57	2.34	1.50	0.86	**4.56***	**6.63***	0.02	0.41
M×W	1	**12.7*****	**6.67***	**14.6*****	1.03	2.68	2.07	0.06	1.15	3.39	2.68	**10.2****	0.13	1.03
F×W	1	**7.26****	**6.07***	**32.3*****	**16.6*****	**22.0*****	0.62	**4.47***	3.12	0.60	**22.0*****	**30.8*****	**8.23****	**14.0****
M×F×W	1	2.94^{+}	0.00	1.12	0.36	0.36	0.09	0.00	0.25	**6.23***	0.36	0.63	0.00	0.07

F-values are shown in the table and those in bold indicate significant treatment effects ($P < 0.05$). $^{+}P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; df, numerator degrees of freedom; Chl-a, chlorophyll a; Chl-b, chlorophyll b; Car, carotenoid; TRV, Total root volume; RSA, root surface area; SRL, specific root length; ARD, average root diameter. There were 16 replicates in each of AMF, P, and W treatments for plant height trait, 8 replicates for plant biomass traits, and 4 replicates for root and photosynthetic traits. Plant height was repeatedly measured over time; effects of time and its interactions with P, W and AMF are not presented in the table.

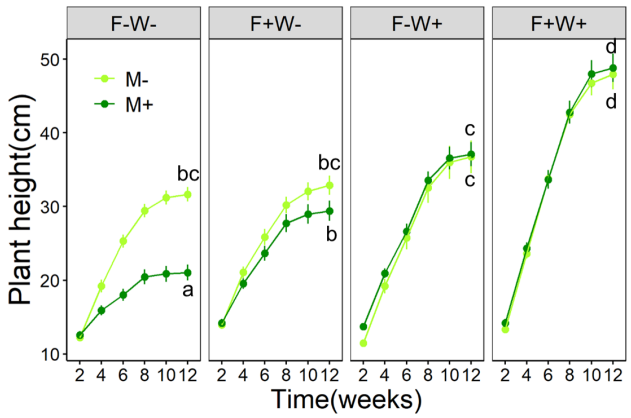

FIGURE 1
Development of plant height over time of *Artemisia ordosica* plants that were inoculated with the AMF species *Funneliformis mosseae* (M+) or not (M-) and that were grown under four different combinations of fertilization (F-: no fertilization; F+: fertilization) and water addition (W-: low soil water; W+: high soil water) treatments in a greenhouse. Light symbols: plants without AMF; dark symbols: plants with AMF. Data at the last time point (Week 12) were separately analyzed across all treatments in plot facets and the scatterplot with identical letters are not significantly different based on Tukey's *post hoc* test. Statistics are shown in Table 1.

content was not affected by water treatment (Table 1, all $p<0.01$, Figures 3A–D). The reduction in leaf chlorophyll b content by additional water occurred only in plants that also received fertilization (Table 1, F × W, $p<0.05$, Figure 3B). When additional fertilizer was supplied, leaf proline content was enhanced by AMF under low water conditions, but reduced by AMF under high water conditions (AMF × F × W interaction, $P < 0.05$, Table 1, Figure 3D).

Root traits – Among the measured plant root traits, SRL was decreased but ARD was increased by fertilization (Table 1, Figures 4A, B). All measured root traits were significantly affected by water addition. But whereas water addition reduced SRL, it enhanced ARD, RSA and TRV. Effects of water addition were stronger in plants that were not fertilized than in fertilized plants (W × F interactions, all $p < 0.01$, Table 1, Figures 4A–D). SRL was enhanced by AMF, but this only occurred when plants did not receive fertilizer supply (Table 1, Figure 4A). The other root traits, including ARD, RSA and TRV were not affected by inoculation with AMF (Table 1, Figures 4A–D) except for ARD that was reduced by mycorrhizal inoculation under low fertilization and water conditions but enhanced by inoculation under high fertilization and water conditions (Table 1, Figure 4B).

Leaf nutritional traits – Concentrations of nitrogen in leaves, stems and roots were strongly enhanced by soil fertilization but

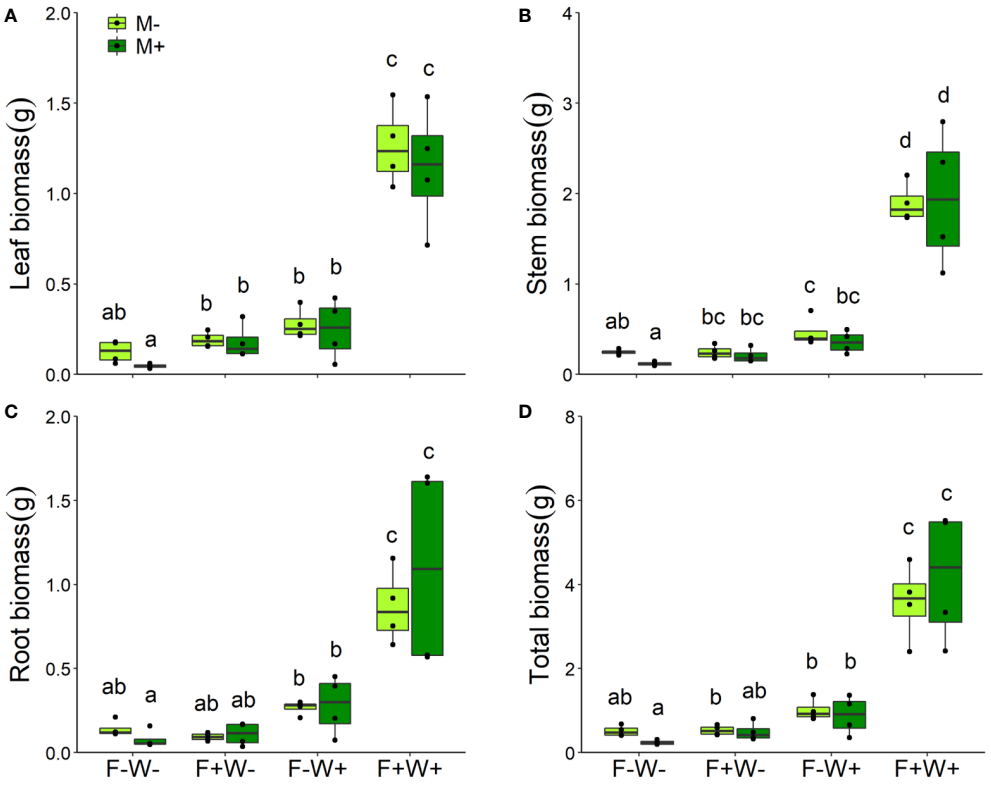

FIGURE 2
Leaf **(A)**, stem **(B)**, root **(C)** biomass and total mass **(D)** of *Artemisia ordosica* plants inoculated with AMF species *Funneliformis mosseae* (M+) or not (M-) and grown under four different combinations of fertilization (F-: no fertilization; F+: fertilization) and water addition (W-: low soil water; W+: high soil water) treatments. Light symbols: plants received no AMF; dark symbols: plants inoculated with AMF. The boxplot shows the 25% and 75% quartiles, the median, whiskers (1.5 times the interquartile range) and the outliers of the samples, and the points in the plot area represent different replicates of the corresponding treatment. Boxplots sharing one or more identical letters are not significantly different based on Tukey's *post hoc* test. Statistics are shown in Table 1.

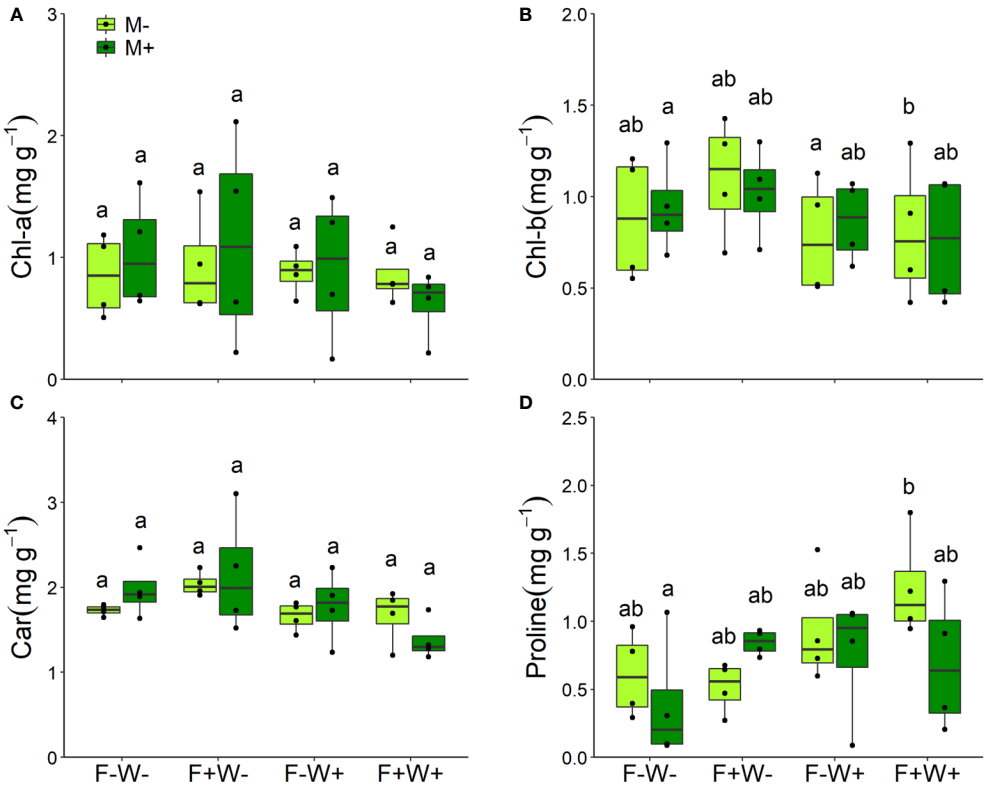

FIGURE 3
Contents of chlorophyll a (Chl-a, **A**), chlorophyll b (Chl-b, **B**), carotenoids **(C)** and proline **(D)** in the leaves of *Artemisia ordosica* plants that were inoculated with the AM fungal species *Funneliformis mosseae* (M+) or not (M-) and grown under four different combinations of fertilization (F-: no fertilization; F+: fertilization) and water addition (W-: low soil water; W+: high soil water) treatments. Light symbols: plants received no AMF; dark symbols: plants inoculated with AMF. The boxplot shows the 25% and 75% quartiles, the median, whiskers (1.5 times the interquartile range) and the outliers of the samples, and the points in the plot area represent different replicates of the corresponding treatment. Boxplots sharing one or more identical letters are not significantly different based on Tukey's *post hoc* test. Statistics are shown in Table 1.

reduced by water addition (Table 2, Figures 5A–C). Especially in roots, effects of fertilization on tissue N concentrations were stronger under low water conditions than under additional water supply (W × F interaction, $p < 0.05$, Table 2, Figures 5A–C). For the concentrations of phosphorus in leaves, stems and roots a similar pattern was observed. These were significantly enhanced by fertilization (Table 2, Figures 5D–F), but in leaves and roots this effect was more strongly observed if plants did not receive additional water (W × F interactions, $p < 0.05$, Table 2, Figures 5D, F). Surprisingly, leaf P concentration was overall significantly reduced by AMF ($p < 0.01$, Table 2, Figure 5D). Although the interaction between AMF and fertilizer treatment was not significant, the suppressive effect of AMF on leaf P tended to be stronger under low fertilization conditions (F-W-: t-test, $t_{[1, 3]} = 4.89$, $p < 0.05$; F-W+: t-test, $t_{[1, 3]} = 2.42$, $p < 0.10$) than under high fertilizer conditions (F+W- and F+W+: both $p > 0.6$).

Herbivore abundance on plants transferred to the field

Abundance of the herbivore *C. aeruginosa* was significantly lower on plants inoculated by AMF than on non-mycorrhizal plants, but this effect was only significant and stronger on non-fertilized plants (mean ± s.e. in insect abundance; AMF: 0.19 ± 0.14; non-AMF: 1.31 ± 0.38; $t_{[1, 15]} = 2.70$, $p < 0.05$) than on fertilized plants (AMF: 1.56 ± 0.57; non-AMF: 2.47 ± 0.87; $t_{[1, 15]} = 1.10$, $p = 0.29$), resulting in a significant two-way interactions between AMF and fertilization treatment (Table 2, M × F: $\chi^2 = 4.00$, $p = 0.045$, Figure 6). In contrast, herbivore abundance was overall higher on plants that had been fertilized, but only when these plants had also received additional watering (Table 2, F × W: $\chi^2 = 9.44$, $p = 0.002$, Figure 6). These treatment effects were not mediated by plant height (a proxy of plant size) at the time of measurement since inclusion of plant height as a covariate in the analyses ($\chi^2 = 0.72$, $p = 0.397$) did not alter the significance of the effects of AMF, fertilizer and watering treatments.

Discussion

Our study examined the role of arbuscular mycorrhizal fungi in modifying plant traits related to growth, physiology and defense, in particular to what extent the role of AMF depends on soil water and phosphorus supply. We found that AMF inoculation repressed plant growth especially under low soil water and nutrient conditions. On the other hand, AMF tended to reduce the incidence of an important herbivorous insect under low soil fertilization conditions and thus alleviate potential damage under adverse environmental conditions, which could have been mediated by the lower leaf P content of AMF-inoculated plants.

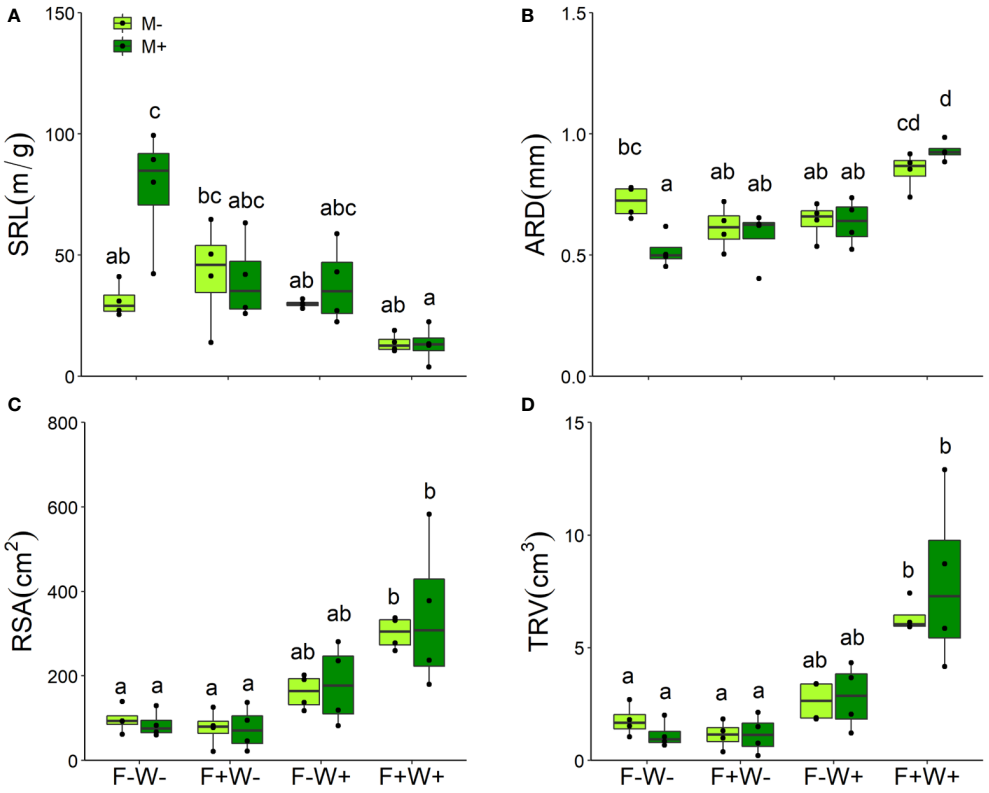

FIGURE 4
Specific root length (SRL, **A**), averaged root diameter (ARD, **B**), root surface area (RSA, **C**), and total root volume (TRV, **D**) of *Artemisia ordosica* plants that were inoculated with the AM fungal species *Funneliformis mosseae* (M+) or not (M-) and grown under four different combinations of fertilization (F-: no fertilization; F+: fertilization) and water addition (W-: low soil water; W+: high soil water) treatments. Light symbols: plants received no AMF; dark symbols: plants inoculated with AMF. The boxplot shows the 25% and 75% quartiles, the median, whiskers (1.5 times the interquartile range) and the outliers of the samples, and the points in the plot area represent different replicates of the corresponding treatment. Boxplots sharing one or more identical letters are not significantly different based on Tukey's *post hoc* test. Statistics are shown in Table 2.

Effects of AMF, soil water and soil P on plant growth

Recent years witness an increasing number of studies recognizing that the outcome of plant-AMF interactions often show a continuum ranging from mutualism to parasitism, depending on the context in which the interactions occur (Hoeksema et al., 2010; Johnson et al., 2015; Jin et al., 2017; Qu et al., 2021). However, these results were mostly obtained from studies using short-lived herbaceous plant species and commercial AMF strains as a model system, and it is yet unclear whether these results apply to plants of different life forms. In the current study, a shrub species, *A. ordosica* was used to measure

TABLE 2 General Linear Mixed Models (GLMM) of the effects of inoculation with arbuscular mycorrhizal fungi (M), fertilization (F) and water addition (W) on plant nitrogen (N) and phosphorus (P) concentrations in leaves, stems and roots of *Artemisia ordosica* plants grown in the greenhouse, as well as generalized linear mixed model ("poisson" distribution) of the effects of M, F, and W treatments on the abundance of insect herbivore *Chrysolina aeruginosa* colonizing each plant in the field.

Source	df	N content			P content			No. *C. aeruginosa*
		Leaf	Stem	Root	Leaf	Stem	Root	
M	1	1.01	1.43	1.64	**8.02****	0.15	0.02	**11.0*****
F	1	**15.4*****	**44.4*****	**6.88***	**389.4*****	**34.8*****	**12.5****	**9.37****
W	1	**11.9****	**39.4*****	**12.6****	**9.35****	0.08	**5.10***	1.29
M×F	1	1.26	1.51	0.83	3.25	2.35	0.87	**4.00***
M×W	1	1.58	**6.60***	0.38	0.08	0.61	0.22	0.61
F×W	1	3.14	3.63	**5.33***	**6.60***	1.36	**5.24***	**9.44****
M×P×W	1	1.92	0.0.33	0.30	0.03	0.03	1.51	0.26

F values for N and P content, and χ^2 values for herbivore abundance are shown in the table and those in bold indicate a significant treatment effect ($P < 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; df, numerator degrees of freedom. There were 4 replicates for data on plant N and P contents, and 8 replicates for data on number of *C. aeruginosa* in each of AMF, F, and W treatments.

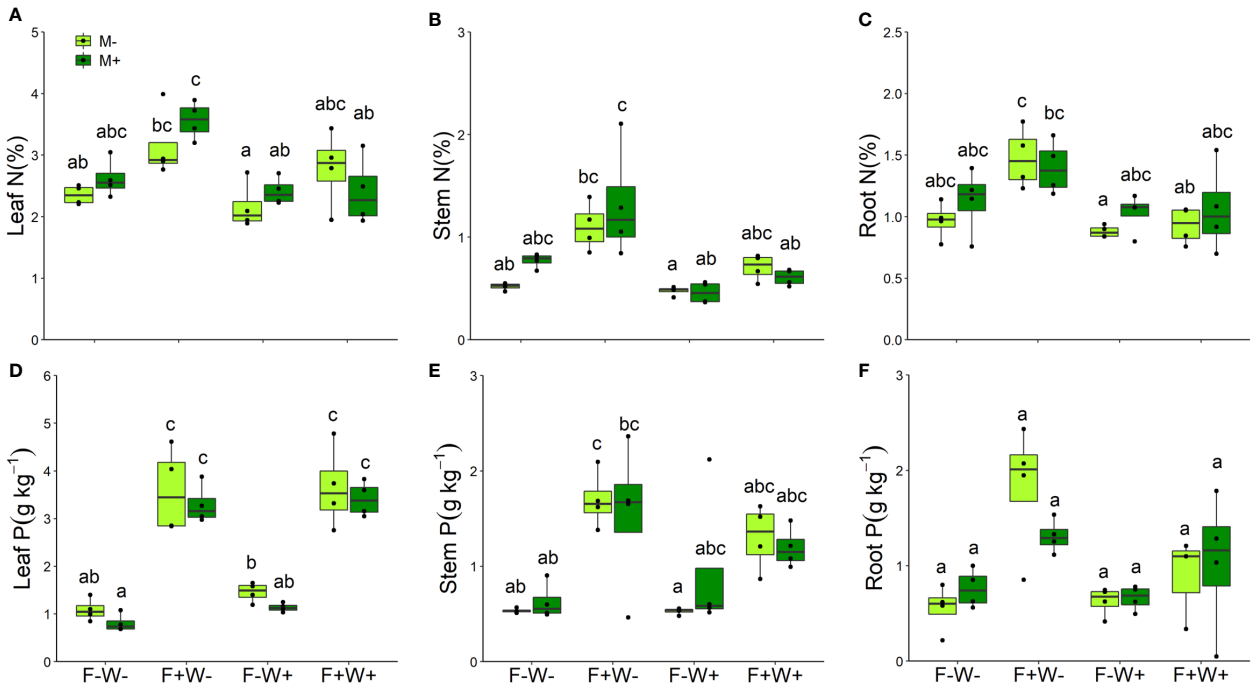

FIGURE 5
Nitrogen **(A–C)** and phosphorus **(D–F)** concentrations of different plant tissues of *Artemisia ordosica* that were inoculated with the AM fungal species *Funneliformis mosseae* (M+) or not (M-) and grown under four different combination of fertilization (F-: no fertilization; F+: fertilization) and water addition (W-: low soil water; W+: high soil water) treatments. Light symbols: plants received no AMF; dark symbols: plants inoculated with AMF *Funneliformis mosseae*. The boxplot shows the 25% and 75% quartiles, the median, whiskers (1.5 times the interquartile range) and the outliers of the samples, and the points in the plot area represent different replicates of the corresponding treatment. Boxplots sharing one or more identical letters are not significantly different based on Tukey's *post hoc* test. Statistics are shown in Table 2.

its responses to AMF under contrasting soil water and soil fertilization conditions. Our results show that inoculation of *A. ordosica* with a strain of the AM fungus *F. mosseae* that was isolated from a comparable sandy, low precipitation habitat as the collection site of the host plant seeds, overall reduced plant height, and the reduction consistently increased over time as plants grew. This result may relate to the overall low root colonization (5% on average) by the mycorrhizal strain, indicating a potentially limited opportunity for beneficial C-P trade in this symbiotic combination (Graham and Abbott, 2000). Such low colonization rate may result in low transfer of water and nutrients to the plant. If, even at low levels of colonization, the AMF would still suppress the plant's direct pathway of P uptake through its own root system, this cost could outweigh the benefits of the indirect pathway provided by mycorrhizal extra-radical mycelia (Smith et al., 2011) and jeopardize the beneficial C-P trade traditionally hypothesized for mycorrhizal symbiosis (Grace et al., 2009; Johnson, 2010). The reason for the overall low root colonization of *A. ordosica* in our experiment is unknown, but low colonization rates of *F. mosseae* have been observed in other plant species as well. For instance, average colonization percentages of a single *F. mosseae* strain varied between 2.6 and 27.0% across a range of tomato cultivars (Steinkellner et al., 2012). Interestingly, the cultivar with the lowest colonization percentage nevertheless showed a significant 30% increase in root dry weight in response to inoculation, whereas the root weight of the cultivar with the highest colonization percentage was not affected by *F. mosseae*. This indicates that, whatever the reason for low colonization is, even low percentages of colonization can significantly affect plant performance, and that the magnitude of AMF effects is not necessarily related to percentage of colonization. It should be noted that despite the low colonization in M+ plants and the unexpected presence of colonization in M- plants, possibly due to

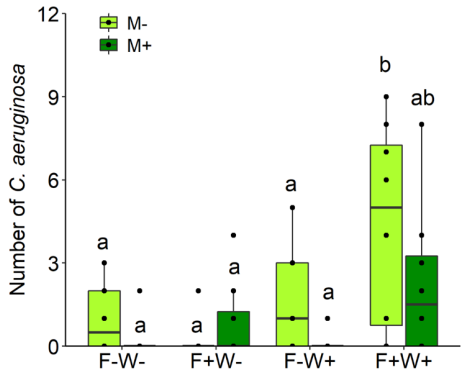

FIGURE 6
Abundance of the insect species *Chrysolina aeruginosa* on the aerial tissues of *Artemisia ordosica* that were inoculated with the AM fungal species *Funneliformis mosseae* (AMF+) or not (AMF-) and grown under four different combination of fertilization (F-: no fertilization; F+: fertilization) and water addition (W-: low soil water; W+: high soil water) treatments. Light symbols: plants received no AMF; dark symbols: plants inoculated with AMF *Funneliformis mosseae*. The boxplot shows the 25% and 75% quartiles, the median, whiskers (1.5 times the interquartile range) and the outliers of the samples, and the points in the plot area represent different replicates of the corresponding treatment. Boxplots sharing one or more identical letters are not significantly different based on Tukey's *post hoc* test. Statistics are shown in Table 2.

cross-contamination, AMF colonization percentages were still significantly higher in M+ plants than in M- plants, indicating that the validity of AMF treatments in our study was not compromised.

Given that *F. mosseae* was one of the dominant AMF species in the study area and that the *F. mosseae* isolate used in the current study originated from a region similar to the region where the plant species *A. ordosica* is commonly found, potential incompatibility between the two partners due to an ecological mismatch should not be a reason for the observed mycorrhizal growth depression (Jin et al., 2017; Řezáčová et al., 2017). Surprisingly, the mycorrhizal plant growth reduction was most strongly observed under low soil water and phosphorus conditions. This is in contrast with other studies showing that AMF inoculation usually alleviates adverse impacts of drought stress (Duc et al., 2018; Liu et al., 2021; Jongen et al., 2022). The mechanism underlying this drought-induced mycorrhizal plant growth reduction is unknown, but it may relate to the observed interactive negative effects of mycorrhizae and drought on root traits, such as specific root length (SRL) or average root diameter (ARD) (Chen et al., 2016). Plants with higher SRL or lower ARD as observed in the current study tend to have greater plasticity in water and nutrient uptake, but they often show less mycorrhizal dependency, and this may be the reason for the mycorrhizal plant growth depression (Eissenstat, 1992). Even more striking was the observation that the mycorrhizal growth reduction tended to be stronger under non-fertilized than under fertilized conditions. Numerous studies have shown that the mycorrhizal growth response becomes more beneficial for plants at lower nutrient levels (e.g. Vogelsang et al., 2006; Klironomos et al., 2011; Johnson et al., 2015), although exceptions have been reported (Püschel et al., 2016; Raya-Hernandez et al., 2020). Perhaps competition for nutrients between AMF and plants occurred under these experimental conditions, which may have aggravated the AMF-induced plant growth reduction (Li et al., 2008).

The mycorrhizal growth reduction was not only observed for plant height but also for plant biomass. Our results showed that inoculation of AMF significantly reduced plant biomass, and, as observed for plant height, the strength of the AMF-induced reduction in plant biomass production depended on soil water and nutrient conditions. Leaf and stem biomass was more strongly suppressed by AMF inoculation under low water or nutrient supply than under more favorable conditions. In addition to the potential competition between AMF and host plants as previously suggested, an alternative explanation for this observation may be that the AMF strain was not adapted to perform optimally with the host under the low water and nutrient conditions since the site of origin of AMF strain used in this study has a higher mean annual precipitation than the experimental region from which the host plants were collected, mimicked by the low water and nutrient conditions. On the other hand, it is interesting to notice that root biomass was not influenced by AMF inoculation. This result indicates that the plant growth response to AMF inoculation was more sensitive to soil heterogeneity in the shoots than in the roots, suggesting the dependence of AMF-host interactions on environmental conditions may differ among plant functional organs (Roesti et al., 2006; Yang et al., 2016).

Many studies have shown that the photosynthetic capacity of mycorrhizal plants is often higher than that of non-mycorrhizal plants (Liu et al., 2015; Zhang et al., 2018; Balestrini et al., 2020). Such improvements can be incurred by enhanced chlorophyll contents, photosynthetic rate and transpiration rate in plants following AMF colonization (Chandrasekaran et al., 2019). However, in our study we did not observe significant effects of AMF inoculation on the concentration of chlorophyll a, chlorophyll b or carotenoids in *A. ordosica*. This result is in contrast to the results of Zhu et al. (2011) who showed that all these photosynthetic traits were higher in maize colonized by the AMF species *Glomus etunicatum* than in non-mycorrhizal plants. The lack of AMF effects on photosynthesis-related traits in our experiment may be explained by the extremely low AMF colonization rate of *A. ordosica* that might be insufficient to systemically induce changes in the plant's light harvesting capacity (Evelin et al., 2009). AMF inoculation also did not significantly enhance leaf levels of the osmolyte proline. Osmolytes are often synthesized under water stress in order to maintain osmotic balance (Furlan et al., 2020), but this was not observed in our experiment. The reasons for this unexpected result are unknown, but it may be related to the unfavorable growing environment in the greenhouse, e.g. low intensity of light, that can prevent stress-induced plant responses (Lin et al., 2019).

Effects of AMF, soil water and soil P on abundance of colonizing herbivores

In this study, we exposed AMF inoculated and control plants grown under different soil water and nutrient conditions to natural herbivores. The relatively lower abundance of the herbivore species *C. aeruginosa* on mycorrhizal than on non-mycorrhizal plants when grown under low fertilizer conditions suggests a reduced attractiveness of AMF-inoculated plants for this insect species. This result complies with the finding that AMF generally induce resistance to leaf chewing herbivores (Pozo and Azcon-Aguilar, 2007; Koricheva et al., 2009; Jung et al., 2012; Meier and Hunter, 2018; Jiang et al., 2021). However, this effect was only observed in plants grown under low soil P, illustrating the context-dependency of the effects of AMF on plant-herbivore interactions. This observation is in line with other studies. For instance, Wang et al. (2020) showed that only in low-nutrient soils AMF colonization was high but aphid infestation was low. Not only soil P content, but also other environmental factors such as light were reported to influence the effects of mycorrhizal inoculation on plant-insect interactions (Qu et al., 2021). Several mechanisms have been proposed to underlie plant defense responses to AMF inoculation, including priming effects or enhanced production of defensive metabolites (Koricheva et al., 2009). In our study, the observed lower incidence of *C. aeruginosa* on AMF-inoculated plants under low fertilization conditions could have been related to the observed lower P concentrations in mycorrhizal plant leaves under these conditions. Even though AMF incurred only a modest reduction in leaf P under these conditions, overall leaf P levels under these conditions were very low, so that further reductions may have had a significant impact on herbivore preference; lower leaf P concentrations generally represent a lower diet quality for herbivores (Real-Santillan et al., 2019). Interestingly, under higher fertilization levels, the preference of the herbivore for non-mycorrhizal over mycorrhizal plants disappeared. Since the overall levels of leaf

phosphorus were much higher under these conditions, this could indicate that once a threshold P concentration of leaves has been reached, the preference of *C. aeruginosa* is no longer driven by P-demand, but by other stoichiometric resource requirements such as a higher N demand under conditions where P is no longer limiting, or by other factors not significantly affected by mycorrhizal inoculation. Leaf nitrogen is another important leaf trait that affects leaf nutritional quality for herbivores either through provisioning of primary metabolites or through N-based secondary metabolite production, but leaf N in our experiment was not significantly affected by mycorrhizal inoculation. An alternative explanation for the lower abundance of the chrysomelid beetle on AMF-inoculated plants grown under low soil fertilization could be the AMF-induced reduction in leaf and stem biomass under these conditions. However, inclusion of plant height as a covariate in the analysis did not account for any variation in herbivore abundance, so this idea is not supported by our data.

Conclusion

In the current study we surprisingly found that effects of AMF on plant growth do not follow the hypothesized pattern that low availability of soil water and nutrient favor the functionality of host-AMF interactions. Mycorrhizal inoculation caused a growth depression in plant height and biomass especially under drought and low-nutrient conditions. Furthermore, we observed that AMF reduced the abundance of a specialist herbivore on plants grown under low soil fertilizer levels, which might be associated with the lower leaf P concentrations in these plants. We thus conclude that plant responses to AMF inoculation may differ in terms of the traits measured and the types of environmental factors the plants experience.

Data availability statement

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

Author contributions

LQ and AB conceived the idea of this study. LQ, ZW and MG performed the experiments, and MW, LQ and AB analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1101932/full#supplementary-material

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Effects of arbuscular mycorrhizal fungus inoculation on the growth and nitrogen metabolism of *Catalpa bungei* C.A.Mey. under different nitrogen levels

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Evidence suggests that arbuscular mycorrhizal fungi (AMF) may promote the growth of woody plants. However, the effects of AMF on nitrogen (N) metabolism in plants, especially trees, and its regulatory mechanism are rarely reported. Here, the effects of AMF inoculation on the growth and N nutrition status of *Catalpa bungei* under different N levels were reported. Three N levels (low, medium, high) and two mycorrhizal inoculation treatments (inoculation with *Rhizophagus intraradices* or not) were used with factorial design. The results showed that medium N could significantly improve the physiological metabolism and growth of *C. bungei* seedlings. However, when N was excessive, growth was significantly inhibited whether inoculated AMF or not. Compared with non-inoculated treatments, AMF inoculation could promote the absorption of N and P, improve photosynthesis under low to medium N levels, thus promoting the growth of seedlings. AMF changed the biomass allocation in seedlings by reducing the stem mass ratio and root/shoot ratio, and increasing the leaf mass ratio. At medium N levels, compared with non-inoculated treatment, AMF inoculation could significantly promote root growth by changing root hormone levels and improving root architecture and root activity. Under N addition, AMF inoculation could improve the absorption and assimilation of N by regulating the expression of key enzyme genes of N metabolism and nitrate transporter genes (*NRT2.4*, *NRT2.5*, *NRT2.7*) in roots, and enhancing the activities of the key enzyme of N metabolism. This study may provide a reference for the application of AMF in the cultivation and afforestation technology of *C. bungei* in Northwest China.

KEYWORDS

woody plant, *Rhizophagus intraradices*, growth characteristics, physiological performance, nitrogen nutrition, gene expression

1 Introduction

Mycorrhizas are mutualistic symbionts formed by the roots of higher plants and a certain kind of fungi in the soil, among which arbuscular mycorrhizas (AM) are the most widely distributed in nature (Das et al., 2022). Arbuscular mycorrhizal fungi (AMF) are a kind of soil beneficial microorganisms (Govindarajulu et al., 2005; Saia and Jansa, 2022), widely distributed in the forest, grassland, farmland and other ecosystems (Zhao et al., 2014; Zhu et al., 2014). Under natural conditions, AMF can form mycorrhizal symbiosis with more than 80% of plants (Ma et al., 2022). After AMF symbiosis with plants, dense hyphae networks can be formed in rhizosphere soil and root cortex cells, which can expand the effective absorption range of the root system, accelerate the transport of mineral elements and water, promote the absorption of soil mineral elements by hosts, regulate metabolic activities in hosts, and promote plant growth (Yang et al., 2014; Li et al., 2022). In addition, this efficient symbiosis can also help host plants cope with a variety of stress, such as disease (Weng et al., 2022), drought (Yang et al., 2014; Chen et al., 2020), salt (Ma et al., 2022) and heavy metal pollution (Riaz et al., 2021).

Nitrogen (N) is an important component of chlorophyll, and the content of chlorophyll directly affects the photosynthetic products. In addition, N is also an indispensable component of proteins, nucleic acids, enzymes and phytohormones in plants (Evangelina et al., 2015; Wang et al., 2021a). In particular, plant hormones, as important signaling substances, play a key role in plant growth, development and physiological response, and often interact with each other in a dynamic equilibrium state in regulating a certain growth process of plants and the adaptation to adversity (Cui et al., 2020). N metabolism is an important physiological activity in plants, including N absorption, reduction and assimilation, amino acid metabolism and transport, which is regulated by the expression of a variety of genes (Huang et al., 2022; Ren et al., 2022). In plant cells, low- or high-affinity nitrate transporters (NRT) and ammonium transporter are mainly involved in the direct uptake of N (Duan et al., 2015). The main pathway of N assimilation is that NO_3^- is firstly reduced to NO_2^- under the action of nitrate reductase (NR), and then reduced to NH_4^+ by the action of nitrite reductase (NiR). NH_4^+ is assimilated by glutamine (Gln) and glutamate (Glu) through the glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway, and then N-containing compounds such as protein and nucleic acid are synthesized under the action of transaminases (Qin et al., 2021; Huang et al., 2022). In addition, glutamate dehydrogenase (GDH) can synthesize Glu under the consumption of NH_4^+ and 2-oxoglutarate (Luo et al., 2013). Therefore, the activity of key enzymes in N metabolism directly reflects the strength of N metabolism in plants. Evidence suggests that AMF can transfer a large amount of N to host plant cells, improve the utilization efficiency of N in the soil, reduce the input of chemical fertilizer, and make a virtuous cycle of the soil ecosystem, thus playing an important role in plant N nutrition (Govindarajulu et al., 2005; Savolainen and Kytöviita, 2022). At present, this has been confirmed in vegetables (Roussis et al., 2022), crops (Tanaka and Yano, 2005) and forage grass (Kang et al., 2020; Khan et al., 2021), but the researches on trees are relatively few. Moreover, the effects of AMF on plant N metabolism and its regulatory mechanism are rarely reported.

Catalpa bungei C.A.Mey. is a valuable and high-quality timber species and a famous ornamental tree species in traditional cultivation in China (Lv et al., 2021; Jian et al., 2022). Because of its excellent wood quality and high economic value, *C. bungei* is widely used in furniture, architecture, technology and other industries (Zheng et al., 2017; Wang et al., 2022). It has strong adaptability and rapid growth, so it is not only conducive to vegetation restoration in Northwest China to popularize and cultivate in a large area, but also provides support for local economic construction (Chen et al., 2021). In recent years, the researches on the fertilization of *C. bungei* have made some progress, mainly by increasing the application of N fertilizer to improve the growth of *C. bungei* plantation (Wu et al., 2015; Wang et al., 2021a). However, it not only requires higher economic costs, but also causes serious environmental pollution (Wu et al., 2017a; Sun et al., 2021). Therefore, it is an important factor to restrict the growth of *C. bungei* that how to apply fertilizer rationally to reduce the waste of resources and improve the efficient and rational use of N fertilizer. In addition, our previous research also showed that under drought stress, AMF could form a symbiotic relationship with *C. bungei*, improve water and nutrient status, and thus improve the drought resistance of *C. bungei* (Chen et al., 2020), which further confirmed the potential of AMF as microbial fertilizer. However, the inoculation effect of AMF may be related to a variety of factors, such as plant species, the genus or species of AMF colonizing roots, and N availability (Wu et al., 2017b), the effects of AMF inoculation on the growth of *C. bungei* under different N application levels have not been reported. Understanding the effects of AMF inoculation on woody plants under different nutrient levels is of great significance for further exploring the effects of the external environment regulating mycorrhizal symbiosis on tree phenotypic traits. Therefore, in this study, the effects of AMF inoculation on the growth, photosynthesis, nutritional status, N metabolism and expression of key genes of *C. bungei* were compared under different N concentrations under potted greenhouse conditions, to explore the effects of N application levels on AMF inoculation. The results can provide a theoretical basis for the development of AMF as microbial fertilizer, and also provide a reference for the application of AMF in the cultivation and afforestation technology of *C. bungei* in Northwest China.

2 Materials and methods

2.1 Mycorrhizal inoculum, test plant and cultivation substrate

The AM fungus *Rhizophagus intraradices* (BGC BJ09) was provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, China. Before inoculation, maize was used as the host plant for propagation, and the inoculum consisted of a sand soil mixture

containing spores, mycelium and infected root segments. Plant material, namely tissue culture seedlings of *C. bungei* "Jinsi" superior clone, was provided by the Henan Academy of Agricultural Sciences. The cultivation substrate was river sand. The screened river sand was washed with clean water (8–9 times), and naturally dried, then sterilized by high-pressure steam (121°C, 2 h) for reserve use.

2.2 Experimental design

The pot experiment was conducted in a two-factor completely randomized block design, including AMF inoculation and N application. Mycorrhizal inoculation included two levels: control treatment without inoculation (NAM) and inoculation with *R. intraradices* (AM). Three levels of N application were set (according to the screening results of the pre-experiment): 0.25 mM (low N), 10 mM (medium or moderate N) and 45 mM NH_4NO_3 (high N), respectively. A total of six treatments were formed, with 20 pots planted in each treatment and 0.75 kg of substrate in each pot, a total of 120 pots. The experiment was carried out in a glass greenhouse of Northwest A&F University.

The seedlings of *C. bungei* with the same growth trend were selected and transplanted into flowerpots (9 cm in diameter and 18 cm in depth, disinfected with 0.5% NaClO) with culture medium, and one plant was planted in each pot. In the inoculated treatment, 3 g of *R. intraradices* inoculum (approximately 200 spores per gram) was evenly spread on the roots of *C. bungei* seedlings during transplanting to fully contact with the roots. In the non-inoculated treatment, the same amount of inactivated inoculum (121°C, 2 h) was added in the same way, and 10 mL of inoculum filtrate (1 μm nylon net) was also added to ensure the consistency of microflora. After transplanting, the water supply was maintained normally (200 mL per plant per week), and Hoagland nutrient solution was supplemented every 10 days until the seedlings grew stably (about 45 days). The root samples of three seedlings were randomly selected to detect the colonization status of *R. intraradices*. After successful colonization, N application was started. 40 mL of 0.25 mM, 10 mM and 45 mM NH_4NO_3 solutions were irrigated respectively every 2 days for one month, during which Hoagland nutrient solution with N removed was supplemented every 10 days to ensure the normal supply of other nutrients. After fertilization treatment, the seedlings were normally supplied with water. After continuing to grow for one month, the seedlings were harvested and sampled for the determination of various physiological, biochemical and molecular biological indexes. The environmental conditions during seedling growth were as follows: day temperature, 20–35°C, night temperature, 10–20°C, a daylight cycle of 12 h, and relative humidity, 40%–85%.

2.3 Measurement of AMF colonization

The roots of seedlings were continuously washed with tap water, and then immersed in 10% KOH solution for 30 min in a water bath at 90°C. After the root segments were transparent, the residual KOH was rinsed with distilled water. Seedling roots were stained with Trypan-Blue (Phillips and Hayman, 1970), then observed with a 200× optical microscope (Olympus Bx43, Japan). The colonization rate of AMF was calculated according to the magnified intersections method (McGonigle et al., 1990).

2.4 Determination of gas exchange parameters and photosynthetic pigments

Before seedling harvest, Li-6400 portable photosynthetic apparatus (Li-COR, USA) was used to measure the gas exchange parameters of leaves, mainly including net photosynthetic rate (Pn, $\mu mol \cdot m^{-2}s^{-1}$), stomatal conductance (Gs, $mol \cdot m^{-2}s^{-1}$), transpiration rate (Tr, $mmol \cdot m^{-2}s^{-1}$), and intercellular CO_2 concentration (Ci, $\mu mol \cdot mol^{-1}$). The third, fourth and fifth leaves on the top of the seedlings were measured on a sunny morning (9:00–11:00). Instantaneous water use efficiency (WUE) was calculated by the ratio of Pn to Tr (Yang et al., 2014). 0.2 g of fresh leaves were weighed, chlorophyll (Chl a and Chl b) and carotenoids were extracted by the acetone direct extraction method, the absorbance at 663 nm, 646 nm, and 470 nm was measured and the pigment contents were calculated, respectively (Gao, 2006).

2.5 Determination of chlorophyll fluorescence parameters

The third, fourth and fifth leaves on the top of the *C. bungei* seedling were selected, and the chlorophyll fluorescence parameters were measured using a modulated chlorophyll fluorescence meter (MINI-Imaging-PAM, Walz, Germany) (Gao, 2006; Huang et al., 2018). The maximum photochemical efficiency (Fv/Fm), actual photochemical efficiency (ΦPSII), potential photochemical efficiency of PSII (Fv/Fo), non-photochemical quenching (NPQ), photochemical quenching (qP) and relative electron transport rate (rETR) through PSII were calculated (Gao, 2006; Huang et al., 2018).

2.6 Growth parameters and biomass allocation

Leaves, stems and roots were harvested separately and then dried to a constant weight at 65°C after 15 min at 105°C, and the biomass (dry weight) was measured. The biomass allocation of each part was calculated according to the following formula (Xiao et al., 2015): leaf mass ratio (LMR) = leaf biomass/total biomass, stem mass ratio (SMR) = stem biomass/total biomass, root mass ratio (RMR) = root biomass/total biomass, root/shoot ratio = underground biomass/aboveground biomass.

2.7 Nutrient absorption and distribution

The dried root, stem and leaf samples were ground into fine powder by mortar. The N concentration in each part was

determined by Kjeldahl, phosphorus (P) concentration was measured by the molybdenum-stibium colorimetric method, and potassium (K), calcium (Ca) and magnesium (Mg) concentrations were determined by flame photometric method, respectively (Bao, 2000).

2.8 Specific leaf area (SLA) and specific leaf weight (SLW)

A single leaf in the same position was collected and the single leaf area was measured using the transparent grid method (Gao, 2006). The leaf was then dried in the oven at 65°C to a constant weight and weighed. The SLA, SLW and leaf area per plant were calculated according to the following formula: SLA = leaf area (cm^2)/leaf dry weight (g), SLW = leaf dry weight (g)/leaf area (m^2), the leaf area per plant = total leaf dry weight (g)/SLW (g/cm^2).

2.9 Root system architecture and root activity

Fresh seedling roots were cleaned under running water and then the root images were scanned with a digital scanner (Epson Expression 10000XL, Epson America, San Jose, CA, USA) at 300 dpi. Then the parameters such as root length, root surface area, average root diameter and root tips were calculated respectively by WinRHIZO root image analysis software (Version 2012b, Regent Instruments Inc., Montreal, QC, Canada) (Zheng et al., 2017). Root activity was determined by triphenyltetrazolium chloride (Gao, 2006).

2.10 Phytohormone levels

The contents of auxin (indole-3-acetic acid, IAA), gibberellin (GA_3), cytokinin (CTK), and abscisic acid (ABA) in roots were determined by high performance liquid chromatography (Pan et al., 2010).

2.11 Determination of key enzyme activities in N metabolism

0.1 g of fresh root samples were fully ground in liquid N_2. The activities of NR, NiR, GS, GOGAT and GDH in roots were measured using the corresponding enzyme-linked immunosorbent assay (ELISA) kits. The corresponding article numbers of the kits are JLC7018, JLC79203, JLC79209, JLC72932, and JLC72820, respectively. Before the enzyme activity assay, bicinchoninic acid (BCA) protein assay kits (JLC-SJ2508) were used to quantitatively measure the protein concentration of each sample. All the above kits were obtained from Shanghai Jingkang Bioengineering Co., Ltd. According to the instructions of the kit, sample addition, incubation (37°C, 30 min), washing, color development, and other processes were carried out. The absorbance was measured at 450 nm and the concentration of enzyme activity in the sample was calculated by the standard curve.

2.12 Expression analysis of genes related to N metabolism

100 mg of fresh root samples were snap-frozen in liquid N_2 and ground into fine powder. The total RNA of the sample was extracted and purified by polysaccharide/polyphenol plant RNA Extraction Kit (NG3021S, HLingene; containing gDNA removal column). The concentration and quality of extracted RNA were determined using a micro nucleic acid protein analyzer (NanoDrop One, Fisher Scientific, USA). The first strand of cDNA was synthesized by reverse transcription kit (RT mix with DNase (All-in-One), US EVERBRIGHT INC), which was used as the template for RT-PCR. With reference to the preliminary transcriptome data (stored in NCBI/SRA database with the accession number PRJNA907402 (http://www.ncbi.nlm.nih.gov/bioproject/907402)) of *C. bungei*, some coding sequences (CDS) of genes related to N metabolism were screened, and gene-specific primers for qRT-PCR were designed using the online primer design tool Primer 3 (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The ubiquitin (UBQ) gene of *C. bungei* was used as the reference gene. The sequence information of specific primers was shown in supplementary data (Table S1). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. qRT-PCR analysis was performed using CFX 96 real-time PCR instrument (Bio-Rad, Hercules, CA, USA) with differently treated cDNA as a template. Each treatment contained 3 biological replicates and 4 technical replicates. The relative expression levels of target genes were calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.13 Statistical analysis

SPSS 25.0 software was used for the statistical analysis of data. One-way analysis of variance (ANOVA) was used to analyze the different treatments, and then the Duncan test was used to make multiple comparisons. The significance level was set as $\alpha = 0.05$. All data were expressed as mean ± standard error (SE). The effects of N application, inoculation and their interaction on the measured indexes were evaluated by two-way ANOVA. The Pearson correlation coefficient was used to evaluate the correlation between AMF colonization and other indicators. Using principal component analysis (PCA), the data were standardized and then computed by function rda () in the vegan library in R (http://www.r-project.org/). Sigmaplot 12.0 software was used for drawing.

3 Results

3.1 AMF colonization

After inoculation with *R. intraradices*, the colonization structures of *R. intraradices*, such as intraradical hyphae,

arbuscules, and vesicles, can be clearly observed in roots at low N (Figure 1A), medium N (Figure 1B) and high N (Figure 1C) levels, while the colonization was not detected in each treatment without inoculation (Figure 1D). After AMF inoculation, with the increase of N concentration, the colonization rates of hyphae, arbuscules, vesicles and total colonization rate in roots of *C. bungei* seedlings increased significantly ($p < 0.05$), reached the maximum at medium N concentration, and decreased markedly at high N levels (Figure 1E).

3.2 Growth parameters

The growth of *C. bungei* seedlings was significantly affected by N application (Figure 2). Whether inoculated or not, with the increase of N concentration, the growth parameters (plant height, basal diameter, leaf number, total biomass) of *C. bungei* seedlings increased significantly, and reached the maximum at medium N levels, but decreased notably at high levels (Figures 2A–D). At low N levels, compared with non-inoculated treatment, AMF inoculation significantly increased the plant height and decreased the basal diameter of seedlings ($p < 0.05$), but had no significant effect on biomass accumulation. At medium N levels, the plant height (Figure 2A), basal diameter (Figure 2B), and total biomass (Figure 2D) of mycorrhizal seedlings were significantly higher than those of non-mycorrhizal seedlings ($p < 0.05$), which were 1.30-, 1.12- and 1.76-fold of those of non-inoculated seedlings, respectively. At high N levels, AMF inoculation notably increased the total biomass, but there were no significant differences in plant height and basal diameter between the inoculated and non-inoculated treatments. In addition, no significant difference in leaf number between inoculated and non-inoculated treatments was detected (Figure 2C).

3.3 Biomass allocation

The biomass allocation of *C. bungei* seedlings changed significantly after inoculation and N application (Table 1). Whether inoculated or not, the leaf biomass, stem biomass and SMR of *C. bungei* seedlings increased first and then decreased with the increase of N concentration, and the LMR increased gradually, while the RMR and root/shoot ratio decreased gradually. Among them, the leaf biomass of mycorrhizal or non-mycorrhizal seedlings reached a significant difference among the three N concentrations, and the stem biomass at medium N levels was significantly higher than that of low or high N levels ($p < 0.05$). Without inoculation, the root biomass was significantly decreased with the increase of N concentration, while it was firstly significantly increased and then markedly decreased after AMF inoculation. At the same N levels,

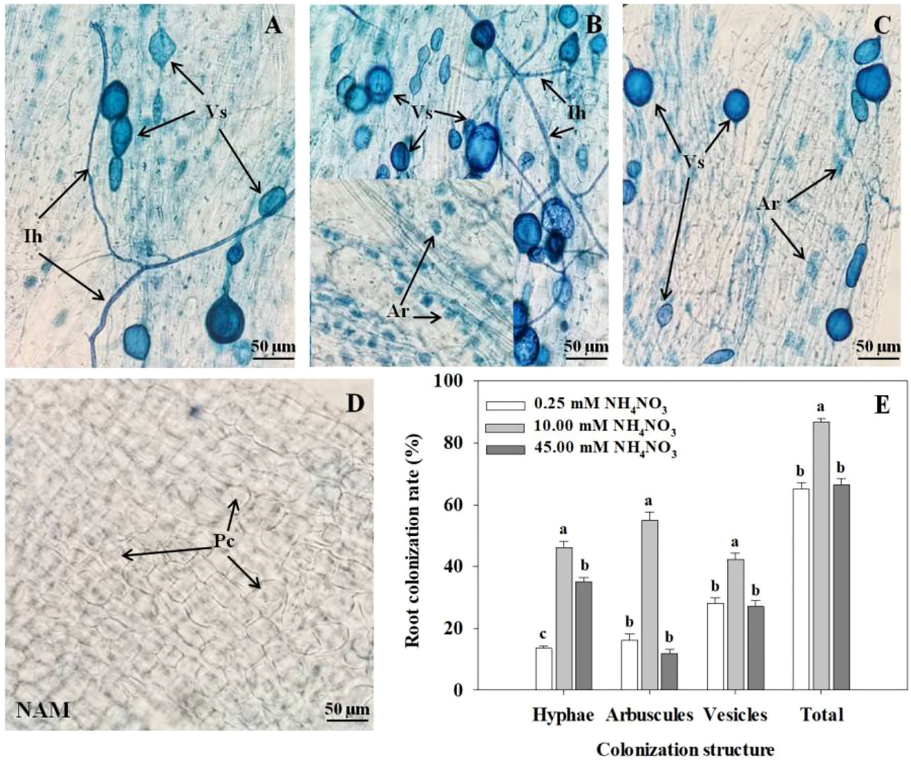

FIGURE 1
Colonization and development of arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* in *Catalpa bungei* seedling roots under **(A)** low N (0.25 mM), **(B)** moderate N (10 mM) and **(C)** high N (45 mM) levels. **(D)** The root of a non-inoculated plant. **(E)** Colonization rate of mycorrhizal *C. bungei* seedlings under different N levels. A, B, C and D, bars = 50 μm. Ih, intraradical hyphae; Vs, vesicles; Ar, arbuscules; Pc, plant cell; NAM, non-AMF-inoculated. Different lowercase letters above the bars indicate significant differences ($p < 0.05$) among different N levels. Values are means ± SE ($n = 6$).

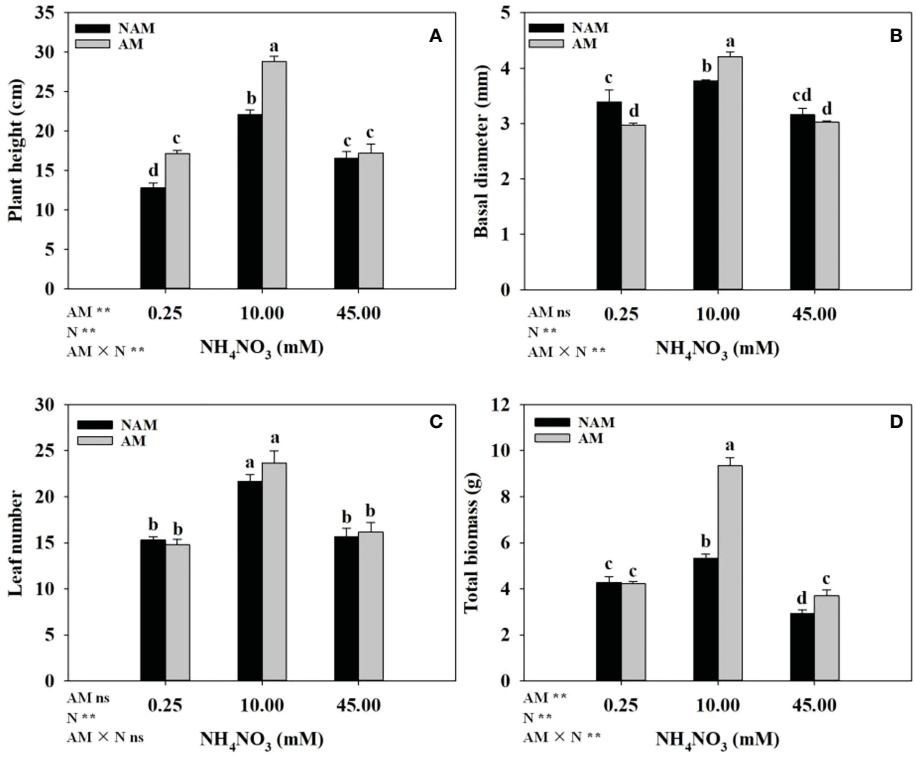

FIGURE 2
Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on **(A)** plant height, **(B)** basal diameter, **(C)** leaf number and **(D)** total biomass of *Catalpa bungei* seedlings under different nitrogen (N) levels. NAM, non-AMF-inoculated; AM, AMF-inoculated. Different lowercase letters above the bars indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE ($n = 6$). Two-way ANOVA output: ns, not significant; *$p < 0.05$; **$p < 0.01$.

the LMR > RMR > SMR, that is, the biomass of inoculated or non-inoculated seedlings tended to be allocated to leaves, followed by roots, and the proportion allocated to stems was the least (leaf > root > stem).

Compared with the non-inoculated treatments, the leaf biomass and LMR of mycorrhizal seedlings were higher than those of non-mycorrhizal seedlings under three N concentrations, while the SMR, RMR and root/shoot ratio were lower (Table 1). After inoculation with AMF, the stem biomass of mycorrhizal seedlings was slightly lower than that of non-mycorrhizal seedlings at low or high N levels, and significantly increased at medium N levels, while the root biomass decreased slightly at low N levels, and was higher

TABLE 1 Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on biomass allocation in different parts of the *Catalpa bungei* seedlings under different nitrogen (N) concentrations.

NH_4NO_3 (mM)	AMF status	Leaf biomass (g)	Stem biomass (g)	Root biomass (g)	Leaf mass ratio	Stem mass ratio	Root mass ratio	Root/shoot ratio
0.25	NAM	1.93 ± 0.12d	0.50 ± 0.03c	1.86 ± 0.18ab	0.45 ± 0.02e	0.12 ± 0.01b	0.43 ± 0.03a	0.78 ± 0.08a
	AM	2.13 ± 0.06d	0.44 ± 0.01cd	1.65 ± 0.12b	0.51 ± 0.02d	0.11 ± 0.00b	0.39 ± 0.02a	0.65 ± 0.06b
10.00	NAM	3.22 ± 0.14b	0.83 ± 0.03b	1.28 ± 0.03c	0.60 ± 0.01c	0.16 ± 0.01a	0.24 ± 0.01b	0.32 ± 0.01c
	AM	6.10 ± 0.21a	1.12 ± 0.03a	2.14 ± 0.12a	0.65 ± 0.00b	0.12 ± 0.00b	0.23 ± 0.01b	0.30 ± 0.01c
45.00	NAM	1.82 ± 0.12d	0.41 ± 0.02cd	0.71 ± 0.06d	0.62 ± 0.02bc	0.14 ± 0.00a	0.24 ± 0.02b	0.32 ± 0.03c
	AM	2.58 ± 0.18c	0.39 ± 0.04d	0.73 ± 0.06d	0.70 ± 0.01a	0.10 ± 0.01b	0.20 ± 0.01b	0.25 ± 0.02c
Significance	AM	**	**	*	**	**	*	*
	N	**	**	**	**	**	**	**
	AM×N	**	**	**	ns	ns	ns	ns

NAM, non-AMF-inoculated; AM, AMF-inoculated; Different lowercase letters within each column indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE (n = 6). Two-way ANOVA output: ns, not significant; *$p < 0.05$; **$p < 0.01$.

than that of non-inoculated seedlings at medium or high N levels. At medium N levels, the leaf, stem, root biomass and LMR of inoculated seedlings were significantly increased ($p < 0.05$).

3.4 Root architecture and activity

N application and AMF inoculation significantly affected the root growth of *C. bungei* (Table 2; Supplementary data, Figure S1). Without inoculation, the root morphological parameters (except the average root diameter) and root activity of seedlings decreased gradually with the increase of N concentration. After inoculation with AMF, all root morphological parameters (except the average root diameter) and root activity of mycorrhizal seedlings showed a trend of first increasing and then decreasing, reaching the maximum at medium N and significantly higher than those at low and high N levels (Table 2). In general, the average root diameter of seedlings was not significantly affected by N application regardless of inoculation status. At the same N levels, there were differences in the effects of non-inoculated and inoculated treatments on seedling root growth (Table 2; Supplementary data, Figure S1). At low N levels, all root morphological parameters (except the average root diameter) and root activity of inoculated seedlings were lower than those of non-inoculated seedlings, and the total root length, length per unit volume, tips, forks, and length of fine roots ($0 < d \leq 0.5$ mm) reached significant differences. However, at medium N levels, all parameters (except the average root diameter) and root activity of mycorrhizal seedlings were significantly higher than those of non-mycorrhizal seedlings ($p < 0.05$; 1.24–1.92-fold). At high N levels, there were no significant differences in all root morphological parameters and root activity between inoculated and non-inoculated seedlings.

3.5 Photosynthetic gas exchange capacity

N application had a certain effect on the photosynthetic gas exchange parameters of seedlings (Table 3). Pn and WUE of non-inoculated (or inoculated) seedlings increased significantly at first and then decreased with the increase of N concentration, and reached the maximum at medium N levels. Under non-inoculated conditions, Gs and Tr decreased gradually with the increase of N concentration, while Ci decreased first and then increased slightly. After inoculation with AMF, Gs and Tr of mycorrhizal seedlings increased first and then decreased with the increase of N concentration, while Ci decreased gradually. Under the same N levels, the photosynthetic gas exchange parameters of inoculated seedlings were all higher than those of non-inoculated seedlings (except Ci at high N levels and Tr at low N levels; Table 3). At medium N levels, Pn, Gs, Ci and WUE of mycorrhizal seedlings were significantly higher than those of non-mycorrhizal seedlings

TABLE 2 Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the root morphological parameters of *Catalpa bungei* seedlings under different nitrogen (N) concentrations.

NH_4NO_3 (mM)	AMF status	Total root length (cm)	Total root surface area (cm^2)	Root projected area (cm^2)	Total root volume (cm^3)	Length per unit volume (cm/m^3)	Average diameter (mm)	Root tips	Forks	LF (cm)	SAF (cm^2)	Root activity [μg/(g·h)]
0.25	NAM	1714.77 ± 131.93b	377.85 ± 28.37b	120.27 ± 9.03b	6.72 ± 0.66b	1714.77 ± 131.93b	0.71 ± 0.03	3994.17 ± 341.38b	7786.67 ± 741.03a	1091.15 ± 107.21ab	100.55 ± 10.56b	87.65 ± 5.95ab
	AM	1389.50 ± 86.62c	321.15 ± 20.89b	102.22 ± 6.65b	6.01 ± 0.39b	1389.50 ± 86.62c	0.75 ± 0.01	2546.00 ± 256.13c	5313.67 ± 301.99b	817.33 ± 61.57c	80.46 ± 3.91b	75.82 ± 6.53b
10.00	NAM	1435.35 ± 114.35c	319.41 ± 19.81b	101.67 ± 6.30b	5.74 ± 0.28b	1435.35 ± 114.35c	0.72 ± 0.02	2654.00 ± 426.77c	5457.17 ± 757.11b	877.72 ± 103.37bc	89.72 ± 8.31b	77.03 ± 6.67b
	AM	2108.03 ± 112.42a	468.75 ± 26.97a	149.21 ± 8.59a	8.40 ± 0.67a	2108.03 ± 112.42a	0.71 ± 0.03	5100.67 ± 526.38a	8972.83 ± 738.95a	1265.28 ± 89.89a	127.49 ± 6.97a	95.54 ± 3.18a
45.00	NAM	944.58 ± 37.28d	217.92 ± 9.17c	69.37 ± 2.92c	4.05 ± 0.32c	944.58 ± 37.28d	0.74 ± 0.04	2488.33 ± 191.44c	4414.17 ± 378.15b	571.44 ± 34.83d	54.16 ± 3.23c	52.63 ± 4.02c
	AM	963.25 ± 57.14d	219.16 ± 9.83c	69.76 ± 3.13c	3.98 ± 0.18c	963.25 ± 57.14d	0.73 ± 0.02	2576.50 ± 327.70c	4190.33 ± 457.00b	567.74 ± 42.45d	54.86 ± 3.54c	53.12 ± 3.14c
Significance	AM	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	N	**	**	**	**	**	ns	**	**	**	**	**
	AM×N	**	**	**	**	**	ns	**	**	**	**	*

NAM, non-AMF-inoculated; AM, AMF-inoculated; LF, length of fine roots ($0 < d \leq 0.5$ mm); SAF, the surface area of fine roots ($0 < d \leq 0.5$ mm). Different lowercase letters within each column indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE (n = 6). Two-way ANOVA output: ns, not significant; *$p < 0.05$; **$p < 0.01$.

TABLE 3 Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the photosynthetic gas exchange parameters of *Catalpa bungei* seedlings under different nitrogen (N) concentrations.

NH_4NO_3 (mM)	AMF status	P_n [μmol/(m^2·s)]	G_s [mol/(m^2·s)]	C_i (μmol/mol)	T_r [mmol/(m^2·s)]	WUE (μmol/mmol)
0.25	NAM	3.98 ± 0.37d	0.22 ± 0.03b	331.46 ± 5.38a	4.90 ± 0.54a	0.94 ± 0.09d
	AM	5.14 ± 0.40cd	0.24 ± 0.03ab	338.51 ± 4.42a	3.10 ± 0.27b	1.72 ± 0.08c
10.00	NAM	6.82 ± 0.45b	0.14 ± 0.02c	295.19 ± 7.13b	3.96 ± 0.41ab	1.86 ± 0.09bc
	AM	8.88 ± 0.32a	0.30 ± 0.02a	324.72 ± 3.93a	4.19 ± 0.27ab	2.24 ± 0.13a
45.00	NAM	5.34 ± 0.48c	0.10 ± 0.01c	298.97 ± 6.07b	3.16 ± 0.31b	1.76 ± 0.09c
	AM	7.03 ± 0.55b	0.14 ± 0.02c	270.28 ± 7.23c	3.45 ± 0.30b	2.09 ± 0.08ab
Significance	AM	**	**	ns	ns	**
	N	**	**	**	ns	**
	AM×N	ns	**	**	**	*

NAM, non-AMF-inoculated; AM, AMF-inoculated; Pn, net photosynthetic rate; Gs, stomatal conductance; Tr, transpiration rate; Ci, intercellular CO_2 concentration; WUE, water use efficiency. Different lowercase letters within each column indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE (n = 18). Two-way ANOVA output: ns, not significant; *$p < 0.05$; **$p < 0.01$.

($p < 0.05$; 1.10–2.14-fold). However, at low or high N levels, only some indexes were significantly different between inoculated and non-inoculated treatments.

3.6 Nutrient uptake and distribution

In general, the N concentration was the highest in *C. bungei* seedlings, followed by K, Ca, and Mg concentrations, while P concentration was the lowest (Figure 3). No matter whether inoculated or not, or the level of N application, the accumulation of N concentration was highest in leaves, followed by roots, and lowest in stems (Figure 3A). P and Mg concentrations were higher in leaves and roots, but lowest in stems (Figures 3B, E). Ca concentration was highest in leaves, followed by stems and lowest in roots (Figure 3D). At low to medium N levels, K concentration in different parts of inoculated (or non-inoculated) seedlings was ranked as root > stem > leaf, while at high N levels, K concentration was the highest in the stem, followed by leaf and root (Figure 3C).

Regardless of AMF inoculation status, with the increase of N application, the N concentration in leaves, stems and roots of *C. bungei* seedlings significantly increased, and the K concentration first decreased and then increased (except the K concentration in roots without inoculation). On the contrary, the Ca and Mg concentrations in seedlings showed a trend of first increasing and then decreasing (except the Ca concentration in leaves), reaching the maximum at medium N levels (Figure 3). The P concentration in leaves of both inoculated and non-inoculated seedlings increased with the increase of N application, while P concentration in stems didn't change significantly. The P concentration in roots increased significantly at first and then decreased markedly with the increase of N application without inoculation ($p < 0.05$), while there was no significant change under inoculation conditions. With the increase of N application, the Ca concentration in leaves didn't change notably under non-inoculated conditions, but increased significantly under inoculation. At the same N levels, the N and P concentrations in leaves, stems and roots, K and Mg concentrations in leaves, and Ca concentrations in roots of mycorrhizal seedlings were higher than those of non-mycorrhizal seedlings (except N concentration in roots under low N condition), while the K and Mg concentrations in stems and roots and Ca concentrations in leaves and stems were lower (except K concentration in roots under low N levels and Mg concentration in stems under high N levels; Figure 3).

3.7 Phytohormones

Both N application and AMF inoculation significantly changed the hormone concentrations and ratios in the root system of seedlings (Figure 4). Without AMF inoculation, with the increase of N application level, IAA concentration and IAA/ABA ratio in roots showed a trend of first decreasing and then increasing, ABA concentration gradually increased, while CTK, GA_3 concentrations, and CTK/ABA, GA_3/ABA ratios increased first and then decreased. After AMF inoculation, the concentrations of IAA, CTK and ABA in roots of mycorrhizal seedlings decreased gradually with the increase of N concentration, while the GA_3 concentration and the ratios of hormones (IAA/ABA, CTK/ABA, GA_3/ABA) showed a trend of increasing first and then decreasing, and the concentrations of IAA, GA_3 and the ratio of GA_3/ABA were significantly different among the three N concentrations ($p < 0.05$). Under the same N levels, the root hormone concentrations (IAA, CTK, GA_3) and ratios (IAA/ABA, CTK/ABA, GA_3/ABA) of the inoculated seedlings were higher than those of non-inoculated treatments (except GA_3 content and GA_3/ABA ratio at high N levels), while the ABA concentration was lower (except ABA content at low N levels; Figure 4). At low N levels, the four hormones (IAA, CTK, GA_3, ABA) of inoculated treatments were about 1.50-fold of those of non-inoculated treatments. At medium N levels, the differences in IAA, GA_3 concentrations, and IAA/ABA, GA_3/ABA ratios between inoculated and non-inoculated treatments were

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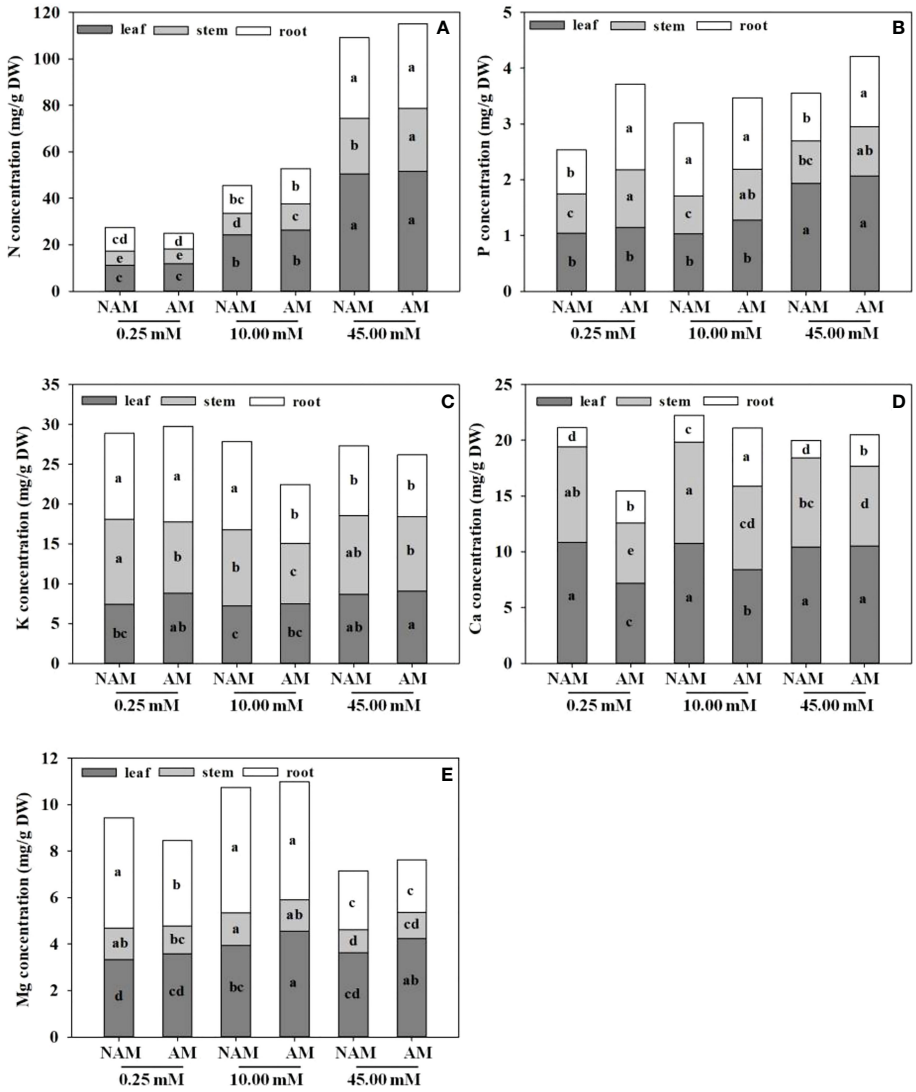

FIGURE 3
Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the **(A)** nitrogen (N), **(B)** phosphorus (P), **(C)** potassium (K), **(D)** calcium (Ca) and **(E)** magnesium (Mg) concentrations of *Catalpa bungei* seedlings under different nitrogen (N) levels. NAM, non-AMF-inoculated; AM, AMF-inoculated. Different lowercase letters above the bars indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE ($n = 6$).

significant, which were 1.50–1.66-fold of those of non-inoculated treatments, respectively.

3.8 Activities of key enzymes for N metabolism in roots

The effects of N application on the activities of key enzymes of N metabolism in the roots of inoculated and non-inoculated seedlings were different (Figure 5). Without inoculation, the activities of NR, GOGAT and GDH in the roots increased significantly at first with the increase of N concentration, reached the maximum at medium N levels, and then decreased (Figures 5A, D, E). The NiR activity decreased gradually (Figure 5B), while GS activity didn't change significantly (Figure 5C). After AMF inoculation, with the increase of N concentration, NR activity in the roots gradually decreased (Figure 5A), NiR and GOGAT activities gradually increased (Figures 5B, D), and GS activity first significantly increased and then slightly decreased (Figure 5C), while the GDH activity didn't change much (Figure 5E). At the same N levels, the activities of key enzymes (NR, NiR, GS, GOGAT, GDH) in the roots of inoculated seedlings were higher than those of non-inoculated seedlings (except NR activity at 10–45 mM; Figure 5). Among them, the activities of NR, GOGAT, GDH at low N levels, NiR, GS at medium N levels, and NiR, GOGAT, GDH at high N levels were significantly different between inoculated and non-inoculated treatments.

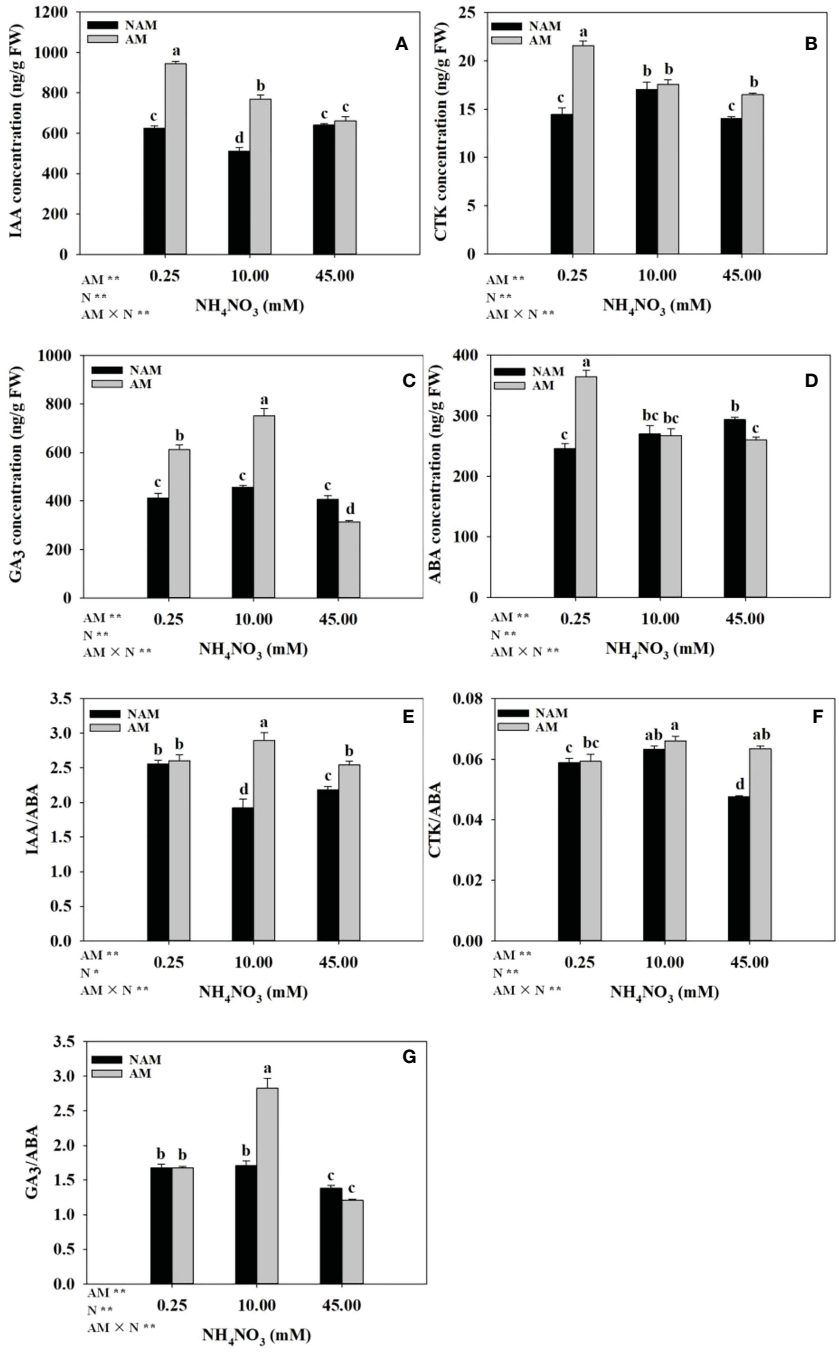

FIGURE 4
Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the phytohormones concentrations and ratios in the root system of *Catalpa bungei* seedlings under different nitrogen (N) levels. **(A)**, indole-3-acetic acid (IAA); **(B)**, cytokinin (CTK); **(C)**, gibberellin (GA_3); **(D)**, abscisic acid (ABA); **(E)**, IAA/ABA; **(F)**, CTK/ABA; **(G)**, GA_3/ABA. NAM, non-AMF-inoculated; AM, AMF-inoculated. Different lowercase letters above the bars indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE ($n = 6$). Two-way ANOVA output: ns, not significant; *$p < 0.05$; **$p < 0.01$.

3.9 Relative expression of genes related to N metabolism in roots

N application significantly affected the expression levels of genes related to N metabolism in seedling roots (Figure 6). Regardless of AMF inoculation status, with the increase of N concentration, the relative expression of *NR*, *Fd-NiR* and *GS* genes in the roots of *C. bungei* seedlings were significantly up-regulated at first and then markedly down-regulated (Figures 6A–C), while the relative expression of *GS1* had no significant change (Figure 6D). Without inoculation, with the increase of N concentration, the relative expression of *NADH-GOGAT1* in roots was firstly significantly up-regulated and then markedly down-regulated (Figure 6E). On the contrary, the relative expression of

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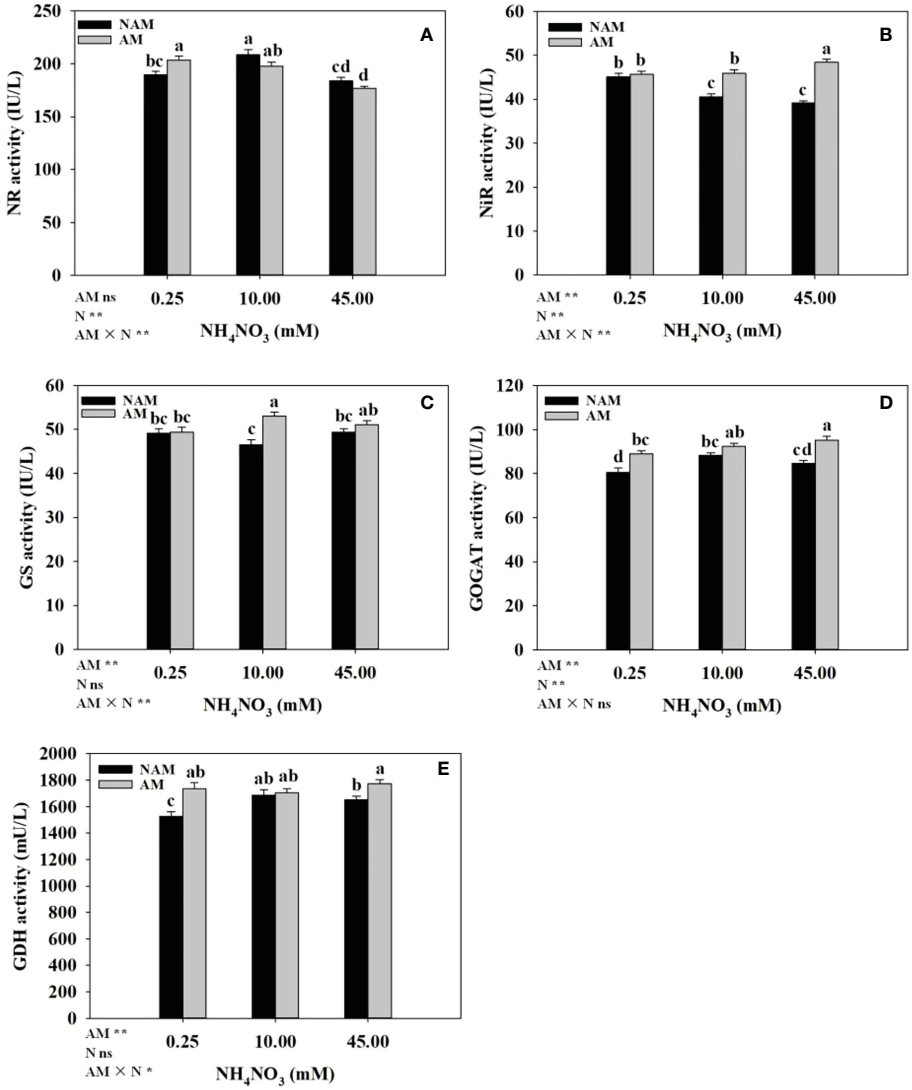

FIGURE 5
Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the activities of key enzymes of nitrogen (N) metabolism in the root system of *Catalpa bungei* seedlings under different N levels. **(A)**, nitrate reductase (NR); **(B)**, nitrite reductase (NiR); **(C)**, glutamine synthetase (GS); **(D)**, glutamate synthetase (GOGAT); **(E)**, glutamate dehydrogenase (GDH). NAM, non-AMF-inoculated; AM, AMF-inoculated. Different lowercase letters above the bars indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE ($n = 7$). Two-way ANOVA output: ns, not significant; *$p < 0.05$; **$p < 0.01$.

NRT2.7 was first down-regulated and then up-regulated (Figure 6L), while the expression of *GDHA* was gradually up-regulated (Figure 6H), and the relative expression of *Fd-GOGAT*, *NADH-GDH*, *GDHB*, *NRT2.4*, and *NRT2.5* were gradually down-regulated (Figures 6F, G, I–K), and *Fd-GOGAT*, *NRT2.4* were significantly different among the three N levels ($p < 0.05$). After AMF inoculation, the relative expression of *NADH-GOGAT1*, *Fd-GOGAT*, *NADH-GDH*, and *GDHB* in roots were firstly up-regulated and then down-regulated with the increase of N concentration, reaching the maximum at medium N levels (Figures 6E–G, I), and the relative expression of *GDHA*, *NRT2.7* was gradually up-regulated (Figures 6H, L). However, the relative expression of *NRT2.4* and *NRT2.5* was gradually down-regulated (Figures 6J, K). At the same N levels, compared with the non-inoculated treatment, the relative expression of *NR*, *Fd-NiR*, *GS*, *NADH-GOGAT1*, *NADH-GDH*, *GDHA*, *GDHB*, *NRT2.4*, and *NRT2.5* in roots of mycorrhizal seedlings were all down-regulated (Figures 6A–C, E, G–K). In addition, at low to medium N levels, the relative expression of most of the above genes reached significant differences between inoculated and non-inoculated treatments. At low N levels, the relative expression of *GS1*, *Fd-GOGAT* and *NRT2.7* in roots of inoculated seedlings was lower than those of non-inoculated seedlings, while at medium to high N levels, the expression of the above three genes was higher (Figures 6D, F, L).

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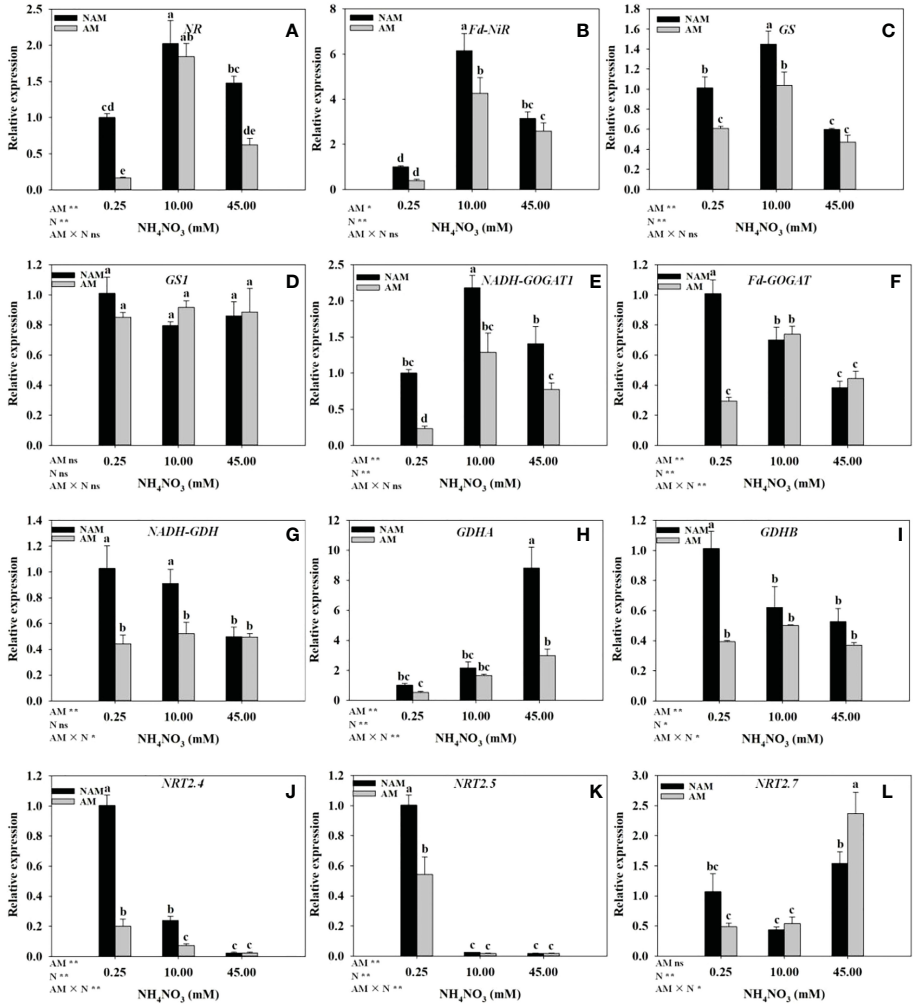

FIGURE 6
Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the relative expression of genes related to nitrogen (N) metabolism in the root system of *Catalpa bungei* seedlings under different N levels. **(A)**, nitrate reductase (*NR*); **(B)**, ferredoxin-nitrite reductase (*Fd-NiR*); **(C)**, glutamine synthetase (*GS*); **(D)**, glutamine synthetase cytosolic isozyme 2 (*GS1*); **(E)**, glutamate synthase 1[NADH] (*NADH-GOGAT1*); **(F)**, ferredoxin-dependent glutamate synthase (*Fd-GOGAT*); **(G)**, NADP-specific glutamate dehydrogenase (*NADH-GDH*); **(H)**, glutamate dehydrogenase A (*GDHA*); **(I)**, glutamate dehydrogenase B (*GDHB*); **(J)**, high affinity nitrate transporter 2.4-like (*NRT2.4*); **(K)**, high affinity nitrate transporter 2.5 (*NRT2.5*); **(L)**, high affinity nitrate transporter 2.7-like (*NRT2.7*). NAM, non-AMF-inoculated; AM, AMF-inoculated. Different lowercase letters above the bars indicate significant differences ($p < 0.05$) among treatments. Values are means ± SE ($n = 3$). Two-way ANOVA output: ns, not significant; *$p < 0.05$; **$p < 0.01$.

3.10 PCA of growth and physiological responses to N application and inoculation

To reveal the key parameters involved in the response patterns of *C. bungei* to N supply levels or inoculation of AMF, PCA was performed using data of morphological and physiological parameters related to growth, photosynthesis, root hormones, and activities of key enzymes of N metabolism (Figure 7; Supplementary data, Table S2). PC1 and PC2 accounted for 38.76% and 15.76% of the variation, respectively. PC1 distinguished the change in the N effect under non-inoculated treatments, while PC2 clearly revealed the effect of N treatment levels after AMF inoculation. Plant height, total biomass, leaf area, photosynthetic pigments, Pn, Fv/Fm and Fv/Fo were key contributors to PC1, while gas exchange parameters (Gs, Ci), and root hormones (IAA, CTK, GA_3, ABA) were important factors to PC2 (Supplementary data, Table S2). In the PCA plot, the greater the distance between the symbols associated with the N treatment levels, the stronger the response of morphological and physiological parameters to changes in N supply levels. After AMF inoculation, the distance between the symbols associated with different N levels was larger than that of non-inoculation treatments, indicating that the inoculation was more sensitive to the change of N supply levels. These results suggested that *C. bungei* seedlings inoculated with and without AMF exhibit distinct morphological and physiological responses in response to N availability.

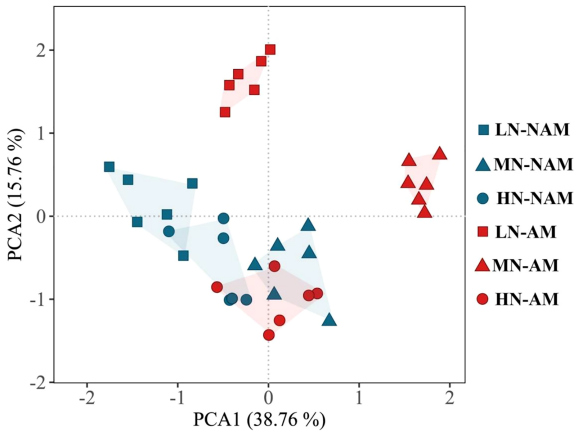

FIGURE 7
Principal component analysis (PCA) plot of growth and physiological characteristics of *Catalpa bungei* under different nitrogen (N) levels and inoculation. LN, low N level (0.25 mM); MN, moderate N level (10 mM); HN, high N level (45 mM); NAM, non-AMF-inoculated; AM, AMF-inoculated.

4 Discussion

4.1 Mycorrhizal colonization and seedling growth

The mycorrhizal colonization rate is an important indicator of close symbiosis (Wang et al., 2021b), which reflects the affinity between fungi and host plants (Li et al., 2022). In general, the total colonization rates under three N levels were higher than 65%, indicating that the root system of *C. bungei* could form a good symbiotic system with *R. intraradices*. Studies have shown that AMF are very sensitive to the nutrient status of their environment (Kong et al., 2022). Our study found that moderate N addition could significantly increase the colonization rate of *R. intraradices* and promote the growth of *C. bungei* seedlings, but excessive N could inhibit mycorrhizal colonization and the growth of seedlings, which was similar to the previous results in *Camellia sinensis* (Zhao et al., 2014), *Sophora japonica* (Yang et al., 2021). Several studies have shown that mycorrhizal symbiosis could promote the growth of woody plants (Turjaman et al., 2006; Outamamat et al., 2022; Tomazelli et al., 2022). However, the effects of AMF on plant growth can be altered by soil N levels. Wu et al. (2017b) showed that inoculation of *R. irregularis* at low N levels significantly promoted the growth of *Populus* × *canadensis*. In the present work, inoculation with AMF at medium N levels significantly promoted the growth parameters (plant height, basal diameter and total biomass), and significant positive correlations ($p < 0.01$) were found between the percentage of mycorrhizal colonization and growth parameters (Supplementary data, Table S3), which also confirmed the positive role of AMF. It could be attributed to the fact that AMF promoted the absorption of nutrients and water in seedlings (Yang et al., 2014). However, when the N concentration was too low or too high, the promoting effect of AMF on *C. bungei* seedlings decreased. Hawkins et al. (2000) reported that under different N concentrations, inoculation of *Glomus mosseae* had no significant effect on the growth of *Triticum aestivum*. However, studies on *Eucalyptus urophylla* (Qin and Yu, 2019) and *P. euramericana* (Rooney et al., 2011) showed that the growth of plants was inhibited after inoculation with AMF. These inconsistent results indicate that in addition to the nutrient supply level of the plant environment, there are also factors such as fungi, host plant species, and the coordination between fungi and hosts, which jointly determine whether AMF function may be effectively performed (Evangelina et al., 2015; Qin and Yu, 2019).

4.2 Biomass allocation

In the process of plant growth, the balance of biomass allocation between underground and aboveground is the key to plant growth, which greatly affects the adaptability and competitiveness of plants (Zhang et al., 2021). In this study, whether inoculated or not, the SMR of *C. bungei* seedlings increased first and then decreased with the increase of N concentration, and the LMR increased gradually, while RMR and root/shoot ratio decreased gradually. These were consistent with the results of N application on biomass allocation in *Acer mono* (Xiao et al., 2015) and *Carya illinoensis* (Wang et al., 2018). The possible explanation is that when the available N supply is insufficient, the carbon content allocated by plants to roots is higher, which is conducive to the absorption of more N by roots. However, when the supply of N is relatively sufficient, the proportion of photosynthate allocated to the aboveground part increases, which is helpful to improve the ability to transform light energy and promote the accumulation of photosynthate (Hermans et al., 2006; Meng et al., 2022). After inoculation with AMF, compared with non-mycorrhizal seedlings, mycorrhizal *C. bungei* seedlings significantly increased the proportion of plant biomass allocated to leaves, but decreased the proportion allocated to stems and roots, thus changing the biomass allocation. Indeed, due to the establishment of AMF, the alleviation of nutrient restriction of host plants may lead to the reduction of root/shoot ratio, which is also confirmed by our results that the percentage of mycorrhizal colonization was negatively correlated with RMR and root/shoot ratio (Supplementary data, Table S3). Alternatively, woody plants tend to distribute aboveground and underground biomass more

unevenly in favor of aboveground growth, and may reflect the ability of AMF to further promote plant growth (Veresoglou et al., 2012). Similar results were also obtained in mycorrhizal *C. sinensis* (Singh et al., 2010).

4.3 Photosynthesis

Photosynthesis is an indispensable part of the life activities of green plants, which can provide the necessary energy source for the metabolism of almost all organisms (Yang et al., 2014; Sun et al., 2021). Our study indicated that moderate N concentration could enhance photosynthetic gas exchange parameters (Pn, WUE), photosynthetic pigment contents (Supplementary data, Table S4), leaf area and SLA (Supplementary data, Figures S2, S3), and chlorophyll fluorescence parameters (except NPQ; Supplementary data, Table S5) of both mycorrhizal and non-mycorrhizal *C. bungei* seedlings. When the N concentration was too low or too high, photosynthesis was inhibited. These results were in accordance with the findings in *Handroanthus heptaphyllus* (Berghetti et al., 2021) and *Cyclocarya paliurus* (Qin et al., 2021). It was suggested that within a certain range, the application of N can increase the leaf area and SLA of plants, enhance the efficiency of capturing solar radiation, and thus enhancing photosynthesis (Seepaul et al., 2016). Mycorrhizal symbiosis also affects photosynthesis (Augé, 2000). After colonizing roots, AMF could maintain the high gas exchange capacity of plants and enhance photosynthesis, which has also been documented in mycorrhizal *Robinia pseudoacacia* (Zhu et al., 2014), *Gleditsia sinensis* (Wang et al., 2019) and *Ilex paraguariensis* (Tomazelli et al., 2022). In particular, in most cases, we also recorded a significant positive correlation between the percentage of mycorrhizal colonization and photosynthetic characteristic parameters ($p < 0.01$, or 0.05; Supplementary data, Table S3). In addition, under the same N levels, the photosynthetic gas exchange parameters (except Tr at low N and Ci at high N), photosynthetic pigment contents (Supplementary data, Table S4), leaf area, SLA (Supplementary data, Figures S2, S3), and chlorophyll fluorescence parameters (Supplementary data, Table S5) of mycorrhizal seedlings were all higher than those of non-inoculated seedlings, and most of them reached significant differences between the inoculated and non-inoculated treatments at low to medium N levels. These results indicated that AMF promoted the photosynthesis of *C. bungei* seedlings, and the effect was more obvious in a moderate level range. On the one hand, this may be related to the promotion of P and Mg uptake by AMF (Zhu et al., 2014; Outamamat et al., 2022). In our study, the results that the percentage of mycorrhizal colonization was positively correlated with leaf Mg concentration ($p < 0.01$, or 0.05) also confirmed the above hypothesis (Supplementary data, Table S3). On the other hand, mycorrhizal plants with higher SLA tended to increase the allocation of leaf N in the photosynthetic system, and had higher photosynthetic capacity and N utilization efficiency (Wu et al., 2017a; Lu et al., 2021).

4.4 Nutrient absorption and distribution

Some studies suggested that in the process of nutrient supply, seedlings would successively be in the three stages of nutrient deficiency, luxury consumption and nutrient toxicity (Timmer, 1997; Salifu and Timmer, 2003). In particular, during the nutrition toxicity stage, the N content still increased with the increase of N application, while the biomass even declined. In the present study, regardless of AMF inoculation status, the N concentration in the leaves, stems and roots of *C. bungei* seedlings increased significantly with the increase of N application, and the N concentration at high N levels was about 3.5–5-fold that at low N levels (Figure 3), but the growth at high N levels was significantly inhibited (Figure 2), which also confirmed the above conclusions. In general, moderate N levels could promote the absorption and accumulation of nutrient elements (N, P, Ca, Mg) in different parts of *C. bungei* seedlings, and similar conclusions have been obtained in other woody plants (Wu et al., 2017b; Qin et al., 2021). Several studies have reported that AMF contribute to the uptake and accumulation of nutrients (P, N, K) in a variety of woody plants under certain conditions (Turjaman et al., 2006; Ouahmane et al., 2007; Outamamat et al., 2022). This may be due to the fact that AMF hyphae are much thinner than plant roots, which can penetrate smaller pores and expand root uptake area, thus improving the plant's access to nutrients, especially those with poor ionic mobility or lower concentrations in soil solutions (Begum et al., 2019; Wang et al., 2019). In the current study, the results showed that AMF inoculation could promote the accumulation of nutrient elements (especially N and P) in seedlings, change the distribution of nutrient elements in different parts, and improve the growth of seedlings at different N levels. Evidence suggests that N and P are closely related to plant photosynthesis. P is an important component of enzymes required for plant photosynthesis and ATP (Shan et al., 2020). While N is the main component of chlorophyll, about 60% of the total N in leaves exists in chloroplasts, which can regulate the activities of enzymes related to the photosynthetic electron transport chain (Berghetti et al., 2021; Qin et al., 2021), and the increase of plant N content within a certain range would promote photosynthesis (Berghetti et al., 2021). Therefore, the promotion of symbiotic plant growth by AMF is closely related to the improvement of plant nutrition and the enhancement of plant photosynthesis, which was in accordance with the results of this study.

4.5 Root architecture and hormone levels

The root system is an important organ of plants, which participates in the acquisition and storage of water and nutrients (Khan et al., 2021). It is generally believed that a low N level can stimulate the root development of woody plants, while a high N level can inhibit root growth (Luo et al., 2013; Song et al., 2019). In this study, low N could significantly stimulate root morphological parameters and root activity of *C. bungei* seedlings without inoculation. However, after inoculation with AMF, moderate N addition significantly promoted the development of mycorrhizal seedling roots. When N was excessive, the growth of seedling roots could be significantly inhibited regardless of inoculation status. These results were in keeping with those of previous studies. Several

studies have shown that AMF colonization can improve the root architecture of woody plants, and directly affect the uptake of soil water and mineral nutrients by plants (Zhang et al., 2016; Gao et al., 2019; Tomazelli et al., 2022). In our study, compared with non-inoculated treatment, AMF inoculation could significantly improve root architecture, enhance root activity, and stimulate root growth of *C. bungei* seedlings under moderate N concentration, while too low or too high N concentrations were not conducive to mycorrhizal effect.

The change of hormone balance in root tissue may be related to the change in root morphology (Hermans et al., 2006). Although auxin is considered to be the major hormone regulating the function of the root meristem, ABA and GA_3 have been proved to play an important role in regulating lateral root formation, such as cell division and cell elongation (Band et al., 2012; Harris, 2015). In the current study, root ABA concentration was negatively correlated with root morphological parameters (except the average diameter), and reached a significant difference with root tips and forks ($p < 0.05$; Supplementary data, Table S6), indicating that ABA level negatively regulated the root development of *C. bungei*. In *Arabidopsis thaliana*, GA_3 could positively control the growth of root apical meristem and the formation of lateral roots (Achard et al., 2009; Ubeda-Tomás et al., 2009). In particular, in our study, GA_3 concentration was significantly positively correlated with root morphological parameters (except the average diameter) and root activity (r was in the range of 0.410–0.657, $p < 0.01$, or 0.05; Supplementary data, Table S6), indicating that GA_3 could significantly promote the growth and development of roots of *C. bungei* seedlings. However, in *Medicago truncatula* (Fonouni-Farde et al., 2019) and *Populus* (Gou et al., 2010), it was found that GA_3 had a negative impact on root growth. These results suggested that the regulation of root structure by phytohormones varies among plant species. In addition, ABA can also exert at least partial effects on root growth by crosstalk with hormone signals such as GA_3 and IAA (Gou et al., 2010; Harris, 2015). In the present study, the ratios of IAA/ABA, CTK/ABA, and GA_3/ABA were significantly positively correlated with most of the root morphological parameters except the average diameter ($p < 0.01$, or 0.05; Supplementary data, Table S6), which also confirmed the above conclusions.

4.6 Activities of key enzymes and expression of genes in N metabolism

Within a certain concentration range, N application could increase the activity of N metabolizing enzymes (NR, NiR, GS, GOGAT, GDH) and up-regulate the expression levels of related genes in woody plant seedlings (Luo et al., 2013; Qin et al., 2021; Sun et al., 2021). In our study, regardless of AMF inoculation status, with the increase of N concentration, the relative expression levels of *NR*, *Fd-NiR*, and *GS* in the roots of *C. bungei* seedlings were significantly up-regulated at first and then notably down-regulated, the expression of *GDHA* was gradually up-regulated. However, with the increase of N concentration, the enzyme activities and the expression trend of other genes were not consistent between mycorrhizal and non-mycorrhizal seedlings. It indicated that, on the one hand, there were differences in N metabolism between mycorrhizal and non-mycorrhizal *C. bungei* seedlings in response to N application. On the other hand, moderate N levels could effectively improve the process of N metabolism. Evidence suggests that AMF can induce genes and enzyme activities related to nutrient transport and assimilation to help host plants absorb and utilize more N and P (Khan et al., 2021; Ren et al., 2022). It is worth noting that in our study, within a certain range of N concentration, AMF inoculation enhanced the activities of key enzymes of N metabolism in the roots of *C. bungei* seedlings, increased plant N concentration, but unexpectedly, the expression levels of most related enzyme genes decreased. The discordance between the enzyme activity and the expression of related genes has been similarly reported in *Oryza sativa* (Cao et al., 2008) and *Zea mays* (Kaldorf et al., 1998). Possible explanations are that, on the one hand, changes in enzyme activity may be the result of integrated regulation by multiple genes (Cao et al., 2008). On the other hand, it is also possible that after mycorrhizal formation in plants, the absorption and transport of N by the mycelial pathway dominated, while the direct root absorption pathway was inhibited (Duan et al., 2016), and the absorption of P by arbuscular mycorrhizal pathway also existed in a similar situation (Smith and Smith, 2011). It has been proved that arbuscules have metabolic activity, especially in plant nutrient exchange and plant respiration (Cox and Tinker, 1976; Kaldorf et al., 1998).

After N addition, the expression of high-affinity transporters *NRT2.1*, *NRT2.2*, and *NRT2.3* in *T. aestivum* roots decreased (Duan et al., 2015), which was in accordance with our result that *NRT2.4* and *NRT2.5* in the roots of *C. bungei* seedlings down-regulated sharply with the increase of N levels. This is not unexpected, since high-affinity NRT genes are dominant in regulating N uptake at low exogenous NO_3^- concentrations (Pérez-Tienda et al., 2014; Duan et al., 2015). The expression of NRT in plants involved in the direct N absorption pathway may also be regulated by AMF colonization in host plants (Duan et al., 2015). In our study, under the same N levels, the relative expression levels of *NRT2.4* and *NRT2.5* in the roots of seedlings inoculated with AMF down-regulated compared with those of non-inoculated treatments. Duan et al. (2015) reported that the expressions of *NRT1.2*, *NRT2.1*, *NRT2.2* and *NRT2.3* in *T. aestivum* roots, except *NRT1.1*, may be locally down-regulated by specific AMF, regardless of whether N was applied. This is in keeping with the results of this study, and also supports the hypothesis that mycorrhizal symbiosis may reduce direct pathway N uptake. In addition, in the present study, the expression of *NRT2.7* was higher at medium to high N levels after AMF inoculation than that in the non-inoculated treatments. Previous studies have also found that *NRT2.3* expression was up-regulated in *Lycopersicon esculentum* after *G. intraradices* inoculation (Hildebrandt et al., 2002). It can be speculated that the regulation of AMF inoculation on the expression of NRT genes in plant roots varies with plant species and gene types (Duan et al., 2016).

5 Conclusions

Medium N could significantly improve the physiological metabolism and growth of *C. bungei* seedlings. Compared with the non-inoculated treatments, inoculation with *R. intraradices* could significantly improve photosynthesis, promote the absorption of N and P, and change root architecture and hormone levels under low to medium N levels. In particular, under N addition, AMF inoculation could improve the absorption and assimilation of N in seedlings by regulating the expression levels of key enzyme genes of N metabolism and nitrate transporter genes in roots, and enhancing the activities of the key enzyme. We preliminarily concluded that *R. intraradices* could improve the growth and performance of *C. bungei*, and moderate N levels contribute to the beneficial effects of this mycorrhizal fungus. However, only one fungus was used in this study, but different fungal species may perform different levels of benefits to plants under variable environmental conditions. Whether the results of this study could be extrapolated to other species of mycorrhizal fungi and/or to *C. bungei* growing in the field still needs further study.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/under the accession number PRJNA907402.

Author contributions

Conceptualization, CW and WC; Formal analysis, WC, XM, PM, and JC; Funding acquisition, YZ and CW; Methodology, WC, XM, JC, PM, XT, GM, and KX; Supervision, CW; Writing–Original Draft Preparation, WC; Writing–Review and Editing, WC, YZ, and CW. All authors contributed to the article and approved the submitted version.

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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

Supplementary material

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The shaping of onion seedlings performance through substrate formulation and co-inoculation with beneficial microorganism consortia

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Introduction: Smart management in crop cultivation is increasingly supported by application of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting microorganisms (PGPM), which sustain soil fertility and plant performance. The aim of this study was the evaluation of the effects of consortia composed of (*Claroideoglomus claroideum* BEG96, *Claroideoglomus etunicatum* BEG92, *Funneliformis geosporum* BEG199, *Funneliformis mosseae* BEG 95, and *Rhizophagus irregularis* BEG140) and PGPM (*Azospirillum brasilense* – AZ, or *Saccharothrix* sp. – S) on onion cultivated in growing media with a composition corresponding to a degraded soil.

Methods: Three types of substrate formulations were used, with peat:sand ratios of 50:50, 70:30, 100:0 (v:v). The analysis of substrate parameters crucial for its fertility (pH, salinity, sorption complex capacity, and elements' content) and characteristics reflecting onion seedlings' performance (fresh weight, stress biomarkers, and elements' content) was performed.

Results: AMF colonized onion roots in all treatments, showing increasing potential to form intercellular structures in the substrates rich in organic matter. Additionally, co-inoculation with PGPM microorganisms accelerated arbuscular mycorrhiza establishment. Increased antioxidant activity and glutathione peroxidase (GPOX) activity of onion roots sampled from the formulations composed of peat and sand in the ratio of 100:0, inoculated with AMF+S, and positive correlation between GPOX, fresh weight and antioxidant activity of onion roots reflected the successful induction of plant acclimatization response. Total phenols content was the highest in roots and leaves of onion grown in substrates with 70:30 peat:sand ratio, and, in the case of roots, it was correlated with AMF colonization parameters but not with antioxidant activity.

Discussion: AMF and PGPM efficiency in supporting onion growth should be linked to the increased onion root system capacity in mineral salts absorption, resulting in more efficient aboveground biomass production. AMF and PGPM consortia were effective in releasing minerals to soluble fraction in substrates rich in organic matter, making elements available for uptake by onion root system, though this phenomenon depended on the PGPM species. Microorganism consortia enhanced onion seedlings' performance also in substrates with lower content of organic carbon through plant biofertilization and phytostimulation

KEYWORDS

Allium cepa L., mycorrhiza, *Azospirillum brasilense*, degraded soil, stress biomarkers

1 Introduction

Microbial interactions in the rhizosphere are intensified by released plant root exudates, which are the main food source for microorganisms, increasing their population density and activity (Raaijmakers et al., 2009; Gavilanes et al., 2021). Plants benefit from root-associated microorganisms through various activities (phytohormones, nutrient supplementation, and pathogen suppression), ultimately increasing growth, health, and yield, thus decreasing the dependence on harmful chemicals and their after-effects (Raaijmakers et al., 2009; Syed and Tollamadugu, 2019). The potential of microbial symbioses is currently considered a valuable contribution to precision agriculture (Ma, 2019). Such inoculation can increase crop yield by enhancing nutrient uptake and pathogen biocontrol (Parnell et al., 2016; Nacoon et al., 2021). Significant changes in the indigenous microflora of soil by introducing single cultures of exogenous microorganisms appear to be complex, and the efforts have not always been successful (Onishchuk et al., 2017; Iturralde et al., 2019). Therefore, the physiological and ecological compatibility of beneficial and effective microorganisms is an important factor that increases the probability of shifting and controlling the "microbiological equilibrium" of the rhizosphere to encourage the growth, yield, and health of crops (Bidondo et al., 2016).

Onion (*Allium cepa* L.) is an economically important crop that is characterized by high environmental and cultivation demands. Onion seedlings grow relatively slowly and develop shallow weakly branched roots without root hairs, which shows a low efficiency in uptaking soil water and nutrients; thus, they are vulnerable to their deficiency since the beginning of the crop cycle (Sekara et al., 2017; Golubkina et al., 2020). Therefore, it is difficult to supply onion seedlings with sufficient amounts of soil nutrients to ensure optimum growth, especially in the early growth stages (Serra and Currah, 2002). According to Lee and Lee (2014) the optimum ranges of soil parameters should be as follows: pH 6.0–6.5; organic matter 25–35 mg kg^{-1}; P 129–168 mg kg^{-1}; exchangeable K, Ca, and Mg, 0.39–0.50, 5.8–6.7, and 2.1–2.7 $cmol_c$ kg^{-1}, respectively. The rate of nutrient uptake depends on the growth stage, as the requirement for N is high during seedling production and subsequent vegetative growth (Mosse et al., 1981; Drost and Koenig, 2002). Simultaneously, high levels of N may cause leaching, denitrification, and increased susceptibility to pests and diseases (Sekara et al., 2017). Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting microorganisms (PGPM) can be successful inoculants when applied at the beginning of the onion vegetation stage to balance the seedling nutritional status (Deressa and Schenk, 2008; Colo et al., 2014). However, the potential benefits justify the investigation of the mechanisms of interactions between AMF, PGPM, and onion plants (Lone et al., 2015; Rozpadek et al., 2016). The research undertaken so far covers the selection of the most effective microorganisms to establish successful symbiose/mutualism in particular environmental conditions and farming systems (Bolandnazar, 2009; Galván et al., 2009; Albrechtová et al., 2012; Caruso et al., 2018), and the implications of these ecological relationships on onion plant performance, especially with respect to bulb yield and quality (Mollavali et al., 2015; Shinde and Shinde, 2016; Fredotovic and Puizina, 2019; Petrovic et al., 2020).

Among the AMF associated with onion roots in different environments and cultivation systems, *Claroideoglomus* spp., *Funneliformis* spp., and *Rhizophagus* spp. are the most widespread (Charron et al., 2001; Bolandnazar et al., 2007; Bolandnazar, 2009; Galván et al., 2009; Mohamed et al., 2014; Mollavali et al., 2015). Concerning the plant growth promoting bacteria, the effects of inoculation with *Azotobacter* sp., *Sphingobacterium* sp., and *Burkholderia* sp. were investigated in onion (Tinna et al., 2020). Hong et al. (2019) demonstrated endophytic infection of cyst-like cells after onion inoculation with *Azospirillum brasilense*. Nevertheless, knowledge regarding the relationship between onions and *Azospirillum* spp. is limited.

Less attention has been paid to AMF and PGPM co-inoculation during the initial stages of onion ontogeny, particularly during transplant production in controlled conditions, where all types of substrates can be used, shaping distinct conditions for the establishment of ecological relationships in the rhizosphere (Joe et al., 2012; Colo et al., 2014; Mohamed et al., 2014; Ma, 2019). However, such experiments provide clear insight in soil–plant–microorganisms system and allow the identification of the most beneficial AMF and PGPM consortia for onions in the juvenile

growth stage. Moreover, the established symbiosis can be continued under field conditions bringing multiple advantages during the overall growing cycle, including increased plant nutrient uptake, imparted biotic and abiotic stress tolerance, and better bulb quality characteristics (Tinna et al., 2020).

The aim of this study was to evaluate the effect of onion inoculation with AMF + *A. brasilense* or *Saccharothrix* sp. consortia on onion seedling development in relation to the substrate formulation. The influence of AMF and PGPM consortia on the growing medium was assessed with respect to the parameters crucial for fertility (pH, salinity, sorption complex capacity, and C, N, P, K, Mg, Na, and Ca concentrations) and onion seedling biochemical characteristics reflecting plant performance (fresh weight, stress biomarkers, and concentration of K, P, Mg, Na, and Ca).

2 Materials and methods

2.1 Material and experimental protocol

The onion (*Allium cepa* L.) cultivar 'Stalagmit' F_1 (Moravoseed, Ltd, CZ) was used for this research. The experiment consisted of seven treatments, each with three replicates (eight plants per replicate). The experimental treatments included three non-inoculated substrate formulations (control) and four substrate formulations inoculated with consortia of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting microorganisms (PGPM). The treatments and abbreviations used in this study are listed in Table 1.

Sowing peat (Klasmann, DE) and sand (local sources) were used. Calcium carbonate in remarkable amounts was used to maintain a pH of approximately 6.5. The remaining substrate parameters are shown in Supplementary Table 1. Before sowing, the substrates were autoclaved at 120°C for 60 min, and then inoculated with AMF and PGPM, namely *A. brasilense* (Tarrand et al., 1978) (CCM 3862) (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic), or *Saccharothrix* sp. (ST2020) (AMF + S). *Saccharothrix* species and strain details are currently confidential because of the patent pending. AMF mix was composed of *Claroideoglomus claroideum* BEG96, *Claroideoglomus etunicatum* BEG92, *Funneliformis geosporum* BEG199, *Funneliformis mosseae* BEG 95, and *Rhizophagus irregularis* BEG140 (Symbiom Ltd., Lanškroun, Czech Republic). The AMF mix contained 145 spores per gram, and it was applied at a dose of 0.015 g per cm^3 of substrate. Inoculation with PGPM was performed on onion seeds soaked for 30 min in *A. brasilense* and *Saccharothrix* sp. (ST2020) suspensions, respectively. *A. brasilense* culture were grown for one week on LuriaAgar media at 24°C (HiMedia Laboratories, Mumbai, India) and ST2020 was grown in a yeast-malt extract liquid medium (ISP2) from the International Streptomyces Project (Shirling and Gottlieb, 1966) at 28°C with agitation at 90 rpm for 10 days. Next, both cultures were homogenized using sterile ceramic beads and the concentration of both suspensions was adjusted to 10^8 CFU/ml in sterile physiological saline (Joe et al., 2012). The substrate parameters selected at the beginning of the experiment are listed in Supplementary Table 1.

TABLE 1 Treatments and their abbreviations used in this study.

Abbreviation	Peat:sand (v:v) ratio	Inoculation
C 50	50:50	–
AMF + AZ 50	50:50	arbuscular mycorrhizal fungi mix (AMF) + *Azospirillum brasilense* (AZ)
C 70	70:30	–
AMF + AZ 70	70:30	AMF + AZ
C 100	100:0	–
AMF + AZ 100	100:0	AMF + AZ
AMF +S – 100	100:0	AMF + *Saccharothrix* sp. ST2020 (S)

2.2 Cultivation conditions

The seeds were sterilized for 10 min in 0.5% sodium hypochlorite, washed with sterile distilled water, and sown in Teku V9 containers (square, 9 × 9 cm; height, 8 cm; volume, 512 cm^3) on 2 April 2020. Seedlings were grown in a phytochamber Fytoscope 4400 (PSI, Czech Republic) at a temperature of 20/18°C (day/night), relative air humidity 80%, light intensity 120 µmol m^{-2} s^{-1} at the germination stage; 18/16°C, 70%, 200 µmol m^{-2} s^{-1}, respectively, at the beginning of the cotyledon stage, and 21/18°C, 75%, and 200 µmol m^{-2} s^{-1}, respectively, after the first leaf stage, with 16 h of daylight. Seedlings were irrigated with a measured volume of tap water. Urea was used for fertilization (5 May, liquid 0.2% solution, 20 cm^3 per pot in irrigation doses), later the fertilizer YaraTera Kristalon 20 + 5 + 10 + 2 (N, P, K, Mg) Azur was applied each week until 18 June as a 0.1% liquid solution at a dose of 20 cm^3 per pot.

2.3 Substrate sampling and analyses

At the end of the experiment (26 June 2020), samples of homogenized substrate (150 g per treatment) were collected, air-dried, and basic parameters were determined, including pH (H_2O and KCl) using the potentiometric method, salinity with the conductometric method, and the sorption complex capacity using Kappen's method. Total N and organic C were determined via elemental analysis using a Vario Max Cube apparatus (Elementar Analysensysteme GmbH, Langenselbold, Germany). The available forms of macroelements (K, P, Mg, Na, and Ca) after extraction with acetic acid were determined by inductively coupled plasma atomic emission spectrometry using a Perkin Elmer Optima 7600 spectrometer (PerkinElmer, US).

2.4 Plant material sampling

Onion plants were collected on 26 June 2020, all leaves were cut with scissors, and roots were completely extracted from the

substrate and washed with distilled H_2O. Samples were stored immediately after harvest in a deep freezer (TSE240VGP, Thermo Fisher Scientific, USA) at temperature −80°C until analysis.

2.5 Fresh weight

Total leaf and root fresh weights (FWs) per plant were measured using a Sartorius A120S balance (Sartorius AG, Germany).

2.6 Staining and microscopy

For colonization analysis, four randomly selected 10 mm long root section per replicate were sampled, fixed in a formaldehyde: ethanol:acetic acid 10%:50%:5% v/v solution (FAA) and stored in the dark at 4°C before staining for microscopy (Schmidt et al., 2008). The roots were then rinsed in distilled H_2O, cleared in 2% KOH for 1 h at 50°C, and washed in distilled H_2O (4 × 3 min). Roots were stained in a tube with a mixture consisting of WGA AF 594 conjugate (Invitrogen, USA) (50 µg ml^{-1}), concanavalin A AF 647 (Invitrogen, USA) (50 µg ml^{-1}), and acid fuchsine (3%) at a ratio of 1:1:1 for 4–5 h at room temperature, rinsed in 1×PBS (4 × 3 min), and incubated for 12 h in 1×PBS to remove all excess stain. Before mounting on the slide, a few drops of Hoechst stain were added to the slides with the roots (Vierheilig et al., 2005). Mycorrhizal colonization was quantification by evaluating four root segments per replication for mycelia, vesicles, arbuscules, and spore presence. Ten observations were made at each root segment, and the calculation of colonization was based on the ratio of segments with AMF structures to roots without fungal structures (Alarcón and Cuenca, 2005).

Confocal microscopy was completed using the LSM 800 (Carl Zeiss, Germany) microscope at 590/617 nm excitation/emission for WGA AF 594, 650/668 nm for concanavalin A AF 647, and 350/461 nm for Hoechst stain. The lens used was a ×20/0.8 NA. Images were processed using the Zen Blue software (Carl Zeiss, Germany).

2.7 Analyses of stress biomarkers

The antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH radical) was measured in onion root and leaf samples. For the extraction, 2.5 g of homogenized onion roots or leaves per repetition was weighed, ground with 10 ml 80% methanol, and centrifuged (3,492×*g*, 10 min, 4°C). The mixture containing 0.1 cm^3 supernatant and 4.9 cm^3 0.1 mM DPPH in 80% methanol was incubated in darkness at 20–22°C after 15 min, the absorbance was measured at λ = 517 nm using a UV–VIS Helios Beta spectrophotometer (Thermo Fisher Scientific, Inc., US). Antioxidant activity was calculated using the following formula: DPPH (%) = ((A0 − A1)/A0) × 100, where A0 is the absorbance of the reference solution and A1 is the absorbance of the test solution (Molyneux, 2004).

Total phenol content was determined using the modified Folin–Ciocalteu colorimetric method (Djeridane et al., 2006). For the extraction, a 2.5 g sample of plant material was ground, mixed with 10 cm^3 of 80% methanol, and centrifuged (3,492×*g*, 15 min, 4°C). The glass tubes were filled with 0.1 cm^3 of the supernatant and 2 cm^3 of sodium carbonate, left for 5 min, and then 0.1 cm^3 of Folin–Ciocalteu's reagent mixed with deionized water (1:1 v/v) was added. After 45 min, phenols were determined by the colorimetric method at 750 nm using a UV–VIS spectrophotometer against a reference solution. The total phenol value was expressed as gallic acid equivalents (mg GAE) per gram of FW.

Glutathione peroxidase activity (GPOX) was measured according to Lück (1962). Plant samples (2.5 g) were ground in an ice bath with 20 cm^3 of a 0.05 M potassium phosphate buffer and centrifuged (3,492×*g*, 15 min, 4°C). The reaction mixture contained diluted supernatant, 0.05 M potassium phosphate buffer, p-phenylenediamine, and hydrogen peroxide. The absorbance at 485 nm was recorded at 60 s intervals for 2 min using a UV–VIS spectrophotometer. GPOX activity is expressed as units (U) per g FW per min.

2.8 Element concentration in plant tissues

To determine the element concentrations in onion roots and shoots, 0.5 g dry weight (DW) of plant material samples were mineralized in a mixture of HNO_3 and H_2O_2 at 1:3 (v:v), then 2 cm^3 HNO_3 per 100 cm^3 distilled water was added. The samples were concentrated 5-fold, and then the concentrations of elements (K, P, Mg, Na, and Ca) were determined by atomic emission spectroscopy (ICP) with an Optima 7600 spectrophotometer (Perkin Elmer, US) using the method described by Pasławski and Migaszewski (2006).

2.9 Statistical analysis

The substrate and plant samples were analyzed for every treatment in three technical replicates, each consisting of eight plants. The data were tested for normality of distribution according to the Shapiro–Wilk method and homogeneity of variances using the Levene test. The ANOVA was applied to test significance levels at $p \leq 0.05$ (*), $p \leq 0.01$ (**), or $p \leq 0.001$ (***) and non-significant (ns), followed by Tukey's honest significant difference (HSD) test to separate means into homogenous groups. The results were also examined using Pearson's correlation coefficient (r) between the analyzed parameters. Principal component analysis (PCA) and cluster analysis (CA) were performed to precisely demonstrate and analyze the data and their relationships. Correlations and PCA were used as supplementary statistical methods that enabled and expanded the analysis of the presented data and made additional relationships visible between the experimental treatments and variables. The results using raw data are presented for PCA because no substantial differences appeared between the raw and standardized data. Modeling was performed with "general regression models (GRM)", a stepwise regression procedure with backward elimination was performed to remove the least significant variables. Multiple regression screening, based on the investigated variables, was performed to determine the most accurate compatibility. Coefficients of determination (R2) and standard

errors of estimation (SEe) were calculated to assess the accuracy of the models. All analyses were performed using Statistica 13.3 (Dell, Inc., USA).

We used multiple linear regression analysis to develop models that describe how the y-variable (FW of leaves, roots, and leaves + roots of onion seedlings) is related to several explanatory variables xn (substrate and plant parameters). After stepwise regression analysis with backward elimination of all investigated parameters variables, did not allow to formulate clear model, so variables affecting simultaneously the FW were screened and two models were developed. The first model for onion FW focused on whole-seedling FW and had the following form: FW of roots + leaves = 1.120 ∗ Mg in substrate − 0.004 ∗ Na in the substrate. The input data were the mean content of elements in the substrate and mycorrhizal parameters. The coefficient of determination is high (R2 = 0.968). This means that the regression line approximates the real data points quite well and that the model can explain approximately 97% of the onion seedling DW variation with $p \leq 0.05$.

The second model was revealed for onion root FW, with input data including only the colonization rate, arbuscule abundance, and vesicle abundance:

$$\text{Roots FW} = 1.18 + 0.102 * \text{colonization rate} - 0.0917 * \text{arbuscule abundance} - 0.046 * \text{vesicles abundance}$$

The coefficient of determination is high (R2 = 0.944). with $p \leq 0.05$. According to multiple regression models, the total onion seedling FW can be predicted based on the Mg and Na content in the substrate, whereas root FW can be predicted based on the analyzed AMF parameters.

3 Results

3.1 Fresh weight of onion as affected by AMF and PGPM application to investigated growing media

The fresh weight (FW) of onion leaves and roots at harvest was 10.94 g and 8.54 g on average, respectively, and exhibited significant dependence on the experimental treatments and plant tissues (Figure 1; Supplementary Table 2). The lowest root FW value was recorded in the AMF + AZ 50 treatment (0.72 g) and the decrease in root FW was 43.3% compared to the control C 50 treatment (1.26 g). The roots of onions sampled from all treatments inoculated with PGPM (AMF + AZ 70, AMF + AZ 100, and AMF + S 100) developed similar root FW as the non-inoculated control with corresponding peat:sand formulations (C 70 and C 100). The FW of onion seedling leaves grown on all substrates inoculated with AMF and PGPM (AMF + AZ 50, AMF + AZ 70, AMF + AZ 100, and AMF + S 100) were significantly higher than in the corresponding controls, where similar peat:sand ratios were used (C 50, C 70, and C 100, respectively). The differences in FW accounted for 18.0% for the AMF + AZ 50 treatment, 22.0% for AMF + AZ 70, 19.7% for AMF + Z 100, and 20.6% for AMF + S 100. Analysis of the total onion seedling FW (leaf + root) revealed that the highest values were recorded for AMF + AZ 100 and AMF + S 100 treatments, 3.40 and 3.57 g, respectively). Onion root FW was

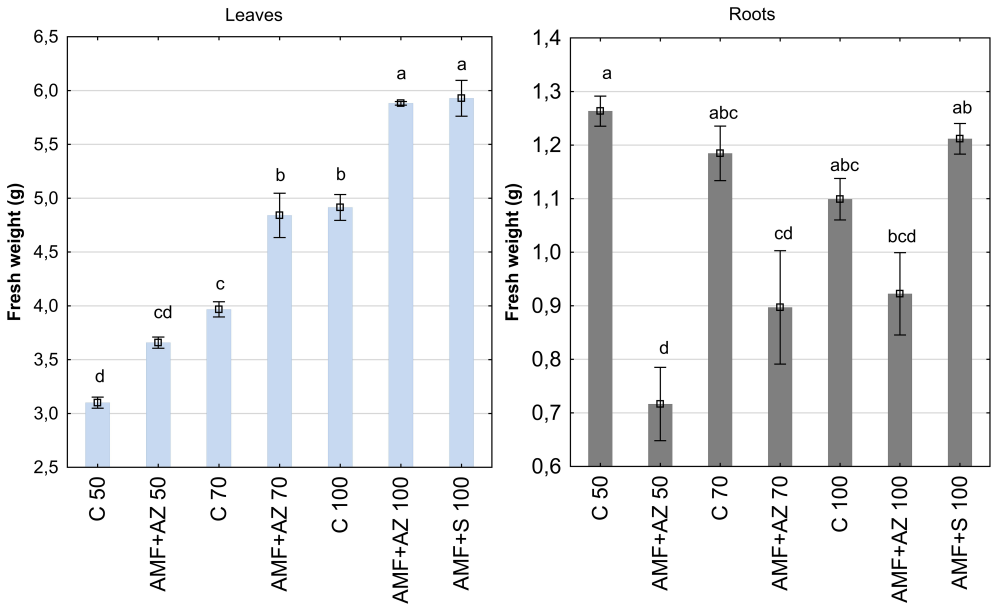

FIGURE 1
Mean values (± SE) of the fresh weight of onion leaves (left) and roots (right). Bars marked with different letters represent different values at $p \leq 0.05$, according to Tukey's test. C 50, peat:sand ratio 50:50 (v:v) without inoculation; AMF + AZ 50, peat:sand ratio 50:50 (v:v) inoculated with arbuscular mycorrhizal fungi (AMF) and *Azospirillum brasilense* (AZ); C 70, peat:sand ratio 70:30 (v:v) without inoculation; AMF + AZ 70, peat:sand ratio 70:30 (v:v) inoculated with AMF and AZ; C 100, peat:sand ratio 100:0 (v:v) without inoculation; AMF + AZ 100, peat:sand ratio 100:0 (v:v) inoculated with AMF and AZ; AMF + S 100 peat:sand ratio 100:0 (v:v) inoculated with AMF and ST2020 (S).

negatively correlated with Ca and Mg content in the leaves (Supplementary Table 3). Leaf FW was positively correlated with Ca and Mg content in roots, but negatively correlated with Na content in roots. Moreover, leaf FW was positively affected by Ca, Mg, Na, K, and P content in the substrate, AMF colonization rate, arbuscule, and vesicle abundance in the roots.

3.2 Root colonization of onion as affected by AMF and PGPM application to investigated growing media

The data in Figure 2 show a high level of root colonization rate in the AMF + AZ 100 and AMF + S 100 treatments (88.3 and 90.0%, respectively). Results of observations performed with confocal microscopy showed successful root colonization of AMF in all inoculated treatments (Figure 3). No colonization was observed in the control treatment.

The arbuscules were present in all treatments inoculated with AMF, with the highest value observed in AMF + AZ 100 and AMF + S 100 treatment (73%) (Figure 2). Vesicles were observed in onion roots sampled from all inoculated substrate formulations in similar amounts 53%–60% (Figure 2). Described parameters indicated the intensive process of mutual symbiosis resulting in the parallel development of AMF, namely colonization rate, arbuscule abundance, and vesicles abundance. This process was additionally confirmed by PCA analysis, showing a close distance between the eigenvectors relevant to colonization rate, arbuscule, and vesicle abundance. Mycorrhization parameters of onion roots (colonization rate, arbuscule abundance, and vesicle abundance) were positively correlated with K content in the substrate. Mycorrhization parameters were positively correlated with antioxidant activity and total phenols in root tissues but negatively correlated with root FW (Supplementary Table 3). Concerning leaf tissues, mycorrhization parameters were positively correlated with leaf FW and Ca, Mg, Na, and P.

3.3 Analyses of stress biomarkers of onion as affected by AMF and PGPM application to investigated growing media

In general, the experimental treatments significantly affected the antioxidant activity of onion seedlings, although no general tendencies were observed. The antioxidant activity, measured as DPPH scavenging activity, was higher in onion seedling roots sampled from the treatments inoculated with AMF and PGPM, namely AMF + AZ 50 (11.2%), AMF + AZ 100 (18.0%), and AMF + S 100 (19.8%), compared to the corresponding controls (C 50 and C 100) at a significance level of 0.05. For onion leaves, the antioxidant activity of plant samples collected from the AMF + AZ 70, AMF + AZ 100 treatments was lower (6.6% and 17.5%, respectively, at a significance level of 0.05) than that of the corresponding controls (C 70 and C 100) (Figure 4A; Supplementary Table 2). The antioxidant activity of onion root extracts was positively correlated with K content in the substrate, AMF colonization rate, arbuscule and vesicle abundance, and root K, Ca, Mg, and GPOX activity (Supplementary Table 3). The antioxidant activity of onion leaf extracts was not affected by soil characteristics or AMF symbiosis, but it was positively correlated with K, Mg, and P content in roots, as well as with GPOX activity in root tissues.

The total phenol content was 35.7% higher in onion seedling leaves than in roots based on the main effect analysis (Figure 4B; Supplementary Table 2). Onion roots sampled from plants

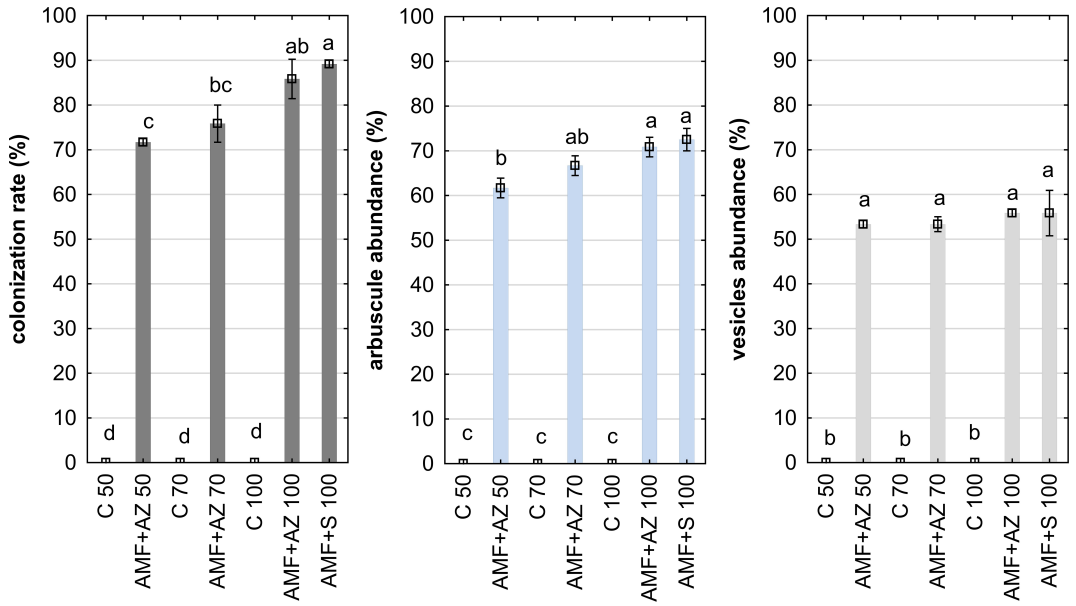

FIGURE 2
Mean values (± SE) of the colonization rate (left), arbuscule abundance (center), and vesicle abundance (right). Bars marked with different letters represent different values at $p \leq 0.05$, according to Tukey's test. Abbreviations are explained in Figure 1 and Table 1.

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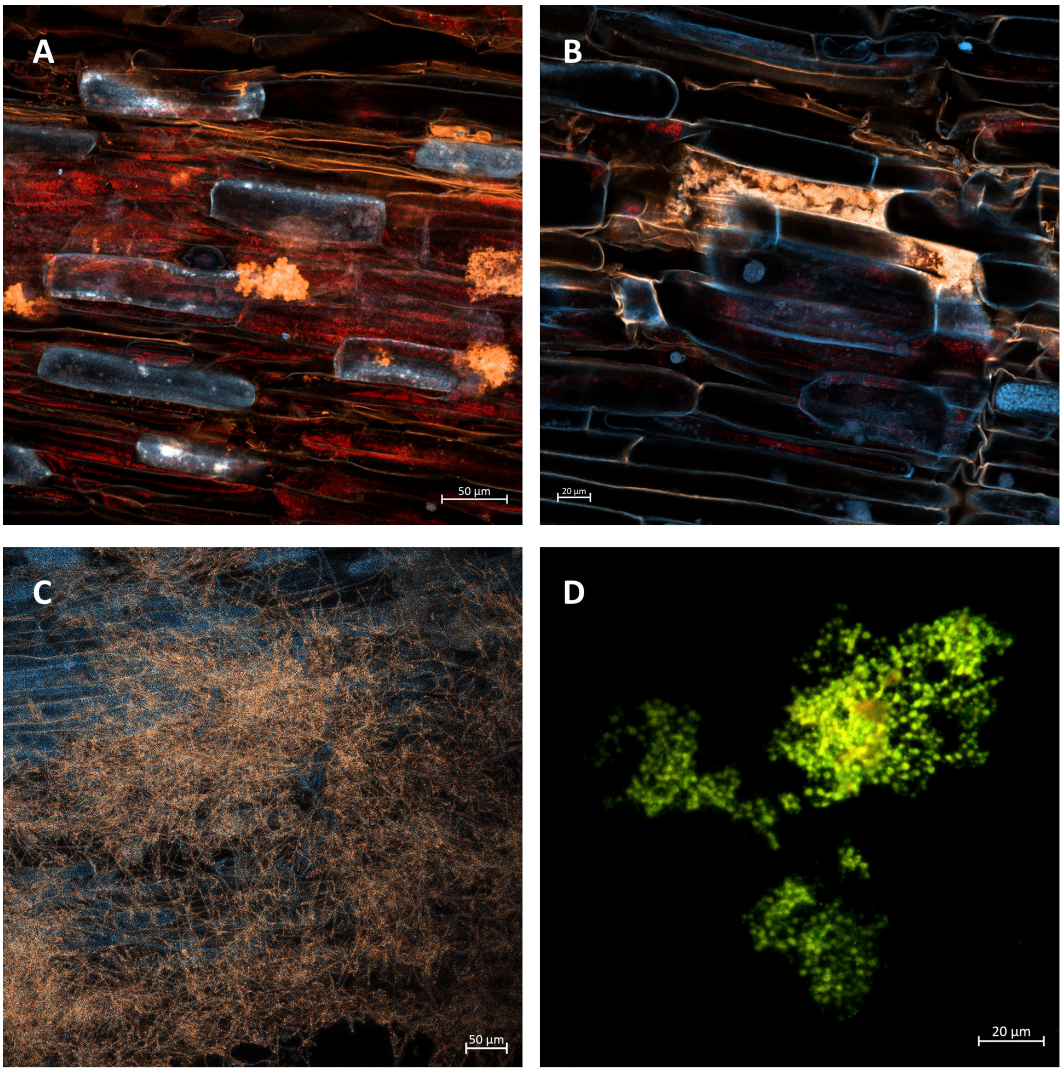

FIGURE 3
(A) Arbuscules (in orange color) developed within the tissue of onion root. Treatment 50 AMF + AZ. Bar = 50 µm. **(B)** Details of arbusculum in particular root cells (orange structure), treated with 100 AMF + AZ. Bar = 20 µm. **(C)** Grid of mycorrhizal mycelia surrounding the onion roots treated with 50 AMF + AZ. Bar = 50 µm. **(D)** Colonies of *Azospirillum brasilense* in the root-hair area. Treatment with 100 AMF + AZ. Scale bar = 20 µm.

inoculated with AMF + AZ 70 accumulated the highest number of phenolic compounds (9.56 g), 26.5% higher than the corresponding uninoculated control (C 70). In onion leaves, the highest number of total phenols was determined in AMF + AZ 50 and AMF + AZ 70 treatments (12.19 and 12.58 r, respectively), but only in the case of AMF + AZ 50, the mentioned compounds were accumulated at 21.5% higher extent than in plant leaves of corresponding control (C 50). Total phenols of onion root extracts were positively correlated with Ca content in roots, as well as K and Na contents in leaves (Supplementary Table 3). A negative correlation was noted between the total phenol content and all elements analyzed in the substrate.

The onion seedling roots showed three-fold higher glutathione peroxidase (GPOX) activity than the leaves, concerning the main effects (Figure 4C; Supplementary Table 2). The highest GPOX activity was noted in root samples from the AMF + S 100 treatment (3,470 µmol tetraguaiacol min^{-1} g^{-1}). The GPOX activity in roots sampled from inoculated AMF + AZ 50 and AMF + AZ 70 treatments was lower (by 5% and 8%, respectively) than that in samples from the corresponding non-inoculated controls (C 50 and C 70). The lowest GPOX activity was noted in onion leaf tissues sampled from treatments with addition of inoculants, namely AMF + AZ 50 and AMF + S 100 (833.3 and 833.8 µmol tetraguaiacol min^{-1} g^{-1}, respectively) as well as from non-inoculated C 100 treatment (825.0 µmol tetraguaiacol min^{-1} g^{-1}). The GPOX activity in onion leaves of AMF + AZ 70 and AMF + AZ 100 treatments was higher (by 22.0% and 26.3%, respectively) than that recorded in plant samples from the corresponding controls (C 70 and C 100). The GPOX activity of onion root extracts was positively correlated with Mg and negatively correlated with Na content in the roots (Supplementary Table 3). No positive correlations were observed between GPOX activity in onion leaf tissues and other soil or plant characteristics. The correlations between the investigated stress biomarkers and the other onion seedling root

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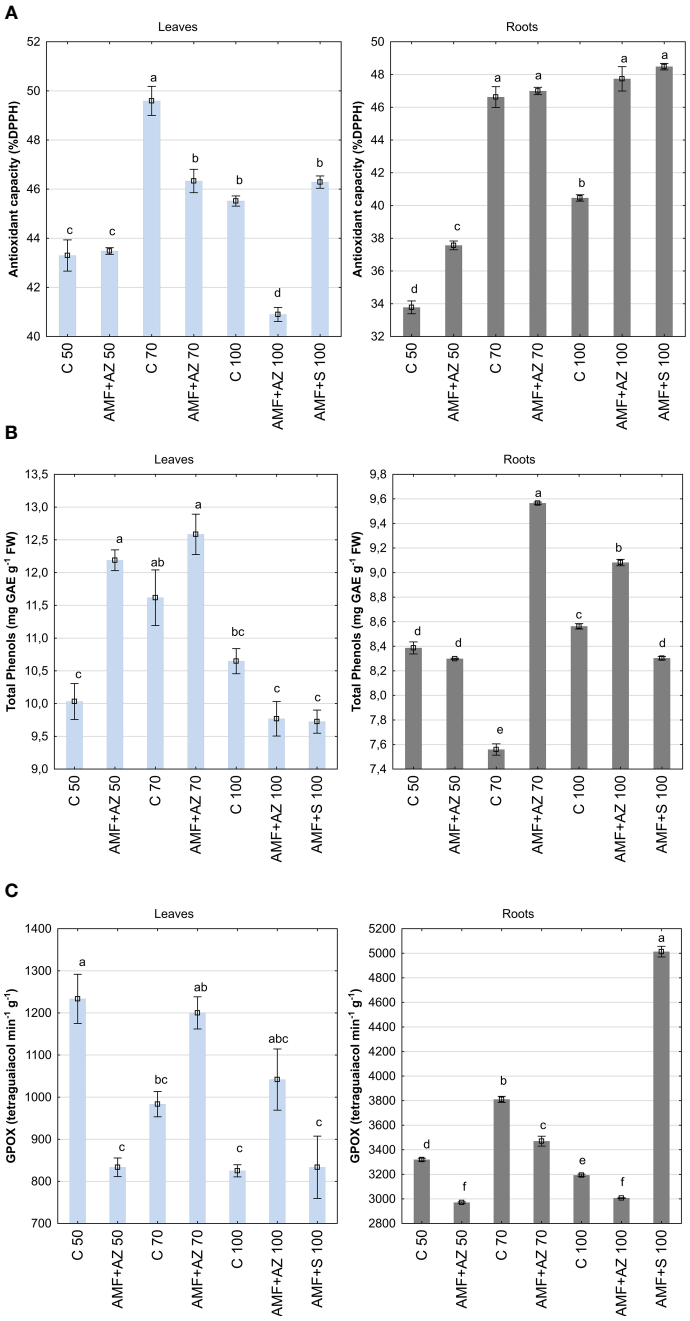

FIGURE 4
Mean values (± SE) of antioxidant activity **(A)**, total phenols **(B)**, and glutathione peroxidase (GPOX) activity **(C)** in the leaves (left) and roots (right) of onion seedlings. Bars marked with different letters are significantly different at $p \leq 0.05$, according to Tukey's test. Abbreviations are explained in Figure 1 and Table 1.

and leaf characteristics are illustrated in Figures 5C, D by the magnitude of the angles between the relevant eigenvectors.

3.4 Element's concentration in onion tissues as affected by AMF and PGPM application to investigated growing media

The mineral content of onion seedlings was significantly affected by substrate composition and microbial inoculation. K and Ca accumulated in higher amounts in onion seedling leaves than in roots. In contrast, Mg, Na, and P contents were higher in the onion seedling roots than in the leaves (Table 2).

The calcium content in the onion leaves and roots ranged from 5,624 to 16,648 mg kg^{-1} DW (Table 2; Supplementary Table 4). Under the conditions of the peat content in the substrate at the level of 50%, the Ca content in the roots was 5,624 mg kg^{-1} DW, while when cultivated on pure peat, this value increased to 7,649 mg kg^{-1} DW. In onion roots cultivated in substrates with inoculation (AMF + AZ 70 and AMF + AZ 100), a significant increase in Ca

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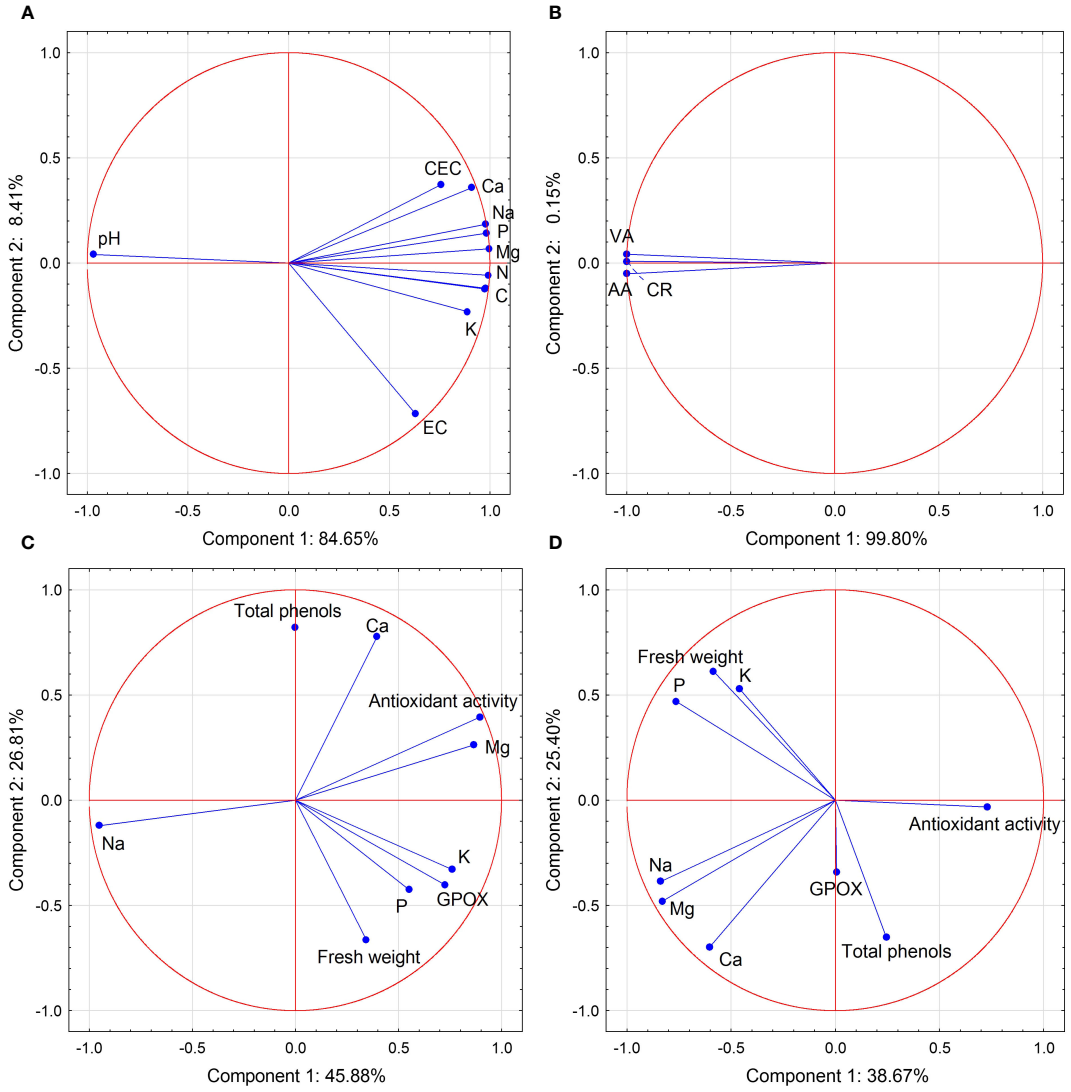

FIGURE 5
Biplot of PCA of the feature space built using data on the biochemical characteristics of the substrate after cultivation **(A)**, root colonization **(B)**, onion roots **(C)**, and leaves **(D)**. Circles represent observations in the principal component space, whereas vectors indicate the contributions of each feature to the first (x axis) and second (y axis) PCs. EC, cation exchange capacity; EC, electrical conductivity; VA, vesicle abundance; AA, arbuscule abundance; CR, colonization rate; GPOX, glutathione peroxidase activity.

content was observed in comparison to the corresponding controls (C 70 and C 100). Concerning the Ca content in onion leaves, a significant increase was found in plant material collected from the AMF-S 100 treatment compared to the C 100 treatment.

The Mg content in the tested plants ranged from 2,498 to 7,785 mg kg^{-1} DW (Table 2; Supplementary Table 4). A significant increase in the concentration of Mg in the onion root tissues by 25%, and in the leaf tissues by 15% was observed as a result of inoculation. The roots collected from the inoculated AMF + AZ 70 and AMF + S 100 treatments contained the highest amount of Mg, but these values were not significantly different from those of the corresponding controls (C 70 and C 100). In the case of onion leaves, inoculation significantly increased Mg content in the AMF + AZ 100 and AMF + S 100 treatments compared to that in the corresponding control (C 100).

In general, approximately 30% more sodium was observed in plants inoculated with substrates than in the corresponding control without mycorrhiza. The level of Na determined in onion roots of the inoculated AMF + AZ 100 and AMF + S 100 treatments was significantly lower than that in the plant material from the corresponding control (C 100). In the case of Na content in onion seedling leaves, no significant differences were noted for the treatments.

There was no difference in the K content of the biomass of plants grown on substrates with and without mycorrhiza. There was also no effect of increasing the proportion of peat in the substrate on the shaping of K content, which ranged from 13,626 to 32,175 mg kg^{-1} DW. Onion roots sampled from the AMF + AZ 70 treatment contained lower amounts of K than the corresponding control (C 70). Similarly, onion leaves of AMF + AZ 70 accumulated less K

TABLE 2 Effects of soil microbial inoculants on mineral content in onion seedling roots and leaves (mg kg^{-1} DW) (mean values ± SD).

Treatment	Ca	Mg	Na	K	P
Roots					
C 50*	5,624 ± 301 b **	3,462 ± 20 d	36,255 ± 224 ab	13,640 ± 14 d	4,354 ± 23 ab
AMF + AZ 50	6,530 ± 97 b	5,352 ± 61 c	37,240 ± 561 ab	14,319 ± 10 cd	3,142 ± 18 b
C 70	6,642 ± 417 b	7,162 ± 283 ab	29,522 ± 351 cd	17,571 ± 312 a	4,475 ± 198 a
AMF + AZ 70	10,927 ± 121 ab	7,785 ± 777 a	28,832 ± 1,968 cd	15,046 ± 417 bc	4,537 ± 259 a
C 100	7,649 ± 206 b	6,636 ± 46 abc	38,285 ± 297 a	15,811 ± 244 b	4,285 ± 54 ab
AMF + AZ 100	16,648 ± 560 a	6,190 ± 818 bc	31,671 ± 4,806 bc	14,954 ± 509 bc	3,759 ± 1038 ab
AMF + S 100	9,981 ± 337 b	7,487 ± 695 ab	25,450 ± 1,823 d	15,923 ± 688 b	4,278 ± 444 ab
Leaves					
C 50	11,356 ± 99 c	3,082 ± 2 b	12,956 ± 214 a	29,185 ± 395 b	2,439 ± 78 c
AMF + AZ 50	14,675 ± 1,444 a	3,996 ± 279 a	13,074 ± 49 a	26,718 ± 738 c	2,843 ± 205 b
C 70	11,355 ± 905 c	3,088 ± 217 b	9,319 ± 210 b	28,941 ± 6 b	2,566 ± 16 bc
AMF + AZ 70	13,448 ± 408 ab	3,687 ± 52 a	12,409 ± 342 a	30,398 ± 320 ab	2,571 ± 93 bc
C 100	8,045 ± 348 d	2,498 ± 32 c	9,714 ± 107 b	29,226 ± 954 b	2,838 ± 189 b
AMF + AZ 100	12,624 ± 217 bc	4,000 ± 50 a	13,518 ± 654 a	31,780 ± 395 a	3,345 ± 60 a
AMF + S 100	13,656 ± 305 ab	3,718 ± 69 a	12,722 ± 440 a	29,674 ± 597 b	2,908 ± 100 b

*Abbreviations are explained in Figure 1 and Table 1.
**Values in each column followed by different letters are significantly different at $p \leq 0.05$, according to Tukey's test.

than those of the corresponding control (C 70); however, in the case of AMF + AZ 100, the dependence was reversed. In the case of K content in onion seedling leaves, no significant differences were noted between the treatments.

The phosphorus content in plant roots ranged from 2,721 to 4,796 mg kg^{-1} DW, whereas that in leaves ranged from 2,360 to 3,405 mg kg^{-1} DW. It was found that the P content was approximately 10% higher inplants collected from substrates subjected to inoculation. P accumulation in onion roots sampled from the inoculated AMF + AZ 50 treatment was lower than that in the samples from the corresponding control (C 50). No significant differences were recorded for the treatments concerning P content in the onion seedling leaves.

The analysis of correlation coefficients indicated a positive relationship between Ca content in the growing medium and P content in the leaves (Supplementary Table 3). Ca content in onion roots was positively correlated with Mg, Na, K, and P in the growing medium and with Mg, K, and P content in leaves.

3.5 Substrate properties after finishing of the growing cycle

The sum of alkaline and acid cations in the sorption complex resulted from the share of the organic fraction in the substrate formulations, and was the highest in the C 100, AMF + AZ 100, and AMF + S 100 treatments, with an organic carbon content of approximately 50%. Total nitrogen was determined in the range of 4.5%–5.0%, and electrical conductivity (EC) 1.38–2.15 mS (Table 3). Organic C was positively correlated with the sum of alkaline and acid cations in the sorption complex and with each of the investigated cations (N, Ca, Mg, Na, K, and P) (Supplementary Table 5).

The contents of Ca, Mg, Na, K, and P forms available to plants in the growing medium analyzed after cultivation were the highest in the formulations composed of peat:sand at a ratio of 100:0 (v:v), namely C 100, AMF + AZ 100, and AMF + S 100 (Table 3). Potassium content in the substrates after plant cultivation ranged from 21 to 147 mg kg^{-1} DW. Moreover, substrates composed of peat:sand in the ratio of 100:0 (v:v), with the addition of beneficial microorganisms (AMF + AZ 100 and AMF + S 100), contained the highest level of K after cultivation. In the case of treatments composed of peat:sand at a ratio of 70:30 (v:v), higher contents of Ca (9.6%), Mg (30.4%), Na (15.1%), and K (53.2%) were determined in growing media inoculated with PGPM (AMF + AZ 70), compared to the non-inoculated control (C 70), whereas P content was similar in the corresponding inoculated and non-inoculated treatments.

A statistically significant correlation was observed between the contents of all examined elements in their available forms in the media. The most common was a negative correlation between the pH measured in KCl and the sum of cations in the sorption complex, as well as the content of Ca, Mg, Na, K, and P forms available to plants (Table 4). These dependencies are illustrated in Figure 5A by the wide angles between the eigenvectors relevant to pH_{KCl} and other soil characteristics.

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TABLE 3 Effects of soil microbial inoculants on substrate physical and chemical characteristics after onion seedling cultivation (mean ± SD).

Treatment		C 50*	C 70	C 100	AMF + AZ 50	AMF + AZ 70	AMF + AZ 100	AMF + S 100
Sum in the sorption complex (mMol Na+ kg^{-1})	alkaline cations (S)	430 ± 24 c**	566 ± 31 b	1,287 ± 56 a	420 ± 42 c	551 ± 57 b	1,198 ± 66 a	1,212 ± 47 c
	acid cations (H)	42 ± 3 c	61 ± 5 b	154 ± 14 a	55 ± 7 bc	54 ± 4 bc	148 ± 9 a	135 ± 16 a
S + H (mMol kg^{-1})		472 ± 38 c	627 ± 52 b	1,441 ± 76 a	475 ± 54 c	605 ± 38 b	1,346 ± 69 a	1,347 ± 96 a
Cation exchange capacity with alkaline cations (%)		88.5 ± 9.5 a	89.5 ± 7.4 a	92.4 ± 6.7 a	83.4 ± 7.2 a	87.1 ± 5.9 a	91.4 ± 6.6 a	88.5 ± 4.7 a
Organic carbon (%)		2.55 ± 0.25 c	7.25 ± 0.32 b	48.16 ± 3.63 a	3.02 ± 0.41 c	7.85 ± 0.52 b	50.25 ± 2.32 a	51.14 ± 6.1 a
Total nitrogen (%)		0.305 ± 0.0251 c	0.610 ± 0.0712 b	4.542 ± 0.315 a	0.312 ± 0.034 c	0.635 ± 0.070 b	4.789 ± 0.366 a	4.991 ± 0.509 a
EC (mS)		0.45 ± 0.031 d	1.51 ± 0.092 b	1.38 ± 0.142 b	0.89 ± 0.102 c	1.58 ± 0.135 b	1.67 ± 0.184 b	2.15 ± 0.182 a
pH	H_2O	7.33 ± 0.52 a	7.18 ± 0.61 a	6.15 ± 0.46 b	7.12 ± 0.42 a	6.89 ± 0.74 a	6.35 ± 0.59 b	6.37 ± 0.76 b
	KCl	7.11 ± 0.29 a	6.75 ± 0.37 b	5.90 ± 0.32 c	6.88 ± 0.41 ab	6.71 ± 0.23 a	6.21 ± 0.41 c	6.24 ± 0.63 c

*Abbreviations are explained in Figure 1.
**Values in each column followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test.

3.6 PCA and cluster analyses

The PCA biplot (Figure 5) shows the contributions of each determined parameter to the first and second PCs separately for soil characteristics, AMF colonization, and onion seedling root and leaf parameters. The data revealed that PC 1 and PC 2 for substrate characteristics accounted for 93.06% of the total variance within the data set, and pH_{KCl} spread below 0 for PC 1, while the remaining factors contributed positively to this component (Figure 5A). All AMF colonization parameters contributed negatively to PC 1 (99.80% of total variance) (Figure 5B). PCA analysis of onion root characteristics showed that all variables, except Na and total phenols, had positive input to PC 1 (84.65% of total variance), while total phenols, antioxidant activity, Ca, and Mg content had positive output to PC 2 (26.81% of total variance) (Figure 5C). In the case of onion leaf characteristics, antioxidant activity and total phenols had a positive input to PC 1, although its share of total variance was only 38.54%. FW, P, and K contents brought positive load to PC 2 (Figure 5D).

In the dendrogram presented in Figure 6, the y-axis shows the distances between the treatments whereas the x-axis represents the analyzed treatments. Based on the cluster analysis (CA) method applied, two main clusters were distinguished based on the substrate characteristics (Figure 6A): the first cluster was formed by substrates with a peat:sand ratio of 50:50 and 70:30 (v:v), and the second was formed with a substrate composed of a peat:sand ratio of 100 (v:v). The CA was obtained from the experimental design. CA analysis of root colonization parameters also distinguished two main clusters: the first was composed of non-inoculated treatments (C 50, C 70, and C 100), and the second was inoculated substrate formulations, with the closest distance between the AMF + AZ 70 and AMF + S 100 treatments (Figure 6B). The root characteristics of

TABLE 4 Effects of soil microbial inoculants on soluble minerals (mg kg^{-1} DW) in the substrate after onion seedling cultivation (mean ± SD).

Treatment	Ca	Mg	Na	K	P
C 50*	2360 ± 121.1 de**	262.2 ± 2.49 e	211.8 ± 9.46 e	21.65 ± 0.531 f	9.35 ± 0.396 e
AMF + AZ50	2219 ± 34.1 de	252.4 ± 2.50 e	219.9 ± 2.48 e	35.30 ± 1.968 e	11.27 ± 0.284 e
C 70	2187 ± 61.4 e	301.6 ± 1.14 d	267.6 ± 2.08 de	33.95 ± 1.535 e	14.87 ± 0.504 d
AMF + AZ70	2397 ± 70.3 d	393.4 ± 4.37 c	307.9 ± 7.56 d	52.01 ± 1.529 d	16.53 ± 1.342 d
C 100	3213 ± 59.4 a	991.0 ± 7.53 a	1500.4 ± 45.50 a	86.21 ± 1.639 c	72.89 ± 1.949 a
AMF + AZ100	2807 ± 47.7 b	976.1 ± 18.02 a	1350.9 ± 38.09 b	150.66 ± 1.529 b	59.31 ± 2.028 b
AMF + S 100	2620 ± 25.4 c	745.8 ± 10.82 b	863.9 ± 8.08 c	101.13 ± 1.724 a	45.99 ± 1.153 c

*Abbreviations are explained in Figure 1 and Table 1.
**Values in each column followed by different letters are significantly different at p ≤0.05 according to Tukey's test.

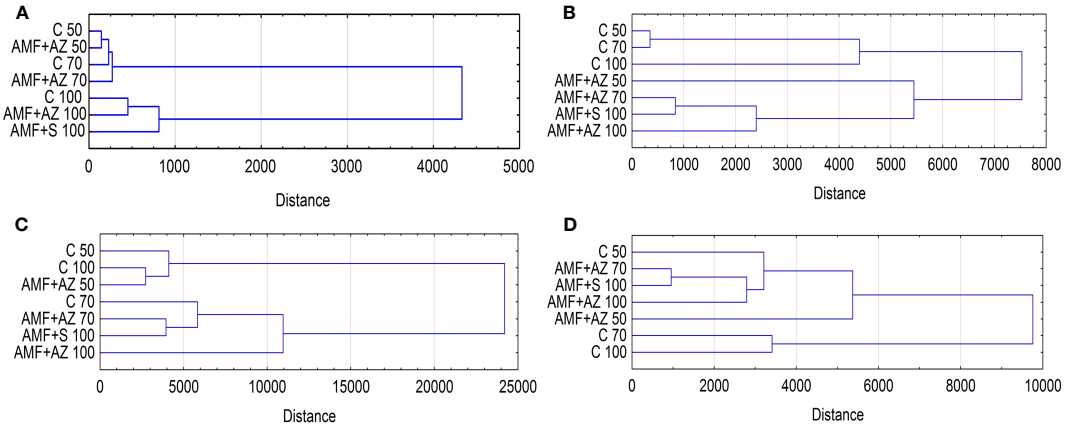

FIGURE 6
Dendrogram (Euclidean distance, Ward's method) showing the extent of distance between experimental treatments based on biochemical characteristics of soil after cultivation **(A)**, root colonization **(B)**, onion root **(C)**, and leaf characteristics **(D)**. Abbreviations are explained in Figure 1 and Table 1.

the CA also form two main clusters. The first included the C 50, C 100, and AMF + AZ 50 formulations, and the second included the remaining treatments. CA covering leaf characteristics resulted in grouping of the investigated substrate formulations into clusters separating C 50 and C 70 from the other treatments, while the closest relationship was recorded for AMF + AZ 70 and AMF + S 100 treatments. In general, the fact that certain objects belong to clusters demonstrates their considerable similarity within the group in question.

4 Discussion

4.1 Fresh weight of onion as affected by AMF and PGPM application to investigated growing media

In the present experiment, the roots of onions from all treatments inoculated with AMF and PGPM had FW similar to that of the uninoculated control treatments in every peat:sand combination. However, the leaf FW of onion seedlings grown on all inoculated substrates was significantly higher than that in the corresponding controls. Albrechtová et al. (2012) confirmed the synergism between AMF and saprotrophic fungi, resulting in a two-fold increase in the onion yield in the presence of organic matter. The mechanism behind the positive effect of inoculation on onion FW can be the synergistic action of AMF, which induces an accumulation of secondary metabolites, vitamins, and minerals (Baslam et al., 2013), and bacteria, such as N-fixing *A. brasilense*, which significantly enhances the length of root hairs and seedling weight by increasing the availability of minerals (Ribaudo et al., 2006). Crops inoculated with *Azospirillum* spp. experience changes in root morphology *via* siderophore production which regulates plant growth as well as vitamins such as thiamine and riboflavin (Sahoo et al., 2014a; Sahoo et al., 2014b). The application of PGPM in vegetables caused a yield increase of above ground crops by 8%–21%, and underground plant parts by approximately 25%–50%, as well as improved nutrient use efficiency by 12%–36% of N, 18%–29% of P, and 9%–15% of K (Rai et al., 2016). The present study confirmed that leaf FW was positively affected by substrate mineral composition, AMF colonization rate, and arbuscule abundance in onion roots. Onion seedling FW was linked to better efficiency but not development of the root system, resulting in higher aboveground biomass production after AMF + PGPM inoculation. Moreover, total seedling biomass can be predicted with the proposed regression equations, including Mg and Na content in the substrate, and root fresh weight can be predicted using regression equations, including AMF parameters (colonization rate, arbuscule abundance, and vesicle abundance).

4.2 Root colonization of onion as affected by AMF and PGPM application to investigated growing media

The results of observations performed with confocal microscopy showed successful root colonization of AMF in onion seedlings in all treatments. However, the colonization rate, arbuscule abundance, and vesicle abundance were the highest in substrate formulations based on the peat medium. Paranavithana et al. (2020) confirmed that AMF colonization of rice roots is more effective in soils rich in organic carbon. The described parameters indicated the intensive process of mutual symbiosis resulting in the parallel development by AMF mentioned morphological structures, i.e., arbuscules, that the primary sites of nutrient interchange between roots and fungi (Akiyama and Hayashi, 2006). Additionally, the bacterial strains used in the inocula formulation could accelerate mycorrhiza establishment and development because of their ability to decompose chitin and chitosan, the two main constituents of AMF spore walls, thus supporting spore germination in the onion rhizosphere (Battini et al., 2016). Although *Saccharothrix* spp. produce dithiolopyrolone derivatives

with antifungal activity (Merrouche et al., 2017), including PGPM in the formulation used in the present study, the presence of antagonism with AMF was not confirmed. In contrast, such consortium application resulted in improved activity of the enzyme, which is related to lignin formation. Cluster analysis revealed a close distance between AMF + AZ 70 and AMF + S 100 treatments with respect to onion root and leaf characteristics; therefore, AMF and *Saccharothrix* sp. The consortium was efficient in substrates rich in organic C, while AMF and *Azotobacter* sp. could be applied to substrates with lower organic C content. According to a study by Zhou et al. (2020), AMF increase plant carbon sequestration. The combination of AMF and *Saccharothrix* sp. was more effective in organic carbon sequestration and substrate colonization, although other substrate characteristics discussed in the previous subchapter should also be considered. For example, the highest soluble K content in the substrate of AMF + S 100 treatments was analyzed after the experiment. Moreover, the very high antioxidant activity and GPOX activity in onion roots sampled from the AMF + S 100 treatment indicated the successful induction of plant acclimatization, probably linked to *Saccharothrix* sp. and AMF interaction. In a previous study, Merrouche et al. (2017) reported the antagonism of *Saccharothrix* against fungi (e.g., *Fusarium* spp.) and bacteria. Based on present results, it can be concluded that *Saccharothrix* versus AMF interactions can evolve from parasitism under less optimal conditions to synergism under optimal conditions, in which competition for mineral resources is low or absent.

4.3 Stress biomarkers of onion as affected by AMF and PGPM application to investigated growing media

Onion seedlings grown in substrates reflecting degraded soil are under abiotic stress, including nutrient imbalances, low water-holding capacity, high concentrations of toxic heavy metals, such as Mn, Fe, and Al, high salinity, and pH fluctuations (Enkhtuya et al., 2000; Lehman et al., 2015). These factors modify the growth, function, and mineral absorption of the root system. AMF and PGPM can improve soil parameters and even compensate for chemical fertilizers, especially in onions, which are described as a highly mycorrhizal-dependent crop (Bidondo et al., 2016). The present study showed a new aspect of this relationship because onion seedling roots grown in substrates inoculated with AMF + AZ 50, AMF + AZ 100, and AMF + S 100 showed higher antioxidant activity than the corresponding controls. The latter indicates the acceleration of anti-stress processes leading to the synthesis of reactive oxygen species scavengers in onion roots. However, in onion leaves of the AMF + AZ 100, and AMF + S 100 treatments, antioxidant activity was significantly lower in leaves than in roots. Antioxidant potency is a measure of the ability of the plants to reverse the toxicity induced by stress factors (Veskoukis et al., 2019). In light of the present results, reinforcing the antioxidant defense of tissues was sufficiently efficient in onion roots in the AMF + AZ 100, and AMF + S 100 treatments, which did not need to be initiated to a similar degree in the onion leaves. AMF and PGPM seem to be moderators of these processes, acting in the rhizosphere by providing mineral nutrients and expressing a variety of enzymes that contribute to the control of cellular ROS levels, as reported by Pozo and Azcón-Aguilar (2007). AMF enhances the concentration of phenolic compounds in the roots (Lokhandwala et al., 2014). In the present study, onion roots sampled from the AMF + AZ 70 treatment accumulated the highest amount of phenol compounds. In onion leaves, the highest number of total phenols was found in AMF + AZ 50 and AMF + AZ 70. Phenolic compounds can scavenge reactive oxygen, which is why the phenolic profile is commonly correlated with the antioxidant activity of plant tissues (Veskoukis et al., 2019). In the present study, total phenols of onion root extracts were positively correlated with AMF (colonization rate, arbuscule, and vesicle abundance), but negatively correlated with antioxidant activity. This phenomenon can be explained by the activity of AMF and PGPM to transform phenols into simple compounds, which are ecologically active in soils through stabilization of free enzymes, modification of the transport and bioavailability of nutrients, and enhancement of element mineralization and humus formation (Raaijmakers et al., 2009).

Additionally, the antioxidant activity of onions is linked to sulfur derivates other than polyphenols (Fredotovic and Puizina, 2019). Moreover, phenols accelerate nutrient uptake by roots by chelating metallic ions and enhancing active absorption sites and soil porosity (Seneviratne and Jayasinghearachchi, 2003). Indeed, the present data revealed a positive correlation between total phenols in onion roots and Ca content in roots, as between K and Na content in leaves. This phenomenon can be explained using polyphenol compounds by AMF as substrates to modify biochemical conditions in the rhizosphere environment (Stewart and Stewart, 2008; Raaijmakers et al., 2009; Fredotovic and Puizina, 2019).

GPOX catalyzes lignin formation and establishes a structural barrier by producing reactive oxygen and nitrogen species (Marjamaa et al., 2009). In the present study, the highest GPOX activity was recorded in root samples from the AMF + S 100 treatment, and the elevated GPOX activity under AMF colonization indicates alleviated oxidative stress and therefore a higher resistance, as was reported by Domokos et al. (2018) for *Artemisia annua* inoculated with *R. irregularis*. Islam et al. (2015) stated that PGPM activated plant antioxidant defense by regulating the activity of GPOX, as a crucial antioxidant enzyme. The next issue is that the GPOX activity in roots sampled from inoculated treatment AMF + AZ 70 was higher as compared to values determined in samples from corresponding controls. AMF and *Saccharothrix* sp. consortium were more effective in organic carbon sequestration of peat media and, in turn, colonization. Moreover, the high antioxidant activity and GPOX activity in onion roots sampled from the AMF + S 100 treatment indicated the successful induction of plant response to stress factors, probably linked to *Saccharothrix* sp. and AMF interaction. In addition, the co-inoculation of onion seedlings with AMF and *A. brasilense* could cause increased GPOX activity in treatments with a lower amount of organic carbon, reflecting abiotic stress. These results are consistent with those previously reported in other plant species, inoculated separately with AMF (Domokos et al., 2018), *A.*

brasilense (Checchio et al., 2021), or *Saccharothrix* spp. (Enebe and Babalola, 2019), but no similar effect has been demonstrated for inoculation with AMF and PGPM consortia.

4.4 Element's concentration in onion Tissues as affected by AMF and PGPM application to investigated growing media

Elemental content in plant products is one of the most important quality parameters (Szeląg-Sikora et al., 2016). The chemical composition of plants determines their nutritional value as well as their suitability for processing or long-term storage (Niemiec et al., 2015). Under the conditions of intensive horticulture, a deficiency of macroelements is very common, which results in a reduction in the yield of plants and deterioration in the usable quality of the crop. Deficiency of macroelements in plants occurs even under conditions of high substrate content (Sikora et al., 2020). The level of bioaccumulation of elements in plants depends not only on their amount in the biotope, but also on their properties (Niemiec et al., 2020a). Increasing the degree of absorption of micronutrients is a strategic aspect of the development of agricultural sciences and production practices (Niemiec et al., 2020b; Seymen et al., 2021). The observed macronutrient contents in the onions of all objects were at the optimal level (Wang et al., 2020; Romo-Péreza et al., 2021); however, samples were taken for analysis in the early stages of development. In the case of macronutrients such as calcium or phosphorus, deficiencies are often visible in the later stages of vegetative development or even in the stage of generative development. An increase in the accumulation of macronutrients in young plants reduces the risk of deficiency at harvest maturity (Bana et al., 2022). Research has demonstrated that co-inoculants of AMF and PGPM enhance the nutrient-use efficiency of fertilizers and reduce chemical fertilizer application rates (Bhardwaj et al., 2014). In general, AMF and PGPM directly affect mineral absorption by the host plant by improving plant growth through nutrient acquisition by the fungus, or indirectly by modifying nutrient mobilization from organic substrates, by enhancing fertilizer use efficiency, or by beneficial association with other microorganisms (Vishwakarma et al., 2017). The analysis of available forms of nutrients in the substrate, their uptake by onion roots, and their translocation to shoots was performed in the framework of the present experiment. The onions collected from the substrate with a peat:sand ratio of 100:0, co-inoculated with AMF + AZ, and AMF + S contained the highest levels of Ca and Mg (but lower Na) in leaves. *Azospirillum*-inoculated plants have been reported to cause acidification of the root surroundings, which increases macronutrient and micronutrient uptake (Dobbelaere and Okon, 2007). El-Batanomy (2009) revealed that a mixture of microbial cultures showed the highest nitrogenase activity and mycorrhizal infection in onion roots. The total essential nutrient content in onion dry shoots increased in mixed inocula of *Azospirillum lipoferum*, *A. chrococcoum*, *Bacillus circulans*, *B. polymyxa*, *Rhizobium* sp., and AMF compared to that in the fertilized control. Moreover, Adesemoye et al. (2009) determined another possible mechanism of PRPM and AMF cooperation; namely, the former can act as a vehicle to spread non-mycorrhizal microorganisms throughout the rhizosphere. Bettoni et al. (2014) demonstrated the increase of P level on onion seedlings inoculated with *Glomus intraradices* and organic matter amendment. In the present study, Ca content in onion roots was positively correlated with other elements in the substrate, as well as AMF colonization parameters, antioxidant activity, total phenols in roots, FW of leaves, and K, Mg, and P content in leaves. Moreover, Ca, Mg, K, P, and Na contents in onion leaves were positively correlated with AMF colonization parameters. Surprisingly, relatively few increases in P uptake into the seedlings were observed, which was one of the strongest effects of AMF inoculation. Growing medium composition had a more significant effect on P availability and uptake than AMF-related accumulation of this element by onion seedlings. Mentioned interdependence was reflected in highest soluble P content after cultivation cycle in substrates based on peat (100%) with and without AMF inoculation. This effect is due to competition between the decomposition products of organic matter and P for soil sorption sites, resulting in increased soil solution P concentrations (Guppy et al., 2005). Additionally, the differences between AMF-inoculated and uninoculated plants can be considered the costs paid by plants for AMF-root associations. According to Al-Karakis (2002), AMF inoculation of garlic plants was highest when soil P was lowest, and decreased with increasing P application. Moreover, AMF selectively uptake or make available essential cations to plants, which act as osmotic equivalents for toxic Na ions (Hammer et al., 2011). Moreover, Na ions are compartmentalized in cell vacuoles and mycorrhizal fungal hyphae to avoid translocation to the shoots (Estrada et al., 2013). The interactions between AMF and PGPM concerning mineral salt uptake and synergism can be managed, but one must be aware that synergetic interactions between these microbes could also be manifested.

4.5 Substrate characteristics

Onions are among the most sensitive crops to soil conditions, especially salinity, which affects plant growth particularly at the seedling stage (Onishchuk et al., 2017). The physiological and chemical properties of the substrate, including its biomass and chemical composition influence plant growth in a multidirectional manner (Niemiec et al., 2020a). In the present study, the available forms of Ca, Mg, Na, K, and P in the substrate analyzed after cultivation were highest in the formulations composed of peat:sand at a ratio of 100:0 (v:v). Uzinger et al. (2020) demonstrated that the combination of compost and plant growth promoting bacteria resulted in higher P and K availability. In the present study, AMF + PGPM was effective in releasing minerals into the soluble fraction in substrates rich in organic matter, although this capacity was dependent on the consortium of microorganisms used. AMF + AZ 100 and AMF + S 100 application resulted in the highest level of K after cultivation, whereas AMF + AZ 70 inoculants increased the soluble forms of Ca, Mg, Na, and K compared to the non-inoculated control. The P content was similar among the treatments mentioned above. A significant and positive correlation between the soluble elements in substrates and organic carbon, while a

negative correlation with pH_{KCl}, confirmed the crucial significance of organic matter and reaction for element availability to plants. This phenomenon has been noted by numerous researchers (Guaya et al., 2020; Niemiec et al., 2020b; Lei et al., 2021). Additionally, in the present study, the mineral soluble forms were not reduced in formulations with higher EC values, particularly in the inoculated treatments. Balliu et al. (2015) stated that uptake of elements was enhanced in inoculated tomato seedlings even under moderate salt stress. Despite the large differences in the organic fraction content of the substrates and the capacity of the sorption complex, slight differences in substrate pH occurred after the experiment. In general, a wide spectrum of soil properties converge to create synergistic effects, leading to the reshaping of microbiome/plant interactions.

5 Conclusions

Alliaceae crops encounter various challenges, and limiting conditions can result in serious economic losses at every growth stage. Research on plant growth-promoting microbes (PGPM) as a potential component of mainstream agriculture is important and essential. Inoculation with AMF and PGPM consortia resulted in significant improvement in onion seedling performance, i.e., higher aboveground biomass production and better stress adaptation in cultivation in substrates with lower organic carbon content. Our results demonstrated that AMF and *Saccharothrix* sp. were effective in substrates containing 70%–100% peat, while AMF and *Azotobacter* sp. can be recommended to inoculate substrates with 50% organic C to enhance onion seedling performance. Differences between AMF- and PGPM-inoculated plants concerning fresh weight, stress biomarkers, and element concentration were considered the costs paid by plants due to AMF–root associations linked with growing medium-related availability of nutrients. The present findings have revealed a significant aspect of soil/plant management, i.e., that organic/beneficial microbe synergy might be the future key for effective nutrient management and sustainable production. Although we demonstrated the benefits of AMF and PGPM inoculation on onion seedlings, the maintenance of symbiosis after transplanting can be the goal of subsequent investigations, as field conditions create new challenges to plant–microorganism interactions.

Data availability statement

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

Author contributions

Conceptualization, RP, AS, AK, and LR. Methodology, RP and LR. Validation, AS and RP. Formal analysis, MN, LR, MJ, and MK. Resources, AS. Data curation, RP and AS. Writing—original draft preparation, RP and AS. Writing—review and editing, LR, MK, MG, MJ, MN, and GC. Supervision, RP. Project administration, AS. Funding acquisition, RP and AS. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1222557/full#supplementary-material

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Enhancing alfalfa photosynthetic performance through arbuscular mycorrhizal fungi inoculation across varied phosphorus application levels

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This study evaluated the effects of arbuscular mycorrhizal fungi inoculation on the growth and photosynthetic performance of alfalfa under different phosphorus application levels. This experiment adopts two-factors completely random design, and sets four levels of fungi application: single inoculation with *Funneliformis mosseae* (Fm, T_1), single inoculation with *Glomus etunicatum* (Ge, T_2) and mixed inoculation with *Funneliformis mosseae* × *Glomus etunicatum* (Fm×Ge, T_3) and treatment uninfected fungus (CK, T_0). Four phosphorus application levels were set under the fungi application level: P_2O_5 0 (P_0), 50 (P_1), 100 (P_2) and 150 (P_3) mg·kg^{-1}. There were 16 treatments for fungus phosphorus interaction. The strain was placed 5 cm below the surface of the flowerpot soil, and the phosphate fertilizer was dissolved in water and applied at one time. The results showed that the intercellular CO_2 concentration (C_i) of alfalfa decreased at first and then increased with the increase of phosphorus application, except for light use efficiency (LUE) and leaf instantaneous water use efficiency (WUE), other indicators showed the opposite trend. The effect of mixed inoculation (T_3) was significantly better than that of non-inoculation (T_0) ($p < 0.05$). Pearson correlation analysis showed that C_i was significantly negatively correlated with alfalfa leaf transpiration rate (T_r) and WUE ($p < 0.05$), and was extremely significantly negatively correlated with other indicators ($p < 0.01$). The other indexes were positively correlated ($p < 0.05$). This may be mainly because the factors affecting plant photosynthesis are non-stomatal factors. Through the comprehensive analysis of membership function, the indexes of alfalfa under different treatments were comprehensively ranked, and the top three were: T_3P_2>T_3P_1>T_1P_2. Therefore, when the phosphorus treatment was 100 mg·kg^{-1}, the mixed inoculation of *Funneliformis mosseae* and *Glomus etunicatum* had the best effect, which was conducive to improving the photosynthetic efficiency of alfalfa, increasing the dry matter yield, and improving the economic benefits of local alfalfa in Xinjiang. In future studies, the anatomical structure and photosynthetic performance of alfalfa leaves and stems should be combined to clarify the synergistic mechanism of the anatomical structure and photosynthetic performance of alfalfa.

KEYWORDS

alfalfa, AMF, phosphorus, net photosynthetic rate, dry matter yield

1 Introduction

Alfalfa (*Medicago sativa* L.) is a perennial legume herb with highly digestible fiber and high protein content, which can be used as hay and silage, making it an indispensable cultivated forage in arid and semi-arid areas of western China (Liu et al., 2021). Phosphorus (P) is an indispensable nutrient element in the process of plant growth and development, such as participating in the conversion and metabolism of chloroplast energy and the transport of leaf photosynthetic products in plant photosynthesis (Nasar et al., 2021). However, phosphorus anions are often adsorbed and precipitated in soil with other cations, resulting in limited availability of phosphorus in plants (Gao et al., 2016; Ning et al., 2019). Studies have shown that phosphorus deficiency acts as a key limiting factor for plant growth, which in turn affects high crop yields (Zhao et al., 2022a). Studies have shown that high concentrations of biogas slurry can promote the mobilization of colloidal phosphorus in rice fields, thereby promoting the absorption of phosphorus by rice (Niyungeko et al., 2018). Therefore, improving the phosphorus use efficiency of crops is of great significance for maintaining sustainable agriculture and crop cultivation.

Arbuscular mycorrhizal fungi (AMF) as a fungus that can establish symbiotic relationships with 80%-90% of the roots of land plants, including angiosperms, gymnosperms, ferns, and bryophytes, it can promote plant growth and induce plant stress resistance. (Frosi et al., 2016; Alotaibi et al., 2021). AMF can form mycelium structure with plant roots, and this structure increases the surface area of plant roots, improves the nutritional status of plants symbiotic, improves its absorption of nitrogen (N), phosphorus and mineral nutrients in the soil and then changes the stress resistance of plants (Sun et al., 2020; Yang et al., 2022), promotes the absorption of water by channel proteins and further improves crop water use efficiency, mineral nutrient nutrition and biomass (Qin et al., 2022). Studies have shown that AMF activates insoluble phosphorus by secreting phosphatases into the soil, and absorbs available phosphorus in the soil through high-affinity phosphorus transporters (Liu et al., 2020). And in the case of CO_2 enrichment, AMF colonization can promote the absorption of phosphorus by plants (AbdElgawad et al., 2022). Therefore, AMF plays an important role in plant uptake and utilization of insoluble phosphorus and other nutrients in soil.

AMF can secrete cytokinins to increase chlorophyll content in plant leaves, thereby improving the yield of mixed sheepgrass (*Leymus chinensis*) and increasing photosynthesis of alfalfa (Shan et al., 2020). Studies have shown that changes in plant chlorophyll content can cause changes in plant photosynthetic activity, which in turn significantly affects plant development, growth and productivity (Zhang et al., 2017b). An important basis for photosynthesis in plants is leaves, and the photosynthetic capacity of leaves and the transport of photosynthetic products of their transport tissues play an important role in crop biomass (Li et al., 2020). Studies have shown that AMF can reduce the toxicity of beryllium to plants and promote photosynthesis of plants (Sheteiwy et al., 2022). Phosphorus application can improve the net photosynthetic rate, leaf chlorophyll content and nitrogen fixation capacity of roots, thereby significantly improving the production performance and nutritional quality of crops (Qi et al., 2013). Other studies have shown that phosphorolytic bacteria can improve plant leaf fence organization and leaf photosynthesis and increase their biomass (Sun et al., 2022). Therefore, fungus and phosphorus application play an important role in promoting the growth of alfalfa and improving photosynthesis.

At present, many studies have focused on inoculating a single AMF plant on alfalfa to improve dry matter yield and photosynthesis (Shan et al., 2020; Zhao et al., 2022a), however, the study on the relationship between photosynthesis and phosphorus interaction of alfalfa by simultaneous inoculation of two fungi under different phosphorus conditions is still missing. Therefore, in this study, the effects of inoculation of two fungi, *Funneliformis mosseae* (Fm) and *Glomus etunicatum* (Ge), on the dry matter yield, photosynthetic gas exchange parameters of alfalfa leaves and phosphorus utilization efficiency of alfalfa under different phosphorus application levels, were studied, in order to provide a theoretical basis for improving the measures for high-quality and high-yield alfalfa and the improvement of scientific and rational fertilization system. We hypothesized that: 1)when the phosphorus treatment was 100 $mg{\cdot}kg^{-1}$, the mixed inoculation of *Funneliformis mosseae* and *Glomus etunicatum* can significantly improve the photosynthetic efficiency and phosphorus utilization efficiency of alfalfa leaves; 2) the effect of mixed inoculation was significantly better than that of non-inoculation; 3) the top three treatments in terms of comprehensive ranking were T_3P_2, T_3P_1, and T_1P_2.

2 Materials and methods

2.1 Experimental materials

AMF uses *Funneliformis mosseae* (Fm) (BGC HK01) and *Glomus etunicatum* (Ge) (BGC GZ03C), the inoculant is a mixture of alfalfa plant roots, mycorrhizal fungal spores and extrarhizosphere mycelium, fungal spore density of 20~30 $piece{\cdot}g^{-1}$, purchased from Qingdao Agricultural Mycorrhizal Research Institute, China. AMF can form a good symbiotic relationship with the roots of most terrestrial plants, construct a root network in the soil, promote the absorption and utilization of nutrients by host plants, and thus plant growth can be promoted. Due to the drought and high light intensity in Xinjiang, WL366HQ was selected as the test plant for alfalfa varieties. The variety has a fall dormancy level of 5, high yield and cold tolerance. It can better adapt to the local environment in Xinjiang and is widely planted in Xinjiang.

Because the climatic conditions in Xinjiang are dry, the precipitation is scarce, and the soil is mostly gray desert soil, the soil used for potting is gray desert soil, which is collected from the 2nd Experimental Station of Shihezi University (44°18′N, 86°03′E), and the soil is naturally dried and sieved through 0.5 cm to remove stones and plant roots from the soil. The physical and chemical properties of the soil are shown in Table 1, the soil is sterilized at 121°C high temperature and humid heat for 2 h, and then naturally

TABLE 1 Basic physical and chemical properties of test soil.

Bulk density ($g \cdot cm^{-3}$)	Alkaline nitrogen ($mg \cdot kg^{-1}$)	Organic matter ($g \cdot kg^{-1}$)	Available phosphorus ($mg \cdot kg^{-1}$)	Total phosphorus ($g \cdot kg^{-1}$)	Available potassium ($mg \cdot kg^{-1}$)
1.47	71.8	24.1	18.3	0.23	135.5

cooled for backup, and the potting substrate is sterilized soil and perlite 3:1 (V:V) mixed to prevent soil compaction.

2.2 Experimental design

The experiment was designed with two factors of complete randomization, with two factors: fungi application and phosphorus application. Fungi application was set at 4 levels: 10 g of *Funneliformis mosseae* (Fm, T_1), 10 g of *Glomus etunicatum* (Ge, T_2), 5 g of *Funneliformis mosseae* and 5 g of *Glomus etunicatum* (Fm×Ge, T_3), with no fungi as the control (T_0). Four more phosphorus levels were set under each application condition: P_2O_5 0 (P_0), 50 (P_1), 100 (P_2) and 150 (P_3) $mg \cdot kg^{-1}$, and a total of 16 treatments were performed with 10 replicates of each treatment, for a total of 160 pots, and the pots were randomly placed.

The potting experiment was carried out in 2021-2022 in the experimental park of the College of Agriculture, Shihezi University (44°18′N, 86°03′E), and the experiment used pots with a size of 23 cm×17 cm×15 cm (pot mouth diameter× pot floor diameter × pot height), each pot containing about 3 kg of soil substrate, and the pots were disinfected with 75% alcohol before sowing. High-quality alfalfa seeds of uniform size and full grains were selected, sterilized with 10% H_2O_2 for 10 min, and then rinsed repeatedly with distilled water, and sown on May 1, 2021, with 10 seeds evenly distributed per pot. After the alfalfa three-leaf stage, five robust and uniform alfalfa seedlings were retained. In order to simulate the natural growth conditions of alfalfa, pots were placed in sunny places, and phosphorus-free Hoagland ‘s nutrient solution and equal amount of water were applied weekly during alfalfa seedlings. The phosphate fertilizer used was monoammonium phosphate (containing P_2O_5 52%, N 12.2%), with its main component being P_2O_5, which has good water solubility and can be better absorbed and utilized by alfalfa, because the phosphate fertilizer had a small amount of N, so a certain amount of urea (containing N 46%) was supplemented under different phosphorus gradients in this experiment to make the N content under different phosphorus gradients consistent to offset the influence of nitrogen content in phosphate fertilizer on the experiment results. The fertilizer is applied with water droplets using the drip irrigation method of fertilizer with water, and the specific fertilization time is June 23, August 1, 2021, and April 16 and June 7, 2022. Alfalfa is harvested at the beginning of flowering (10%), on July 14, August 23, 2021, May 15, and June 30, 2022. Due to the insufficient environmental conditions for the first measurement of photosynthetic gas exchange parameters, the indicators were measured in the second stubble.

2.3 Alfalfa dry matter yield

In pots, three pots of alfalfa plants with uniform growth in the second stubble of the two years were selected, and the aerial part of the alfalfa plant was cut 2 cm from the surface of the potted soil for weighing, and the fresh weight was recorded. The fresh sample of alfalfa was dried at 105°C for 30 min, and then dried to constant weight at 65°C, the moisture content was determined and the dry matter yield was converted, the calculation formula was as follows: Dry matter yield = plant fresh weight × (1-moisture content), where moisture content refers to the calculation based on plant fresh weight.

2.4 Alfalfa photosynthetic pigment content

Chlorophyll is responsible for capturing light energy and driving the photosynthetic process. It is the most important pigment in photosynthesis. Chlorophyll a and chlorophyll b in fresh alfalfa leaves were extracted by absolute ethanol method (Cui and Yang, 2011), and the absorbance values were determined by spectrophotometer, and the total chlorophyll a, chlorophyll b and chlorophyll content in alfalfa leaves were calculated.

2.5 Alfalfa photosynthetic gas exchange parameters

Under cloudless conditions with sufficient light, the photosynthetic gas exchange parameters of well-grown alfalfa leaves were randomly determined by measuring the first flowering stage (10%) of the second stubble of two-year potted alfalfa on August 20, 2021 and June 27, 2022 at 11:00~13:00 a.m. The healthy intact and fully expanded leaflets in the middle of the third triple compound leaf of alfalfa plants from top to bottom were selected, and the photosynthetically active radiation (PAR) was controlled at about 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and each treated sample was measured in 3 replicates for analysis and averaged. Specific photosynthetic gas exchange parameters include net photosynthetic rate (P_n, which represents the rate of carbon dioxide absorption and assimilation during photosynthesis, $\mu mol \cdot m^{-2} \cdot s^{-1}$), stomatal conductance (G_s, which is the degree of stomatal opening, $mmol \cdot m^{-2} \cdot s^{-1}$), transpiration rate ($T_r$, which represents the amount of water transpiration per unit leaf area in a certain period of time, $mmol \cdot m^{-2} \cdot s^{-1}$), intercellular CO_2 concentration (C_i, which reflects the utilization efficiency of CO_2 by plants, $\mu mol \cdot mol^{-1}$), light use

efficiency (LUE, which is an index to measure the light use efficiency and production level of crops in a certain area, mmol·mol^{-1}) and alfalfa leaf instantaneous water use efficiency (WUE, which reflects the water utilization capacity of plant leaves, μmol·mmol^{-1}), in which the light energy use efficiency and alfalfa leaf instantaneous water use efficiency are calculated by formulas, the specific calculation formula is as follows: LUE=P_n/PAR; WUE=P_n/T_r.

2.6 Statistical analysis

Microsoft Excel 2010 was used for data processing, DPS 7.05 software (Data Processing System, China) was used for statistical analysis of data, two-factor experiment in a completely randomized design was used to statistically analyze the interaction between fungus treatment (T), phosphorus treatment (P) and fungus phosphorus (T×P), multiple comparisons were made by Duncan's new complex polarity difference method, and the mapping software used Origin 2021 (OriginLabOriginPro, USA).

Because the performance of each treatment is different in different indexes, it is not comprehensive to evaluate the optimal bacterial phosphorus treatment with any single index (Zhang et al., 2017a). The membership function analysis method is used to comprehensively evaluate the optimal treatment, and the specific formula is: UX(+) = (X_{ij}-X_{imin})/(X_{imax}-X_{imin}); UX(-) =1-UX(+), where X_{ij} is the measurement value of the jth index in the ith treatment; X_{imax} and X_{imin} are the maximum and minimum values of the jth indicator in all treatments, respectively. UX(+) is the value of the positive indicator membership function, and UX(-) is the value of the negative indicator membership function. The range of membership function values is 0-1.

3 Results

3.1 Effects of different treatments on the dry matter yield of alfalfa

According to the test results, dry matter yield of alfalfa exhibited an increase at lower P doses (P_0-P_2) and a decrease at higher doses (P_2-P_3) within the same fungal treatment condition (Figure 1). Except for P_1 treatment significantly larger than P_0, P_2 and P_3 in 2022 ($p < 0.05$), the dry matter yield of alfalfa treated with phosphorus treatment reached the highest value in P_2 treatment, which was 25.87 g·pot^{-1} in 2021, which was 58.2% higher than that of T_0P_0 treatment. In 2022, it would be 26.68 g·pot^{-1}, which is 59.35% higher than that of T_0P_0 treatment. The dry matter yield of alfalfa under P_2 treatment exhibited a statistically significant increase compared to other treatments ($p < 0.05$).

When the phosphorus treatment was 100 mg·kg^{-1}, the dry matter yield of double inoculated Fm and Ge alfalfa exhibited a statistically significant increase compared to no fungus treatment ($p < 0.05$), and the maximum value of T_3 treatment was reached under P_2 treatment. The effect of simultaneous inoculation of Fm and Ge was significantly better than that of single inoculation ($p < 0.05$). The dry matter yield of alfalfa under the treatment of fungus (T), phosphorus treatment (P) and fungus phosphorus interaction (T×P) in two years showed extremely significant differences ($p < 0.01$).

3.2 Effects of different treatments on photosynthetic pigment content of alfalfa

There were obvious AMF × phosphorus interactions for chlorophyll a, chlorophyll b, and total chlorophyll contents.

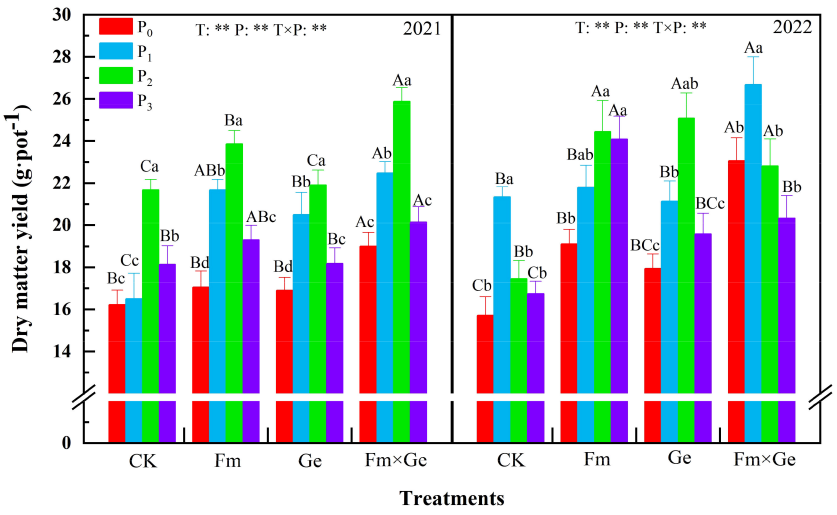

FIGURE 1
Dry matter yield of alfalfa under different treatments from 2021 to 2022. Note: T_0, T_1, T_2, and T_3 represent CK, *Funneliformis mosseae* (Fm), *Glomus etunicatum* (Ge), and *Funneliformis mosseae* × *Glomus etunicatum* (Fm×Ge), respectively. P_0, P_1, P_2, and P_3 represent 0 mg·kg^{-1}, 50 mg·kg^{-1}, 100 mg·kg^{-1}, and 150 mg·kg^{-1}, respectively. Different capital letters indicated significant difference in different fungus treatments under the same phosphorus application conditions ($p < 0.05$), differences small letters mean significant difference under the same fungus application conditions ($p < 0.05$). ** indicates significant difference extremely ($p < 0.01$). The value of n is 48.

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According to the test results, chlorophyll a, chlorophyll b, and total chlorophyll contents demonstrated an increase at lower P doses (P_0-P_2) and a decrease at higher doses (P_2-P_3) within the same fungal treatment condition (Figure 2). Except for the T_0 treatment in 2021, the chlorophyll b content of alfalfa was significantly higher in the P_3 treatment ($p < 0.05$), the chlorophyll a and total chlorophyll content was significantly higher in the P_3 treatment ($p < 0.05$) under T_0 condition in 2022, the chlorophyll b content was significantly higher in the P_1 treatment ($p < 0.05$). The chlorophyll a, chlorophyll b and total chlorophyll contents of alfalfa was the highest when most of the phosphorus treatment was 100 mg·kg^{-1}, and the P_2 treatment exhibited a statistically significant increase compared to the P_0 treatment ($p < 0.05$).

Under the condition of double inoculation of Fm and Ge fungal (T_3), the chlorophyll a, chlorophyll b and total chlorophyll contents of alfalfa at the phosphorus treatment of 100 mg·kg^{-1} (P_2) in 2021 and 2022 exhibited a statistically significant increase compared to the P_0 treatment ($p < 0.05$). In 2021, the total chlorophyll content, chlorophyll a content and chlorophyll b content of alfalfa treated with T, P treatment and T×P showed extremely significant differences ($p < 0.01$), and in 2022, the chlorophyll a and total chlorophyll content of alfalfa between T, P treatment and T×P treatment showed extremely significant differences ($p < 0.01$). In 2022, the chlorophyll b content T and P treatment showed significant differences ($p < 0.05$).

3.3 Effects of different treatments on photosynthetic gas exchange parameters of alfalfa

Effects of two years of different phosphorus treatments on photosynthetic gas exchange parameters of alfalfa (Figure 3).

3.3.1 Net photosynthetic rate

According to the test results, the net photosynthetic rate (P_n) values of alfalfa exhibited an increase at lower P doses (P_0-P_2) and a decrease at higher doses (P_2-P_3) within the same fungal treatment condition. Except for 2021, the net photosynthetic rate demonstrated a statistically significant increase in the P_1 treatment compared to P_0, P_2, and P_3 under T_2 treatment conditions ($p < 0.05$), the rest of the treatments demonstrated a statistically significant increase in the P_2 treatment compared to P_0, P_1, and P_3 ($p < 0.05$). Phosphorus treatment led to a significant increase in photosynthetic parameters compared to the non-phosphorus treatment ($p < 0.05$). Under the same phosphorus treatment, FmGe treatment > Fm/Ge treatment > CK treatment.

3.3.2 Transpiration rate

According to the test results, the transpiration rate (T_r) values of alfalfa exhibited an increase at lower P doses (P_0-P_2) and a decrease

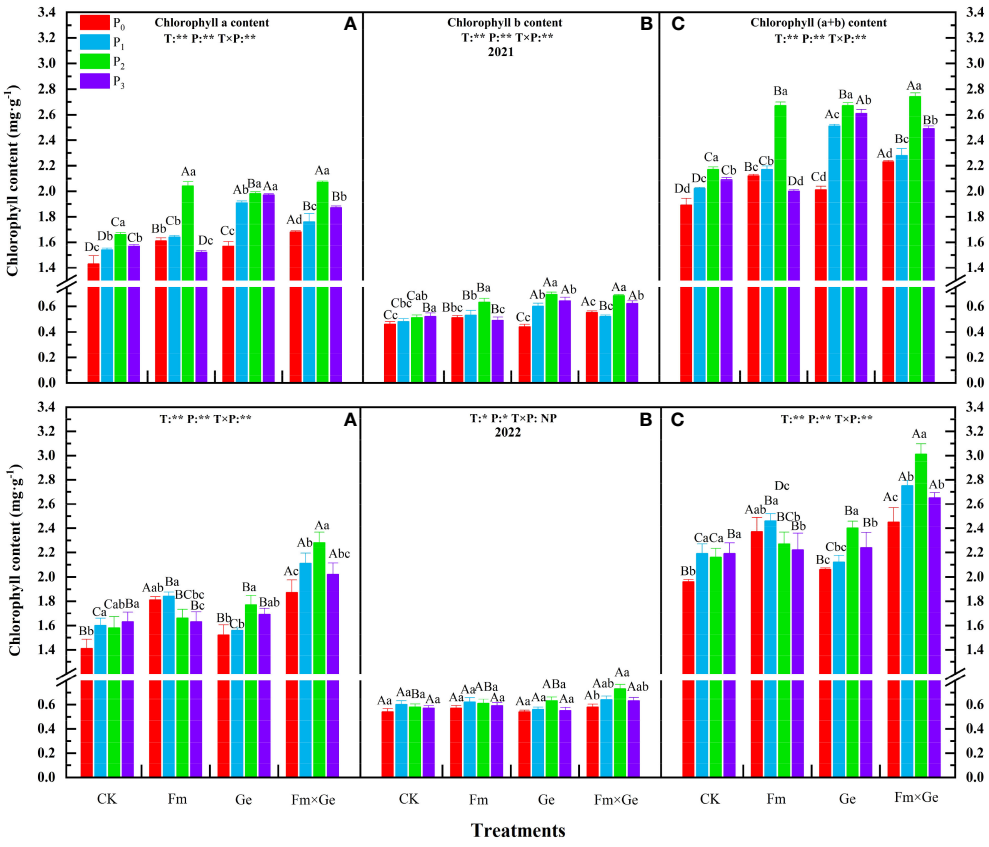

FIGURE 2
Chlorophyll content under different treatments from 2021-2022. **(A–C)** in the figure represent chlorophyll a content, chlorophyll b content, and total chlorophyll content, respectively. Different capital letters indicated significant difference in different fungus treatments under the same phosphorus application conditions ($p < 0.05$), differences small letters mean significant difference under the same fungus application conditions ($p < 0.05$). ** indicates significant difference extremely ($p < 0.01$). The value of n is 48.

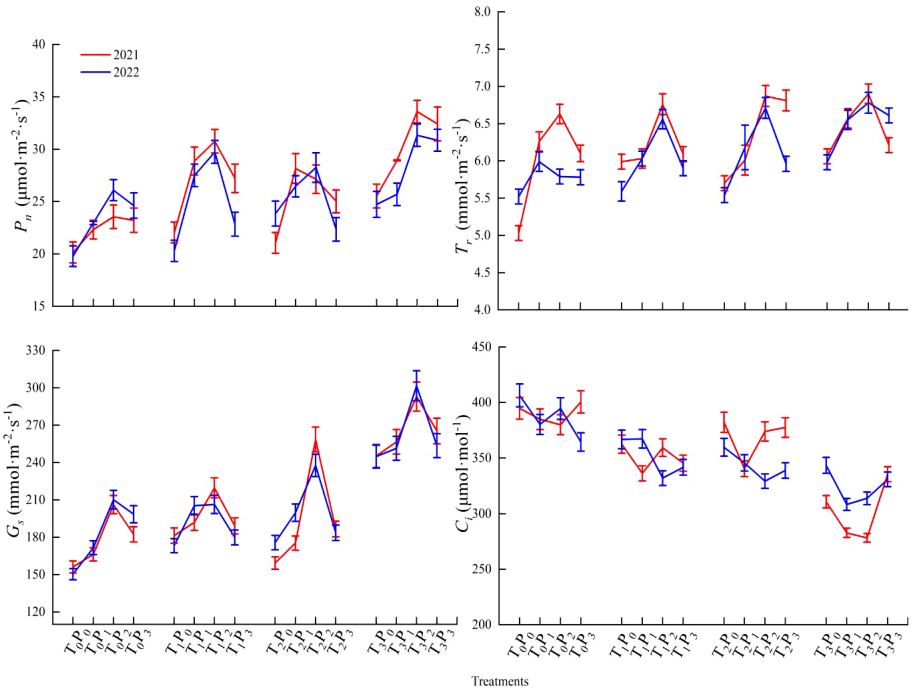

FIGURE 3
Photosynthetic gas exchange parameters of alfalfa under different treatments from 2021 to 2022.

at higher doses (P_2-P_3) within the same fungal treatment condition. The transpiration rate values of alfalfa demonstrated a statistically significant increase in the P_2 treatment compared to P_0, P_1, and P_3 ($p < 0.05$). Phosphorus treatment led to a significant increase in photosynthetic parameters compared to the non-phosphorus treatment ($p < 0.05$). Under the same phosphorus treatment, FmGe treatment > Fm/Ge treatment > CK treatment.

3.3.3 Stomatal conductance

According to the test results, the stomatal conductance (G_s) values of alfalfa exhibited an increase at lower P doses (P_0-P_2) and a decrease at higher doses (P_2-P_3) within the same fungal treatment condition. The stomatal conductance demonstrated a statistically significant increase in the P_2 treatment compared to P_0, P_1, and P_3 ($p < 0.05$). Phosphorus treatment led to a significant increase in photosynthetic parameters compared to the non-phosphorus treatment ($p < 0.05$). Under the same phosphorus treatment, FmGe treatment > Fm/Ge treatment > CK treatment.

3.3.4 Intercellular CO_2 concentration

The intercellular CO_2 concentration (C_i) value of alfalfa exhibited an increase at lower P doses (P_0-P_2) and a decrease at higher doses (P_2-P_3) within the same fungal treatment condition. Except for 2021, the intercellular CO_2 concentration of P_1 exhibited a statistically significant decrease compared to other treatments under T_1 and T_2 treatment, P_3 exhibited a statistically significant decrease compared to other treatments under T_0 treatment in 2021 and P_1 exhibited a statistically significant decrease compared to other treatments under T_3 treatment in 2022, the rest was significantly lower in P_2 treatment ($p < 0.05$). Under the same phosphorus treatment, T_0 treatment exhibited a statistically significant increase compared to other treatments ($p < 0.05$).

3.4 Effects of different treatments on light energy use efficiency and instantaneous water use efficiency of alfalfa leaves

3.4.1 Light energy use efficiency

According to the test results, the light use efficiency of alfalfa in two years exhibited an increase at lower P doses (P_0-P_2) and a decrease at higher doses (P_2-P_3) within the same fungal treatment condition (Figure 4). The light use efficiency of alfalfa under P_2 treatment exhibited a statistically significant increase compared to other treatments ($p < 0.05$).

When the phosphorus treatment was 100 mg·kg^{-1}, the light use efficiency of alfalfa under T_3 treatment exhibited a statistically significant increase compared to T_0 treatments ($p < 0.05$). The effect of simultaneous inoculation of Fm and Ge was significantly better than that of single inoculation ($p < 0.05$). The light use efficiency of alfalfa under the fungus treatment (T), phosphorus treatment (P) and fungus phosphorus interaction (T×P) in two years showed extremely significant differences ($p < 0.01$).

3.4.2 Instantaneous water use efficiency

According to the test results, the instantaneous water use efficiency of alfalfa in two years exhibited an increase at lower P doses (P_0-P_1) and a decrease at higher doses (P_1-P_3) within the same fungal treatment condition (Figure 4). Except that under T_0 condition, the instantaneous water use efficiency of alfalfa leaves

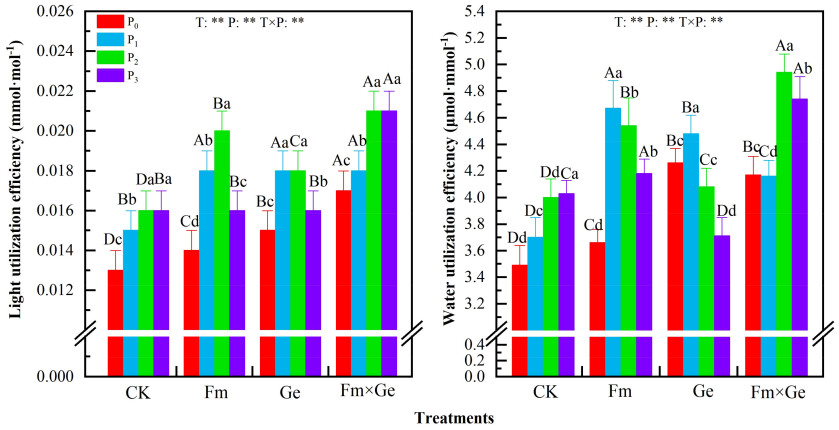

FIGURE 4
Light use efficiency and Water use efficiency of alfalfa leaves under different treatments from 2021-2022. Different capital letters indicated significant difference in different fungus treatments under the same phosphorus application conditions ($p < 0.05$), differences small letters mean significant difference under the same fungus application conditions ($p < 0.05$). ** indicates significant difference extremely ($p < 0.01$). The value of n is 48.

under P_3 treatment exhibited a statistically significant increase compared to other treatments ($p < 0.05$), and under T_3 condition, the instantaneous water use efficiency of alfalfa leaves under P_2 treatment exhibited a statistically significant increase compared to other treatments ($p < 0.05$). The instantaneous water use efficiency of alfalfa was significantly higher in the P_1 treatment ($p < 0.05$).

When the phosphorus treatment was 100 mg·kg^{-1}, the instantaneous water use efficiency of alfalfa under T_3 treatment exhibited a statistically significant increase compared to T_0 treatment ($p < 0.05$). The effect of simultaneous inoculation of Fm and Ge was significantly better than that of single inoculation ($p < 0.05$). The instantaneous water use efficiency of leaves of alfalfa under the fungus treatment (T), phosphorus treatment (P) and fungus phosphorus interaction (T×P) in two years showed extremely significant differences ($p < 0.01$).

3.5 Correlation analysis of alfalfa indicators under different treatments

Pearson correlation analysis showed that except for the instantaneous water use efficiency of alfalfa leaves and intercellular CO_2 concentration, the other indexes were extremely significantly positively correlated each other ($p < 0.01$) (Figure 5). Alfalfa leaves were extremely positively correlated with net photosynthetic rate and light use efficiency ($p < 0.01$), while

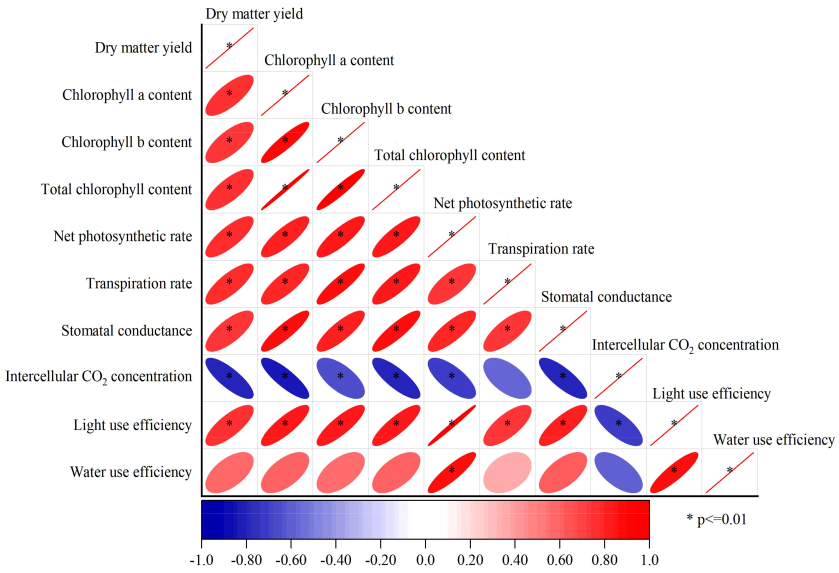

FIGURE 5
Correlation analysis of alfalfa indexes under different treatments. *Significant correlation was found at the 0.01 level (bilateral).

alfalfa leaf water use efficiency was positively correlated with dry matter yield, chlorophyll a, chlorophyll b, total chlorophyll content and stomatal conductance ($p < 0.05$), but not significantly positively correlated with transpiration rate ($p > 0.05$). Intercellular CO_2 concentration (C_i) was extremely negatively correlated with dry matter yield, chlorophyll a content, chlorophyll b content, total chlorophyll content, leaf net photosynthetic rate, stomatal conductance and light use efficiency ($p < 0.01$), and intercellular CO_2 concentration was negatively correlated with transpiration rate and instantaneous water use efficiency of alfalfa leaves ($p < 0.05$).

3.6 Comprehensive evaluation of membership functions

In order to avoid one-sidedness in the analysis of a single index and evaluate the optimal phosphorus and fungal application treatment (Sun et al., 2019), the membership function analysis evaluated eight key indicators of alfalfa performance (Table 2). The net photosynthetic rate, transpiration rate, stomatal conductance, light energy use efficiency and instantaneous water use efficiency of alfalfa leaves were all positive indicators, while the intercellular CO_2 concentration was negative. The top three treatments in terms of comprehensive ranking were T_3P_2, T_3P_1, and T_1P_2.

4 Discussion

4.1 Effect of AMF inoculation on the dry matter yield of alfalfa under different phosphorus application conditions

Alfalfa, as a perennial legume forage, has always been known for its high quality and high yield. Dry matter yield is one of the important representatives of alfalfa production performance index. The intricate relationship between phosphorus application and AMF inoculation significantly shapes alfalfa's dry matter yield. Studies have shown that phosphorus application and AMF inoculation can significantly increase the phosphorus use efficiency and leaf chlorophyll content of alfalfa, thereby increasing the aboveground biomass of plants (Mickky et al., 2018). The results showed that, with the increase of phosphorus application, the dry matter yield of alfalfa increased at the lower dose of P (P_0-P_2), while it decreased at the higher dose of P (P_2-P_3), and the application of phosphorus fertilizer in alfalfa was better than that without phosphorus fertilizer ($p < 0.05$). When the amount of phosphorus (P_2O_5) reached 150 mg·kg^{-1}, the dry matter yield of alfalfa decreased, which may be due to the high concentration of phosphorus, which caused a significant decrease in the diversity of AMF in the mycorrhizal community of alfalfa root and outside the root, and the colonization rate also decreased, which affected plant growth and reduced plant dry matter yield (Hinsinger, 2001). Therefore, the appropriate phosphorus application level is helpful to increase the yield.

In addition, high concentrations of phosphorus were found to damage the branches, vesicles and root fungal activity of maize (*Zea mays* L.) roots after AMF inoculation and phosphorus application (Chen et al., 2015.). In this study, under the condition of phosphorus application of 100 mg·kg^{-1}, Fm × Ge treatment was significantly higher than that of the control group ($p < 0.05$). It can be seen that inoculation of the two fungi has a significant positive effect on the growth of alfalfa, the main reason may be that AMF inoculation can significantly reduce the content of phosphorus phytate in alfalfa plants, and the arbuscular mycorrhizal fungal mycelium can absorb phosphorus from the soil and transfer it to the roots of alfalfa (Sun et al., 2022). Studies have shown that inoculation of sweet potatoes (*Ipomoea batatas* (L.) *Lam.*) with arbuscular mycorrhizal fungi is able to exhibit higher levels of photosynthetic pigments, enhance chlorophyll fluorescence and net photosynthetic rate under dehydration stress (Yooyongwech et al., 2014), resulting in better growth and yield traits (Yooyongwech et al., 2016). Therefore, the increase in the dry matter yield of alfalfa is due to the accumulation of photosynthetic products and the increase of available phosphorus content in the soil.

4.2 Effect of AMF inoculation on photosynthetic characteristics of alfalfa under different phosphorus application conditions

Photosynthesis is the basis for the formation of organic matter, oxygen and dry matter yield in plants, and improving plant photosynthetic efficiency and chlorophyll content is of great significance for increasing the accumulation of plant dry matter yield and improving the maintenance of plant nutritional quality (Sun et al., 2022). Phosphorus has a very close relationship with the photosynthesis of plants. Studies have shown that ATP and enzymes in plant photosynthesis require the participation of phosphorus, and the increase of phosphorus in plants is beneficial to the progress of plant photosynthesis, and the assimilation of CO_2 is directly related to the growth, development and photosynthesis of plants (Balestrini et al., 2020; Valkov et al., 2020). The results showed that, with the increase of phosphorus application, the net photosynthetic rate, transpiration rate and stomatal conductance of alfalfa increased at the lower dose of P (P_0-P_2), while it decreased at the higher dose of P (P_2-P_3), which may be because when the phosphorus content in the soil is insufficient for the phosphorus requirement of plants, the low phosphorus content will affect the opening and closing of the stomata in the leaves of plants, limit the regeneration of ribulose diphosphatase in alfalfa leaves (Zhang et al., 2016), and hinder the synthesis of ribulose diphosphate, which in turn affects the progress of alfalfa photosynthesis (Qi et al., 2013), photosynthesis is limited by the regeneration ability of RuBP in Calvin cycle. Rubisco is the rate-limiting enzyme of photosynthetic carbon assimilation, and its activity directly affects the photosynthetic rate of light saturation(Qi et al., 2013) and the application of phosphate fertilizer helps to improve the photosynthesis of alfalfa. When the phosphorus application rate reached 150 mg·kg^{-1}, the excess phosphorus impaired the photosynthesis process of alfalfa plants, which in turn reduced the net photosynthetic rate of alfalfa (Song et al., 2021). Therefore, reasonable fertilization strategies can help improve plant photosynthesis.

TABLE 2 Comprehensive evaluation of alfalfa indexes.

Index	Dry matter yield	Total chloro-phyll content	Net photosynthetic rate	Transpiration rate	Stomatal conductance	Intercellular CO_2 concentration	Light use efficiency	Water use efficiency	Average	Rank
T_0P_0	0.000	0.000	0.000	0.290	0.000	1.000	0.000	0.000	0.161	16
T_0P_1	0.343	0.189	0.214	0.545	0.109	0.829	0.171	0.171	0.321	13
T_0P_2	0.418	0.253	0.389	0.599	0.381	0.874	0.127	0.127	0.396	12
T_0P_3	0.171	0.226	0.317	0.427	0.258	0.217	0.786	0.786	0.399	11
T_1P_0	0.246	0.337	0.096	0.331	0.167	0.658	0.343	0.343	0.315	14
T_1P_1	0.669	0.411	0.658	0.484	0.315	0.536	0.466	0.466	0.501	7
T_1P_2	0.950	0.575	0.823	0.885	0.415	0.478	0.528	0.524	0.652	3
T_1P_3	0.665	0.195	0.405	0.462	0.217	0.458	0.545	0.545	0.437	9
T_2P_0	0.168	0.116	0.199	0.000	0.099	0.718	0.283	0.283	0.233	15
T_2P_1	0.564	0.411	0.588	0.525	0.238	0.453	0.550	0.550	0.485	8
T_2P_2	0.874	0.642	0.618	0.968	0.658	0.530	0.468	0.468	0.650	5
T_2P_3	0.338	0.526	0.298	0.709	0.221	0.597	0.405	0.405	0.437	10
T_3P_0	0.588	0.437	0.413	0.478	0.637	0.300	0.703	0.703	0.532	6
T_3P_1	1.000	0.621	0.588	0.822	0.700	0.000	1.004	1.004	0.717	2
T_3P_2	0.972	1.000	1.000	1.000	1.000	0.004	1.000	1.000	0.872	1
T_3P_3	0.496	0.679	0.934	0.726	0.738	0.358	0.644	0.644	0.652	4

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The results showed that when the amount of double fungi and phosphorus application was 100 mg·kg^{-1}, the light use efficiency and the instantaneous water use efficiency of leaves of alfalfa increased at the lower dose of P (P_0-P_2), while it decreased at the higher dose of P (P_2-P_3), and the net photosynthetic rate of leaves was significantly positively correlated with the light use efficiency and instantaneous water use efficiency ($p < 0.05$), which may be because phosphorus application and double fungus can improve the drought resistance of alfalfa. The lower the decrease of net photosynthetic rate, the less damage to photosynthetic organs in plant leaves, the higher the activity of photosynthetic cells, and the stronger the drought resistance of plants. Water use efficiency is an important symbol to judge the drought resistance of plants, which can reflect the water consumption and water use ability of plants. The higher the water use efficiency is, the stronger the adaptability of plants to drought is (Tang et al., 2023). Studies have shown that plants reduce chlorophyll content and stomatal conductance during drought stress, which affects plant photosynthetic capacity, which in turn reduces biomass (Naylor et al., 2018). Therefore, the appropriate bacterial fertilizer strategy is helpful to improve the drought resistance of plants.

Chlorophyll content is a key factor affecting the photosynthetic efficiency of alfalfa, and participates in the process of absorption, transfer and conversion of light energy (Zhu et al., 2017). Therefore, the chlorophyll content of plants plays an important role in their growth. The results showed that AMF inoculation of *Robinia* spp. seedlings significantly improved leaf area, carboxylation efficiency, chlorophyll content, net photosynthetic rate and effective photochemical efficiency of PSII (Mo et al., 2016), thereby maximizing their biomass (Zhu et al., 2014). Arbuscular mycorrhizal fungi can modulate the source-library relationship by enhancing the exchange of carbohydrates and mineral nutrients with the plant host, thereby stimulating the plant host to increase the rate of photosynthesis and increasing the carbon demand of arbuscular mycorrhizal fungi (Zhao et al., 2022b). Therefore, inoculation of arbuscular mycorrhizal fungi helps to increase chlorophyll content, promote photosynthesis, and thus increase crop yield.

4.3 The membership function comprehensively evaluates the optimal phosphorus application mode of different treatment combinations

Determining the best bacterial phosphorus treatment for the agricultural environment will help to improve the economic benefits of alfalfa. The effects of inoculation of Fm and Ge on dry matter yield, chlorophyll content and photosynthesis of alfalfa were different, and the membership function analysis and evaluation method was to comprehensively evaluate the performance of different treatments on each index and screen out the optimal phosphorus sterilization treatment among all treatments (Sun et al., 2019). In this study, the optimal combination of different treatments was ordered by T_3P_2 treatment (Table 2), indicating that when the phosphorus application rate was 100 mg·kg^{-1}, the photosynthetic efficiency and dry matter yield of mixed inoculation of *Funneliformis mosseae* and *Glomus etunicatum* were maximized. Simultaneous inoculation of two fungi under suitable phosphorus application conditions can effectively promote the uptake of available phosphorus in the soil by plants, and then increase the dry matter yield of alfalfa.

5 Conclusion

Compared with no fertilization, the amount of phosphorus (P_2O_5) applied was 100 mg·kg^{-1}, and the mixed inoculation of *Funneliformis mosseae* and *Glomus etunicatum* could significantly improve the photosynthetic efficiency and chlorophyll content of alfalfa leaves, thereby increasing the dry matter yield of alfalfa. This study clarified the optimal coupling mode of bacterial phosphorus for further improvement of alfalfa yield. It is the focus of future research to combine the anatomical structure and photosynthetic performance of alfalfa leaves and stems, and to clarify the synergistic mechanism of the anatomical structure and photosynthetic performance of alfalfa.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

DX: Conceptualization, Software, Writing – original draft, Writing – review & editing. XA: Data curation, Investigation, Methodology, Software, Writing – review & editing. IL: Supervision, Writing – review & editing. CM: Conceptualization, Writing – review & editing. QZ: Formal Analysis, Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing.

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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.

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Comparative proteomic analysis identifies proteins associated with arbuscular mycorrhizal symbiosis in *Poncirus trifoliata*

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Citrus, one of the most widely cultivated fruit crops in the world, relies on arbuscular mycorrhizal fungi (AMF) to absorb nutrients and water from soil. However, the molecular mechanism of AM symbiosis (AMS) in citrus in general have largely been understudied. Here, using a TMT labeling proteomic approach, we identified 365 differentially expressed proteins (DEPs) in roots of *Poncirus trifoliata* (a common citrus rootstock) upon *Rhizophagus irregularis* colonization as compared with uninoculated roots, of which 287 were up-regulated and 78 were down-regulated. GO analysis revealed that the DEPs were mainly involved in biological processes such as negative regulation of endopeptidase inhibitor activity, negative regulation of endopeptidase, one-carbon metabolic process and carbohydrate metabolic process. KEGG enrichment analysis indicated that the DEPs were mainly involved in regulating metabolic pathways such as fatty acid biosynthesis, phenylpropanoid biosynthesis and carbon metabolism. Furthermore, 194 of the 365 DEPs were found to be associated with AMS-responsive genes by association analysis with our previous transcriptomes data, which highlighted the important roles of these proteins in AMS. One of the 194 DEPs, neutral ceramidase (PtNCER), was further chosen for function analysis via RNAi interfering its homologous gene *MtNCER* in a mycorrhizal model plant *Medicago truncatula*, which confirmed a positive role of NCER in AM establishment. Our results provided basic data and key candidate genes for genetic improvement of efficient nutrient uptake through AM establishment in citrus and other crops.

KEYWORDS

arbuscular mycorrhizal symbiosis, citrus, proteomic, NCER, Poncirus trifoliata, Rhizophagus irregularis

Introduction

Arbuscular mycorrhizal symbiosis (AMS) is a mutually beneficial association formed between the roots of most land plants and arbuscular mycorrhizal fungi (AMF) (Lanfranco et al., 2016). This symbiotic relationship plays a vital role in enhancing plant nutrient acquisition, promoting plant growth and development, and improving plant tolerance to various biotic and abiotic stresses (Pozo and Azcón-Aguilar, 2007; Parniske, 2008; Smith and Smith, 2011). The establishment of AMS involves intricate molecular interactions between the host plant and AMF, leading to the formation of specialized structures called arbuscules, and special interfaces for bidirectional nutrient transfer called peri-arbuscular membrane (PAM) within the root cortex cells of the plant (Harrison, 1999).

Citrus is one of the most important fruit crops in the world. Due to their high heterozygosity, polyembryony and long juvenility, citrus plants usually propagate through grafting to maintain the excellent characteristics of the variety and reduce juvenility, such as *Poncirus trifoliata*, which is a popular rootstock for the citrus industry in China (Zhu et al., 2019; Huang et al., 2021). However, most citrus are cultured in hilly and mountainous areas with barren soil and few root hairs (Cao et al., 2013; Zhang et al., 2013). This seriously affects the nutrient absorption of citrus roots and the formation of fruit yield and quality. It is believed that the absorption of soil nutrients by citrus roots largely depends on symbiotic AMF (Wu et al., 2016). Indeed, studies had shown that AMF can significantly improve the growth performance of citrus (Chen et al., 2014; Xiao et al., 2014). Therefore, understanding the molecular mechanisms underlying symbiotic interaction is crucial for promoting citrus nutrient absorption. However, the molecular mechanism of AM symbiosis (AMS) in citrus was poorly studied. The study of the complex molecular dialogue between citrus and AMF remains a challenging task.

The interaction between host plants and AMF is highly complex, as the establishment of AMS involves numerous molecular and cellular developmental processes (Gutjahr and Parniske, 2013). These processes are significantly influenced by host plant root proteins responding to AMF colonization (Javot et al., 2007; Bravo et al., 2016; Floss et al., 2017). Some key proteins have been identified as essential for establishing AMS, including phosphate transporter protein PT4 (Phosphate Transporter 4), lipid biosynthetic enzyme FatM (Fat required for AMS), ABC transporters proteins STR1 (Stunted Arbuscule 1) and STR2 (Stunted Arbuscule 2), GRAS domain transcription factors such as RAM1 (Required for Arbuscular Mycorrhization1), RAD1 (Required for Arbuscule Development 1), AP2 domain transcription factors like CBX1 (CTTC Motif-Binding Transcription Factor 1), WRI5a/b/c (WRINKLED5a/b/c) and MYB (v-myb avian myeloblastosis viral oncogene homolog) domain OsPHR2 (Phosphate Starvation Response 2) transcription factor (Javot et al., 2007; Gobbato et al., 2012; Gutjahr et al., 2012; Xue et al., 2015; Bravo et al., 2017; Jiang et al., 2017; Jiang et al., 2018; Xue et al., 2018; Shi et al., 2021).

Transcriptomics analysis is a powerful tool for dissecting the interactions between plants and microorganisms. However, mRNA abundance often has a weak correlation with its corresponding protein levels, resulting in the transcriptome not fully reflecting the actual physiological role of the genes (Vogel and Marcotte, 2012). Proteomes represents the ultimate regulatory processes, providing a wealth of additional information on plant-microbe interactions (Feussner and Polle, 2015; Domingo et al., 2023). Proteomic analysis enables the systematic identification and quantification of proteins expressed in plant roots in response to AMF colonization, providing insights into the functional dynamics of the plant-microbe interaction at the protein level (Jia et al., 2019). Consequently, proteomic approaches have emerged as powerful tools for studying the molecular interactions between plants and AMF. For instance, a protective effect of AMS against arsenic stress in *Pteris vittata* was observed through the proteomic approach performed by Bona et al. (Bona et al., 2011). In addition, proteomic analysis of *Elaeagnus. angustifolia* roots revealed that the abundance of 170 proteins were significantly influenced after AMF inoculation under salt stress, which were mainly involved in the amino acid metabolism, lipid metabolism, and glutathione metabolism. (Chang et al., 2022).

In this study, proteomic analysis of *P. trifoliata* (most common rootstock of cultivated citrus) was conducted for the first time using tandem mass tags (TMT) labeling coupled with LC-MS/MS approach. 365 differentially expressed proteins (DEPs) in response to colonization by *R. irregularis* was identified. Among these DEPs, 194 were associated with differentially expressed genes (DEGs) from our previous transcriptome data. Furthermore, a reverse genetic analysis was performed to validate further the symbiotic functions of neutral ceramidase (NCER), which was conserved in mycorrhizal plant species. Our work aimed to reveal the key proteins of *P. trifoliata* in response to AMF and the majority of proteins conserved in AM-host plants. Our results provided novel basic data and insights for the underlying molecular mechanisms of AMS in citrus.

Materials and methods

Plant materials and mycorrhizal inoculation

Seeds of trifoliate orange [TO, *Poncirus trifoliata* L. Raf.] were surface-sterilized by washing in 1 M NaOH for 15 min and in 3% sodium hypochlorite for 10 min, followed by rinsing with distilled water. The treated seeds were soaked in water for two days and the water was changed once a day. The seeds were placed in Petri dishes and covered with sterilized gauze for 1 week in the dark (28°C), and then sown in autoclaved vermiculite and placed in a greenhouse for 1 month. Seedlings were transplanted into sterile quartz sand in 6 pots of 4 plants per pot (18 × 18 cm) and grown under greenhouse conditions (25/22°C day/night). To inoculate AMF, 400 spores of *R. irregularis* DAOM197198 were placed below the seedlings for each pot. The *R. irregularis* DAOM197198 spores were obtained from Agronutrition, Toulouse, France (https://www.agronutrition.com/en). Plants without AM inoculation were used as controls. Plants were watered twice a week with 250 ml half-strength Hoagland

solution containing 20 μM phosphorus as described previously (Ji et al., 2023). After 6 weeks post-inoculation, the *P. trifoliata* roots were well colonized and arbuscules were observed, and therefore plants were sampled at this time. Lateral roots from mycorrhizal and non-mycorrhizal plants were harvested for proteomic analysis with three biological replicates, respectively labeled as AM and NM, each containing 4 plants roots.

Medicago truncatula genotypes A17 seeds were scarified with sulfuric acid for 4 min and surface-sterilized with 10% sodium hypochlorite for 2 min. After rinsing 5 times with sterile water, seeds were vernalized for 1 d at 4°C and germinated at 25°C for 24 h in darkness. One-day-old seedlings were transferred to new 9 cm Petri dishes containing Färhaeus medium grown at 16 h light (25° C)/8 h dark (22°C). Hairy root transformation was performed according to Limpens et al. (Limpens et al., 2004). The transformed plants were individually transplanted into cones with a vermiculite: sand (1:4 V/V) mixture. To inoculate AMF, 400 spores of *R. irregularis* DAOM197198 were placed below the seedlings for each cone. Plants were watered twice a week with modified Hoagland medium containing 20 μM phosphorus (Javot et al., 2011). Plants were harvested 5 weeks after inoculation.

Protein extraction, digestion, and TMT labeling

The roots of citrus were ground into powders in liquid nitrogen. And then, the powders were suspended with solution containing 10% (w/v) TCA, 65mM DTT. After precipitation at -20°C for 1 h, the samples were centrifuged at 10,000 rpm at 4 °C for 45 min and the supernatant was discarded. The pellet was then lysed by SDT buffer (4% (w/v) SDS, 100 mM Tris-HCl at pH 7.6, 0.1M DTT) to extract proteins (Wiśniewski et al., 2009). Then the protein concentration was determined by using the BCA Protein Assay Kit (Beyotime). Protein digestion was performed according to procedure as described previously (Bai et al., 2019). Extracted proteins (200 μg) for each sample were digested according to the filter-aided sample preparation (FASP) procedure (Wiśniewski et al., 2009). After trypsin digestion, the peptides were desalted on a Strata X C18 SPE column (Phenomenex, Torrance, CA, USA) and vacuum dried. The peptide mixture was recombined in 0.5 M TEAB and processed according to the TMT kit (Thermo Fisher) protocol. The labeled peptides from each group were mixed and classified using AKTA Purifier 100. The dried peptide mixture was reconstituted and acidified with 2 mL of buffer A (10 mM KH2PO4 in 25% ACN, pH 3) and loaded onto a Poly SULFOETHYL 4.6 × 100 mm column (5 mm, 200 Å, PolyLC Inc., MD, Columbia, USA). Peptides were eluted at a flow rate of 1 mL/min with a gradient of 0-10% for 0-25 min, 10-20% to 25-32 min, 20-45% to 32-42 min, 45-100% to 42-47 min, and at 100% from 47-52 min and 52-60 min using buffer B (10 mM KH2PO4, 500 mM KCl, 25% ACN, pH 3). During the elution process, the absorbance value of 214 nm was monitored, and the eluted fractions were collected every 1min and lyophilized and desalted by C18 Cartridge (EmporeTM SPE Cartridges C18, Sigma, Roedermark, Germany). All samples were stored at -80°C for LC-MS/MS analysis.

LC-MS/MS analysis

Approximately 10 μL of each fraction was injected for nano-LC-MS/MS analysis using an Q-Exactive MS (Thermo Scientific) equipped with Easy nLC1000 (Thermo Scientific). The peptide mixture was loaded onto a C18 column (Thermo Scientific EASY trap column) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% ACN and 0.1% formic acid) at a flow rate of 250 nL/min. The peptides were eluted with a gradient of 0–35% buffer B from 0 to 50 min, 35 to 100% buffer B from 50 min to 58 min, and 100% buffer B from 58 min to 60 min. After chromatographic separation, the samples were analyzed by mass spectrometry (MS) using Q-Exactive mass spectrometer. The peptides were analyzed in a positive ion mode and MS spectra were acquired using a data-dependent top 10 methods dynamically choosing the most abundant precursor ions from the survey scan (300-1800 m/z) for higher-energy collisional dissociation (HCD) fragmentation. Determination of the target value was based on predictive automatic gain control (AGC). Dynamic exclusion duration was 40.0 s, survey scans were set at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200. Normalized collision energy was 30 eV and the underfill ratio was defined as 0.1%.

Bioinformatic analysis

The LC-MS/MS data were searched against the UniProt Sus scrofa database for peptide identification and quantification using Mascot 2.2 (Matrix Science) embedded in Proteome Discoverer 1.4. Peptides were identified using the following options. Peptide mass tolerance, ± 20 ppm; fragment mass tolerance, 0.1 Da; max missed cleavages, 2; fixed modifications: carbamidomethyl (C), TMT 10-plex (N-term), TMT 10-plex (K); variable modifications: oxidation (M), TMT 10-plex (Y). The score threshold for peptide identification was set ≤0.01 false discovery rate (FDR).

DEPs were screened according to the criteria with fold change up-regulation greater than 1.5-fold or down-regulation less than 0.67-fold and a p-value < 0.05. OmicsBean software was used to analysis Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of these DEPs.

Phylogenetic analyses

The deduced amino acid sequences of PtNCER was used as BLASTp queries against 22 plant species databases in Phytozome (https://phytozome-next.jgi.doe.gov/) with a cut-offs of *E*-value<1e-50. All the sequences were aligned with the MAFFT software, and a phylogenetic tree was constructed by using the Neighor-joining method. The sequences were aligned by MAFFT software. All the peptide sequences are listed in Supplementary Table 5.

Plasmid construction and hairy root transformation

For promoter-GUS analysis, the *MtNCER* promoter was cloned from Medicago A17 genomic DNA, and the *MtNCERpro* : GUS vectors were constructed using Golden Gate cloning. For RNAi analysis, a ~375-bp coding sequence of *MtNCER* (*Medtr3g079190*) was cloned into the pDONR221 vector using gene-specific primers, followed by recombination into the pK7GWIWG2(II) binary vector containing DsRed as a visual selection marker (Limpens et al., 2004) using Gateway® LR ClonaseTM (Invitrogen). An empty construct CHEAP-pK7GWIWG2 (II) was used as a negative control in the hairy root transformation assays (Limpens et al., 2004). All the recombinant plasmids introduced into *Agrobacterium rhizogenes* strain MSU440 and the positive Agrobacterium cells were used for hairy root transformation in *M. truncatula* A17.

WGA and GUS staining, microscopy and quantification of AMF colonization

For promoter-GUS analysis, GUS and WGA Alexa Fluor 488 co-staining was conducted as follows. Transgenic roots were harvested 5 week after inoculation with *R. irregularis* immerged in GUS staining buffer (3% sucrose, 0.5 mM EDTA, 2 mM potassium-ferrocyanide, 2 mM potassium-ferricyanide and 1 mg/mL X-Gluc in 100 mM PBS, pH7.0), and vacuum-infiltrated for 10 min followed by incubation at 37°C in darkness for 3-5h, and then washed three times in water. The roots incubated in 10% (w/v) KOH at 90°C for 8 min, washed twice in water, socked in 1M HCl for 20 min, incubated in ×1 PBS buffer for 15 min, and then incubated in ×1 PBS containing 2 mg/L WGA 488 (Invitrogen, Waltham, MA, USA) at 4°C overnight. GUS and WGA 488 imaging were performed using a Nikon fluorescence microscope.

For RNAi analysis, the *M. truncatula* transformed roots were cleaned with water, and WGA 488 staining was conducted as indicated above. Roots were cut into 1-cm fragments, and the level of colonization was calculated according to the previously reported method of Trouvelot et al. (1986) using a Nikon fluorescence microscope.

RNA extraction and gene expression detection

Total RNA extraction and quantitative real-time PCR were performed as previously described (Yu et al., 2023). The transcript levels of genes were normalized using the widely used housekeeping gene *Pt7g003560.1* (*PtrActin*) as a reference. For *M. truncatula* samples, the widely used housekeeping gene *Medtr6g021800* (*MtEF1α*) was used as the reference. The relative expression levels were calculated by $2^{-\triangle\triangle ct}$ with three biological replicates for each sample. All the qRT-PCR data was analyzed by T-test using a Graphpad Prism 9 software. All the primer sequences for qRT-PCR analysis were listed in Supplementary Table 8.

Results

Proteomic analysis identified 365 P. *trifoliata* proteins responsive to *R. irregularis* colonization

To investigate the change of protein levels in response to AMF colonization in the commonly used citrus rootstock *P. trifoliata*, we inoculated the roots of *P. trifoliata* with or without *R. irregularis* for 6 weeks, and then checked the AMF colonization level to ensure the AM establishment. Results showed that, in the *R. irregularis* inoculated roots, the frequency of AMF colonization (F%) in the roots was ~89%, with an arbuscular abundance (A%) of ~29%, and well-developed arbuscules were also observed (Figures 1A, C). As expected, no WGA488 staining (a fungal cell wall staining) was observed in the uninoculated roots of citrus rootstock (Figure 1B).

Total proteins were extracted from the roots of citrus rootstock inoculated or uninoculated with *R. irregularis*. A proteomic approach using tandem mass tags (TMT) labeling coupled with LC-MS/MS technology was employed. A total of 5139 expressed proteins were found from 6 root samples (3 inoculated (ZK-AM) and 3 uninoculated (ZK-NM) samples, Supplementary Table 1). A total of 365 proteins were identified as DEPs with expression level of fold change > 1.5 and $p < 0.05$, of which 287 proteins were up-regulated and 78 were down-regulated in mycorrhizal roots relative to nonmycorrhizal controls (Supplementary Table 2). To obtain a visualized view of the comparative proteomic results, the up-regulated DEPs (red dots) and down-regulated DEPs (blue dots) were shown in a volcano plot (Figure 2A), and expression profile of each DEP was shown by a hierarchical clustering heat map (Figure 2B). It was worth noting that 10 proteins in roots were up-regulated over 6 folds by *R. irregularis* colonization, including Pt1g014080.1, Pt5g009680.1, Pt9g018680.1, Pt3g010560.2, Pt8g001290.1, Pt2g025350.1, Pt1g019080.1, Pt6g018480.1, Pt7g019310.1 and Pt3g011180.3. Especially, the protein Pt9g018680.1 showing the up-regulated folds (8.08 folds) was an orthologous to MtPT4 (Medtr1g028600), a symbiotic phosphate transporter strongly induced by AMF and essential for symbiotic phosphate transport (Javot et al., 2011). The protein Pt1g019080.1 showing the up-regulated folds (7.30 folds) was an orthologous to MtKIN5 (kinase 5), which was conserved for AMS identified through phylogenomics (Bravo et al., 2016).

To evaluate the reliability of the proteomic data, we examined the expression level of 8 up-regulated proteins by qRT-PCR, including PtNIP5;1 (nodulin 26-like intrinsic proteins5;1), PtPT4, PtSLC35 (solute carrier family 35), PtKIN5 (kinase 5), PtLHT1 (lysine histidine transporter 1), PtLRR (leucine rich repeat), PtSBT (subtilisin-related protease) and PtRlpA-like (rare lipoprotein A-like). The results showed that the transcription levels of all the

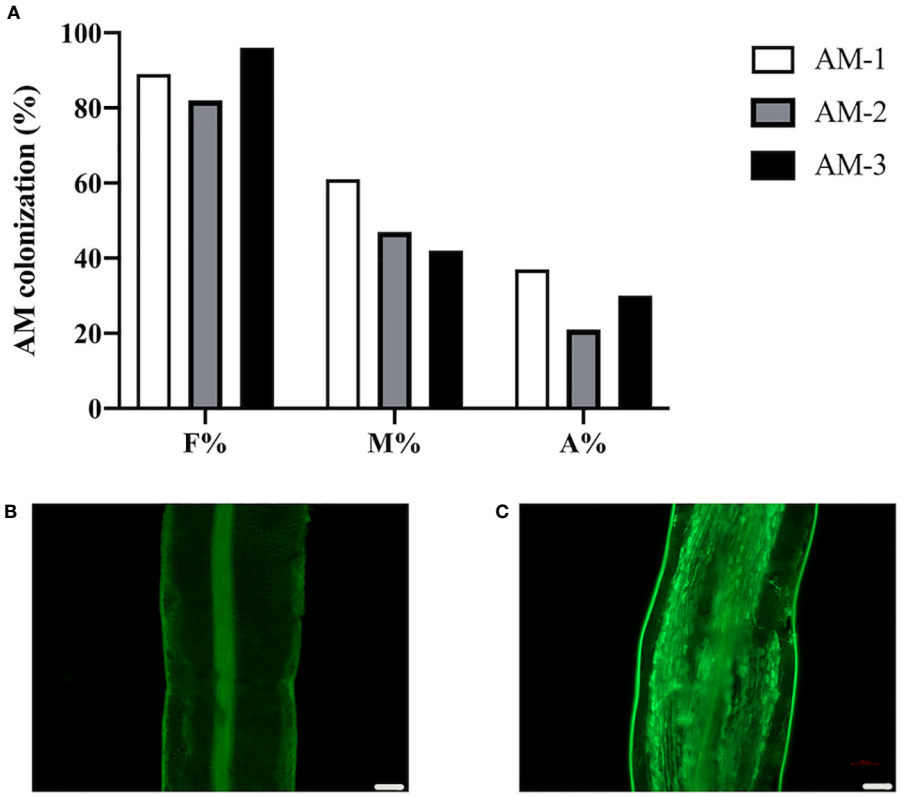

FIGURE 1
Arbuscular mycorrhizal symbiosis of *P. trifoliata*. **(A)** Quantification of mycorrhization level of three plant roots of *P. trifoliata* (named AM-1, AM-2 and AM-3 respectively) at 6 weeks post inoculation with *R. irregularis*. F%, frequency of analyzed root fragments that are mycorrhizal; M%, intensity of infection in whole roots; A%, arbuscule abundance in whole roots. **(B)** WGA staining of *P. trifoliata* roots after 6 weeks without inoculation with *R. irregularis* **(C)** WGA staining of *P. trifoliata* roots after 6 weeks inoculation with *R. irregularis* (Scale bars, 100 μm).

tested proteins were also largely up-regulated in *R. irregularis* colonized samples as compared with controls (Figure 2C).

Enrichment analysis of the DEPs

To understand the potential biological processes of the DEPs in AMS, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed, respectively. GO terms were assigned to the three main categories: biological process (BP), cellular component (CC) and molecular function (MF). In BP category, DEPs were significantly enriched in negative regulation of endopeptidase activity process, one-carbon metabolic process, carbohydrate metabolic process, chitin catabolic process and response to biotic stimulus. In CC category, DEPs were notably enriched in the intracellular membrane-bounded organelle, integral component of plasma membrane, extracellular region, cell wall and chloroplast, and so on. In MF category, DEPs were observably enriched in endopeptidase inhibitor activity, serine-type endopeptidase activity, iron ion binding, transferase activity, transferring acyl groups other than amino-acyl groups, and phosphatase activity (Figure 3A).

The KEGG pathway enrichment analysis revealed that the 365 DEPs were significantly enriched in fatty acid biosynthesis, phenylpropanoid biosynthesis, fatty acid metabolism, carbon metabolism, biosynthesis of secondary metabolites, pyruvate metabolism, glycolysis/Gluconeogenesis, biosynthesis of cofactors, amino sugar and nucleotide sugar metabolism and citrate cycle (TCA cycle). As shown in Figure 3B, the most significantly enriched pathway was fatty acid biosynthesis.

Comparison between the proteome data and our previous transcriptome data

To investigate transcriptional regulation of the formation and functioning of *P. trifoliata* during AM symbiosis, the RNA-seq of citrus rootstocks with or without *R. irregularis* were carried out in our previous study (Ji et al., 2023). A total of 3750 genes were identified as DEGs (Ji et al., 2023). To comprehensively identify the proteins involved in the formation and function of *P. trifoliata* during AM symbiosis, we carried out a correlation analysis between the proteome data and previous transcriptome data. By comparing 3750 DEGs and 365 DEPs, 194 proteins with their corresponding transcripts were successfully correlated. The 194 proteins were identified as key DEPs in response to AMF (Supplementary Table 3). Of these 194 key DEPs, 19 proteins associated with a set of conserved genes in five AM host plants including *Medicago truncatula*, *Poncirus trifoliata*, *Lotus japonicus*, *Oryza sativa* and

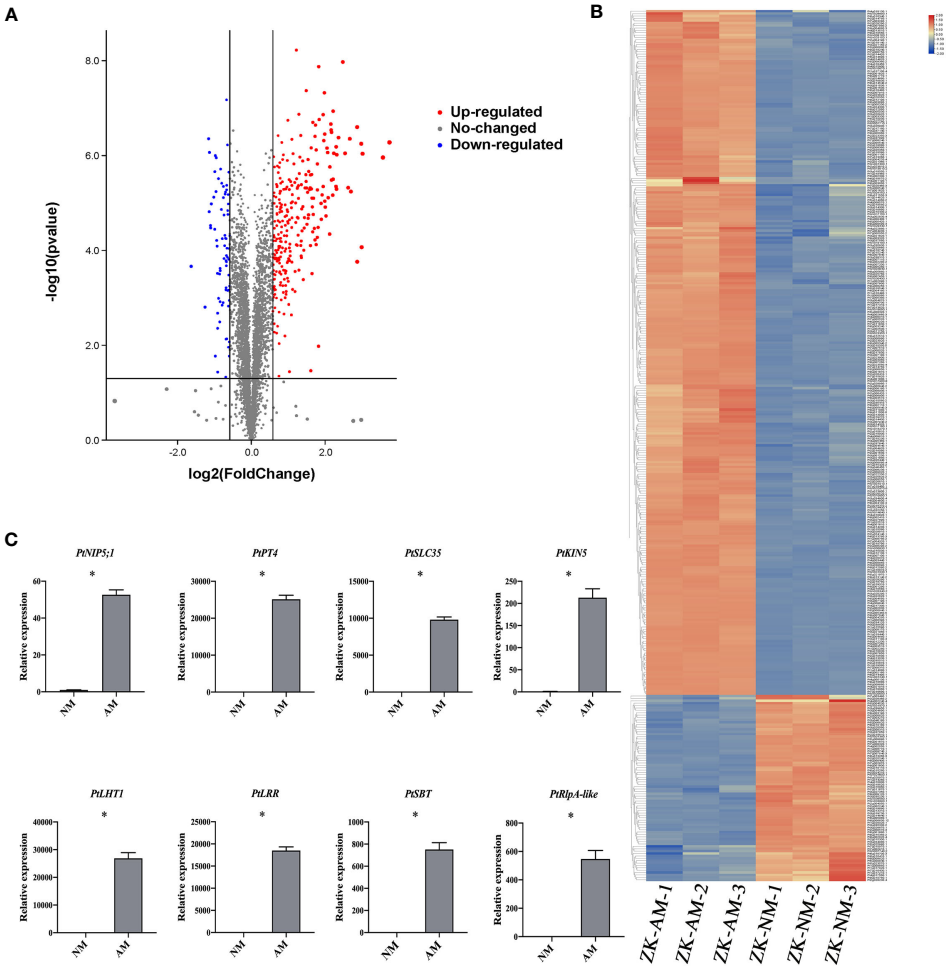

FIGURE 2
Identification of arbuscular mycorrhizal-responsive differentially expressed proteins from *P. trifoliata*. **(A)** Volcano plots of differentially expressed proteins (DEPs) between ZK-NM (roots of *P. trifoliata* uninoculation with AMF) and ZK-AM (roots of *P. trifoliata* inoculation with AMF) groups. The red dots are significantly up-regulated proteins, the blue dots are significantly down-regulated proteins, and the gray dots are no-changed proteins. **(B)** The heat-map for cluster analysis of DEPs between ZK-NM and ZK-NM. Up-regulated proteins are shown in red, down-regulated proteins are shown in blue. **(C)** qRT-PCR measurement of the expression of *PtNIP5;1 (Pt5g009680), PtPT4 (Pt9g018680), PtSLC35 (Pt2g025350), PtKIN5 (Pt1g019080), PtLHT1 (Pt2g010500), PtLRR (Pt4g009840), PtSBT (Pt8g004250)* and *PtRlpA-like (Pt5g018060)* genes in ZK-NM and ZK-AM. Expression is normalized against *Pt7g003560.1* using the $2^{-\triangle\triangle Ct}$ method. Error bars represent standard errors for three biological replicates. Asterisks represent significant differences (Student's *t*-test: $*p < 0.05$).

Solanum lycopersicum, were up-regulated by AMF (Supplementary Table 6) (An et al., 2018). As shown in Figure 4A, a positive correlation between the transcript and protein abundance was observed (Pearson correlation = 0.466). Previous studies have shown that the Pearson correlation coefficient of proteome and transcriptome association analysis is about 0.40 (Vogel and Marcotte, 2012). These results further indicated that the DEPs identified by proteomic analysis of *P. trifoliata* mycorrhizal played key roles in the symbiosis between citrus and AMF.

GO enrichment analysis of the 194 key DEPs were performed to explore the potential biological function. The top 20 GO term enrichment of 194 key DEPs were shown in Figure 4B. In terms of the BP category, 59 of the 194 key DEPs were mainly enriched in monocarboxylic acid metabolic process, oxidation-reduction process, monocarboxylic acid biosynthetic process, carboxylic acid metabolic process, oxoacid metabolic process, organic acid metabolic process, fatty acid metabolic process, purine nucleoside diphosphate metabolic process, purine ribonucleoside diphosphate metabolic process, ribonucleoside diphosphate metabolic process, pyruvate metabolic process, fatty acid biosynthetic process and nucleoside diphosphate metabolic process. In CC category, 4 of the 194 key DEPs were primarily enriched in integral component of plasma membrane. Regarding the MF category, 132 of the 194 key DEPs were mainly enriched in catalytic activity, oxidoreductase activity, serine-type peptidase activity, serine hydrolase activity, serine-type carboxypeptidase activity and monooxygenase activity (Figure 4B; Supplementary Table 4). The KEGG pathway enrichment analysis of the 194 key DEPs was shown in Figure 4C. Of these proteins, 61 were significantly enriched in fatty acid synthesis, pyruvate metabolism, glycolysis/Gluconeogenesis, citrate cycle (TCA cycle), biotin metabolism, amino sugar and nucleotide sugar metabolism, propanoate

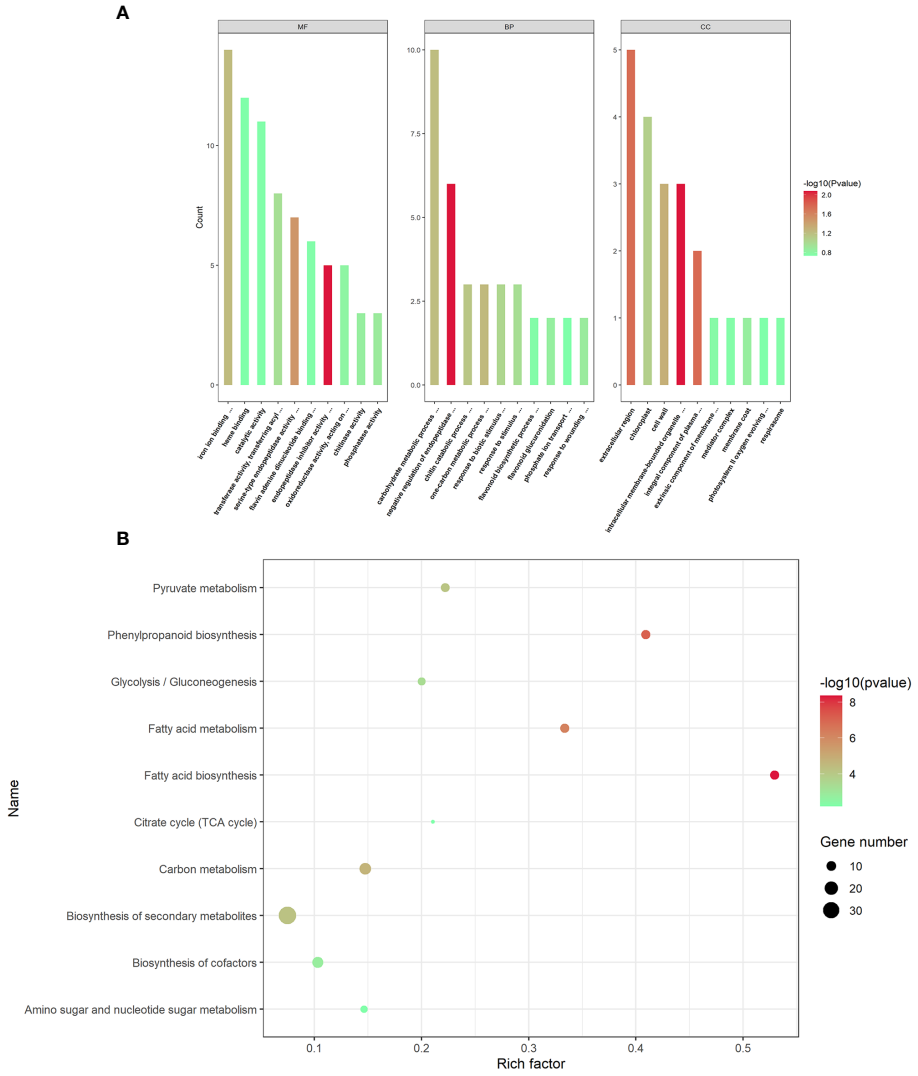

FIGURE 3
GO functional annotations and KEGG pathway enrichment analyses of the differentially expressed proteins. **(A)** The top GO terms in the biological process (BP), cell component (CC) and molecular function (MF) categories enriched with the differentially expressed proteins (DEPs). The color of the histogram represents the value of *p*-values. **(B)** The top 10 KEGG pathway enrichments of *P. trifoliata* DEPs. The size of the circle represents the number of DEPs enriched in the KEGG pathway. The color of the circle represents the value of *p*-values.

metabolism, purine metabolism and phenylpropanoid biosynthesis pathways (Figure 4C; Supplementary Table 5). Among these pathways, key DEPs related to fatty acid synthesis accounted for the highest proportion at 18%.

Functional analysis of NCER in AMS

Our combined proteomic and transcriptomics of *P. trifoliata* revealed that 194 key DEPs may play key roles in the symbiosis. To further verify this result, we focused on the protein neutral ceramidase (PtNCER: Pt5g001130), which was induced by AMF and enriched in arbuscules for functional analysis. NCER is a ceramidase involved in sphingolipid metabolism. Sphingolipids are required for maintaining the arbuscular mycorrhizal endosymbionts (Moore et al., 2021). As a result of the low efficiency and long period of genetic transformation of citrus, it is difficult to test PtNCER function in citrus using RNAi or stable overexpression methods. *Medicago truncatula* was the mycorrhizal model plant. Considering that the function of genes involved in AMS is highly conserved in plants, to quickly verify the function of PtNCER, its putative homologues (MtNCER; Medtr3g079190) in *Medicago truncatula* was first verified. Previous research has found that MtNCER was highly induced in cells containing arbuscules, which were isolated by laser microdissection (Supplementary Figure S1) (Zeng et al., 2018). According to the 'Noble MtGEA V3 by LIPME' resource in the Expression Atlas (https://lipm-browsers.toulouse.inra.fr/pub/expressionAtlas/app/mtgeav3/zz.complete_dataset), the result found that *MtNCER* gene was only expressed in roots colonized with AMF (Supplementary Figure S2).

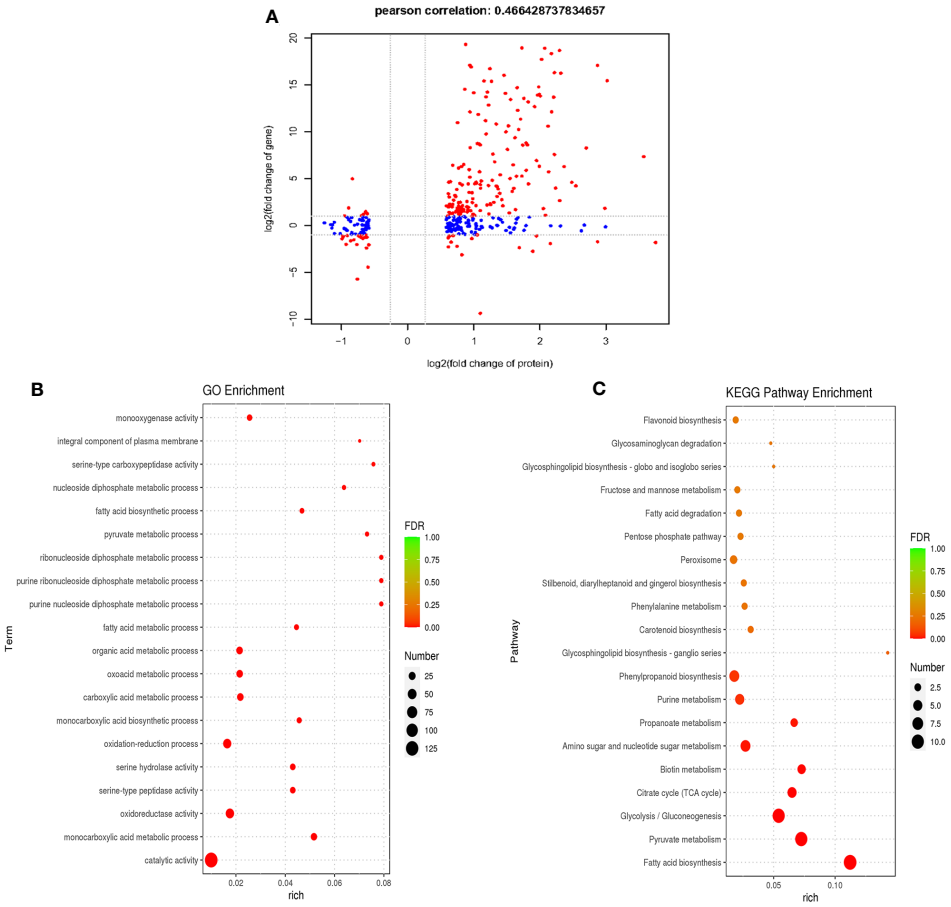

FIGURE 4
Combined analysis of differentially expressed proteins of proteome and differentially expressed genes of transcriptome. **(A)** Pearson correlation analysis of differentially expressed proteins (DEPs) and differentially expressed genes (DEGs) (Pearson correlation = 0.466). The red dots are proteins that are significantly differentially expressed in both proteome and transcriptome, the blue dots are proteins that are significantly differentially expressed in proteome. **(B)** The top 20 GO terms of *P. trifoliata* 194 key DEPs. The size of the circle represents the number of key DEPs enriched in the GO terms. **(C)** The top 20 KEGG pathway enrichments of *P. trifoliata* 194 key DEPs. The size of the circle represents the number of key DEPs enriched in the KEGG pathway. The color of the circle represents the value of False Discovery Rate (FDR).

Promoter-GUS analysis shown that GUS activity in roots expressing the *MtNCERpro* : GUS construct significantly increased upon inoculation with AMF, while no GUS activity was observed in the absence of *R. irregularis* inoculation (Supplementary Figure S3). Further microscopic observations showed that the GUS signals were particularly strong in root cortical cells containing arbuscules and hyphae (Figures 5A, B). The results indicated that the *NCER* gene was induced by mycorrhizal colonization.

Then, 70 homologous protein sequences from 20 plant species were obtained to perform phylogenetic analysis using MAFFT (Supplementary Table 7). Notably, phylogenetic analysis revealed that PtNCER and MtNCER proteins were clustered on clade unique to AM host plants, which further emphasizing their potentially important roles in AM symbiosis (Supplementary Figure S4). To investigate whether the MtNCER protein plays functional role in AMF colonization, transgenic *M. truncatula* roots expressing RNAi construct targeting *MtNCER* were generated. qRT-PCR analysis suggested that the expression of *MtNCER* were decreased by approximately 75% compared with control roots (Figure 5D). Reduction of *MtNCER* significantly affected colonization levels by *R. irregularis*. We observed that roots of the *MtNCER* RNAi plants exhibited a significantly lower level of both AMF colonization frequency (F%), AMF colonization intensity (M%) and arbuscule abundance (A%) compared with empty control (EV) roots (Figures 5C, F, H). As shown in Figure 5E, EV plant roots formed normal growth with an orderly arrangement of hyphae after *R. irregularis* invaded root cortex cells, while the colonization of *R. irregularis* in *MtNCER*-RNAi plant roots was inhibited, and the hyphae were observed close to the epidermal cells but did not invade (Figure 5G). Consistently, transcript levels of *MtPT4* (*MtPT4*, a marker gene for AMS) and *RiEF1α* (*XM_025321412.1*, a housekeeping gene representing AMF abundance in roots) were decreased in *R. irregularis*-inoculated roots of *MtNCER* RNAi compared with EV roots (Figure 5D). These results demonstrated that NCER was required for AMF colonization during symbiosis establishment.

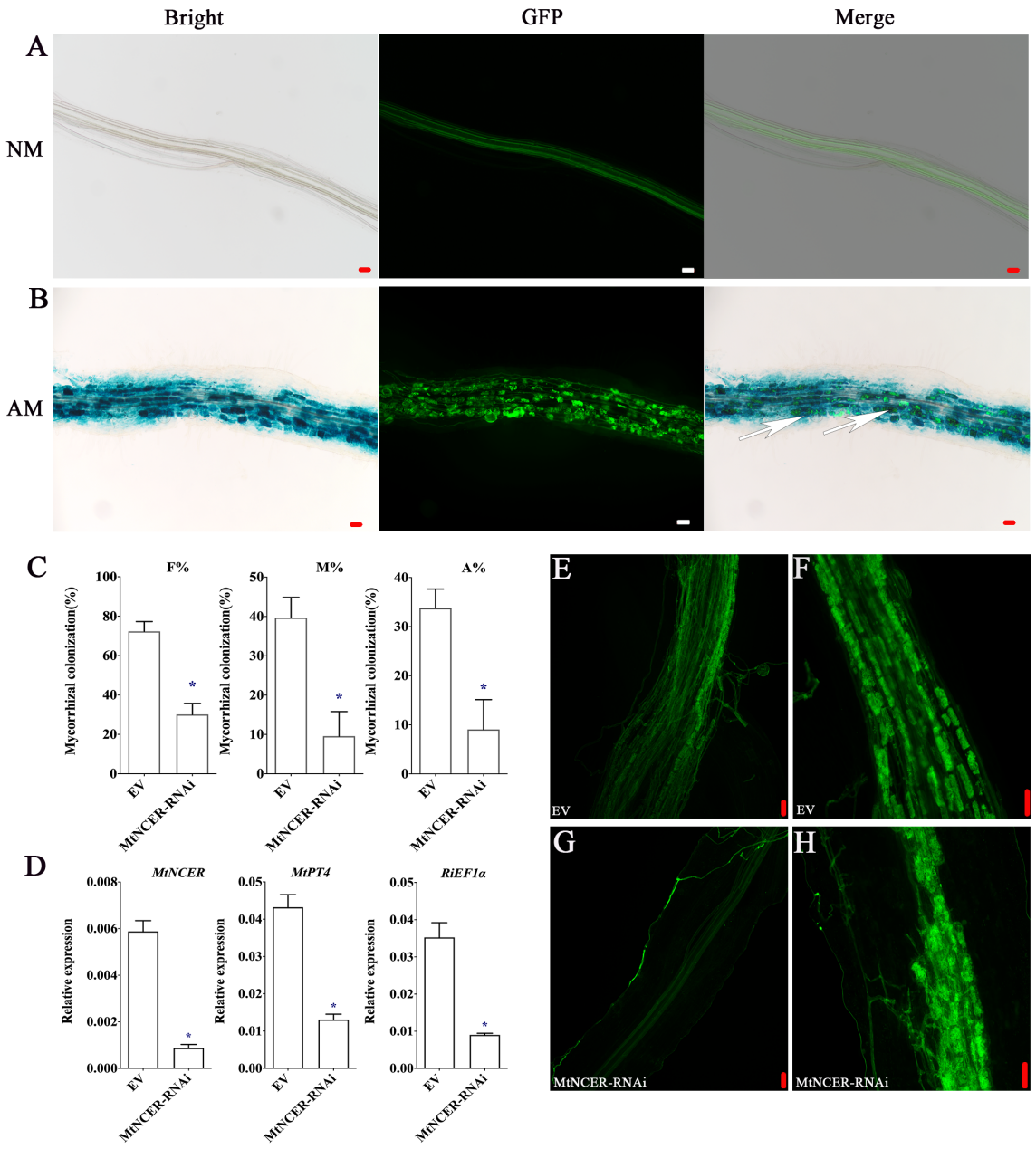

FIGURE 5
Functional analysis of NCER in arbuscular mycorrhizal symbiosis. **(A)** GUS (Bright) and WGA-Alexa Flour 488 (GFP) staining of the Medicago hairy root transformed with the *MtNCERpro : GUS* vector and uninoculated (NM) or **(B)** inoculated (AM) with *R. irregularis*. Right images showed merge of GUS and WGA-Alexa Flour 488 staining. The white arrows point to arbuscule-containing cells. **(C)** Quantification of AMF colonization levels in EV and *MtNCER*-RNAi. F%, frequency of analyzed root fragments that are mycorrhizal; M%, intensity of infection in whole roots; A%, arbuscule abundance in whole roots. **(D)** Expression levels of *MtNCER (Medtr3g079190)*, *MtPT4 (Medtr1g028600)* and *RiEF1α (XM_025321412.1)*. Expression is normalized against *MtEF1α* using the $2^{-\triangle\triangle Ct}$ method. Error bars represent standard errors for three biological replicates. Asterisks represent significant differences (Student's *t*-test: $*p < 0.05$). **(E, F)** WGA-alexa488 staining of mycorrhization in roots of EV plants. **(G, H)** WGA-alexa488 staining of mycorrhization in roots of *MtNCER*-RNAi plants (Scale bar, 50 μm).

Discussion

The establishment of symbiosis between plants and arbuscular mycorrhizal fungi (AMF) is a multifaceted process involving numerous molecular and cellular developmental mechanisms within plants (Nasir et al., 2021; Ho-Plágaro and García-Garrido, 2022). These processes facilitate signal transduction and nutrient exchange, which are essential for maintaining the symbiotic relationship (Fabiańska et al., 2019; Kameoka and Gutjahr, 2022; Luo et al., 2023). Proteomics or transcriptomics are effective methods to solve biological problems. To date, transcriptomics analyses of plants involved in AMS, including *Medicago truncatula*, *Oryza sativa*, *Solanum lycopersicum* and *Poncirus trifoliata*, have identified thousands of differentially expressed genes (Gutjahr et al., 2015; Sugimura and Saito, 2017; An et al., 2018; Zeng et al., 2018). However, investigations into protein-level

changes following AMF inoculation have predominantly focused on herbaceous plants such as Medicago (Talukdar et al., 2009), with limited reports available for woody plants, especially citrus. In this study, proteomic analyses of citrus rootstock *P. trifoliata* after colonization of AMF *R. irregularis* were performed, and 365 DEPs responding to AMF (287 up-regulated and 78 down-regulated) were identified for the first time. In addition, 194 key DEPs (constituting 53% of total DEPs) were found by association with DEGs previously identified through transcriptomic analysis of citrus rootstock *P. trifoliata* involved in AMS. Our combined analysis of multi-omics data provides pivotal basic data for the in-depth analysis of the molecular mechanism of AMS, and 194 overlapping DEPs provided reliable candidate genes involved in mycorrhizal symbiosis.

The absorption of nutrients and water is essential for plant growth and development. The interface for nutrient sharing between plants and AMF is provided by Periarbuscular membrane (PAM), a new plasma membrane that surrounds the arbuscules during symbiotic interaction between plants and AMF (Xue et al., 2018). The unique membrane structure of PAM is adjacent to the plasma membrane of cortical cells and represents an extension of the plasma membrane to a certain extent (Roth et al., 2019). Although it shares some proteins with the regular plasma membrane, PAM also possesses some unique membrane transporters. Membrane transporters reside in the PAM, mediating nutrient transfer from fungi to roots. The uptake of nutrients by plants depends on these membrane transporters (Pumplin and Harrison, 2009). Therefore, it is important to explore the specific membrane transporters of PAM for the molecular mechanism of AMS. Our results shown that 11 DEPs were predicted to be membrane transport, including Pt1g017100.4, Pt2g023350.3, Pt2g026610.1, Pt3g018050.1, Pt3g031530.1, Pt4g020070.1, Pt5g009680.1, Pt7g011830.1, Pt8g001290.1, Pt9g018680.1 and PtUn031030.1. Of 11 DEPs, Pt4g020070.1 and Pt9g018680.1 have high homology with MtPT4 (a phosphate transport protein), which is essential for the establishment of AMS, and is localized in PAM (Javot et al., 2007; Gutjahr and Parniske, 2013). These results indicated that Pt4g020070.1 and Pt9g018680.1 proteins were involved in phosphate transport and participated in AMF symbiotic in citrus. As one of the essential substances of AMS, water absorption plays a key role in AMS. Pt5g009680.1 (NIP5;1: nodulin 26-like intrinsic proteins5;1) has been previously identified as an aquaporin, which regulates cell membrane water permeability (Zhang et al., 2021). In our research, NIP5;1 was regulated by *R. irregularis* in citrus rootstock *P. trifoliate*, which indicted that NIP5;1 play an important role in water absorption during citrus arbuscular mycorrhizal interactions. The oligopeptide transporter (OPT) superfamily has been demonstrated to be involved in the development of root nodules in Medicago. (Seabra et al., 2012). Pt1g017100.4 belongs to OPT and is also found in DEPs, which suggested that AMS and nodule symbiosis has some similar formation mechanisms. Several studies over the past decade have reported ATP-binding cassette (ABC) transporters to be important players in the beneficial symbioses (Gutjahr et al., 2012; Kretzschmar et al., 2012; Banasiak et al., 2020). It was interesting to note that Pt7g011830.1 homologous protein AMN3 (ABC subfamily B transporter for mycorrhization and nodulation 3), common symbiotic ABC transporter, were expressed early during infection by rhizobia and AMF in Medicago (Roy et al., 2021). Among the DEPs, Pt7g011830.1, a homologous protein of AMN3, was identified as an ABC transporter. This result suggested that Pt7g011830.1 transporter is crucial to the development of AMS in citrus rootstock *P. trifoliata*. Based on the above description, a large number of transporters responding to mycorrhizal induction were identified, which also preliminarily suggested that transporters play an important role in AMS of citrus rootstock *P. trifoliata*.

AMF establish symbiotic interactions with plants, providing the host plant with minerals, in exchange for carbohydrates. Carbohydrates are required to sustain intraradical AM fungal growth (Roth and Paszkowski, 2017). Previous researches have provided evidences that plants primarily deliver carbohydrates to AMF in form of fatty acids (Jiang et al., 2017; Luginbuehl et al., 2017). During plant-AMF symbiosis, genes (such as FatM) are induced, leading to the synthesis of large amounts of fatty acids within the plant (Brands et al., 2018). Fatty acids are transported to the AMF through transport proteins (such as STR) located on the PAM (Jiang et al., 2017). In this study, the enrichment analysis of KEGG pathway of 194 key DEPs showed that fatty acid biosynthesis pathway was emerged as the most significantly altered pathway (Figure 3B). This result suggested that under conditions of symbiosis between AMF and woody plants, such as citrus, carbohydrates were transported to the fungus mainly in form of fatty acids. In fatty acid biosynthesis pathway, 11 proteins, including Pt4g005460.1, Pt3g007940.2, PtUn033150.1, Pt1g022510.1, Pt9g012200.1, Pt6g013760.3, PtUn010890.1, Pt8g012510.1, Pt2g001630.1, Pt5g008320.1 and Pt2g019580.2, were identified. Among these identified proteins, FatM, Pt2g019580.2 homologous protein in Medicago, was reported to be required for symbiosis (Bravo et al., 2017). The homologous protein of PtUn033150.1, which was involved in fatty acid biosynthesis, has been proved to be an essential component of the mycorrhiza-induced regulon (Martino and Crawford, 2021). PtUn010890.1 is a beta-hydroxyacyl-(acyl-carrier-protein) dehydratase (FabZ) involved in the synthesis of palmitate (C16:0), which only detected in the plasma membrane fraction of AM roots in Medicago (Aloui et al., 2018). These results suggested that proteins associated with fatty acid biosynthesis play a potential role in the maintenance of arbuscular development during AMS of *P. trifoliata*.

Sphingolipids metabolism is required for maintaining the endosymbionts of arbuscular mycorrhizal (Moore et al., 2021). Sphingolipids have long chain saturated fatty acids, creating less fluidity and a more orderly microenvironment (Gault et al., 2010). Together with cholesterol, sphingolipids establish a microstructural domain called lipid rafts. These lipid rafts rich in sphingolipids serve as the major platform recruiting proteins, achieving effective signal transduction, protein sorting, and membrane fluidity regulation, which are crucial for cellular processes (van Meer et al., 2008). Ceramide is a simple sphingolipid that usually exists in low concentrations in plants and is involved in various life activities of cells (Coant et al., 2017). In Arabidopsis, studies have shown that ceramides are cleaved by ceramidase to form

a sphingosine base, which is recognized by downstream receptor kinases and activates the downstream immune response (Kato et al., 2022). The accumulation of ceramides induced the synthesis of reactive oxygen species (ROS) leading to programmed cell death (PCD) (Bi et al., 2014). The NCER protein is a unique ceramidases associated with sphingolipid metabolism (Ali et al., 2018). In our research, interfering with the NCER gene resulted in a reduced rate of AM colonization and a decrease in arbuscule abundance, without affecting arbuscule morphology (Figures 5E-H). We demonstrated the positive role of NCER in AM establishment. Thus, we speculated that the NCER protein affects plant immune response by influencing the accumulation of substrate ceramide during mycorrhizal interactions, thereby indirectly affecting the attachment or invasion of root epidermal cells by extraradical hyphae in the early stage of AMS. An important task in the future is to determine how NCER affects the invasion of root epidermal cells by hyphae. The mutant of NCER can be further utilized to observe whether its AMF colonization frequency and the accumulation level of ceramides are affected, thus determining the mechanism of NCER in mycorrhizal symbiosis.

In conclusion, our proteomic analysis of *P. trifoliata* roots colonized by *R. irregularis* has unveiled a set of DEPs, providing key basic data and candidate genes for uncovering the molecular mechanism of AMS in citrus. The DEPs spanned various functional categories and signaling pathways, providing valuable insights into the metabolic and cellular processes involved in AMF colonization. The positive role of NCER in the establishment of AM was confirmed, which laid a foundation for its application to the beneficial interactions between citrus plants and AMF, and ultimately to improve the effective nutrient uptake and citrus yields.

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

HY: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. CJ: Investigation, Writing – review & editing, Methodology. ZZ: Writing – review & editing, Investigation. MY: Writing – review & editing, Investigation. YL: Writing – review & editing, Supervision. SX: Supervision, Writing – review & editing. ZP: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1294086/full#supplementary-material

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Dictyophora indusiata and *Bacillus aryabhattai* improve sugarcane yield by endogenously associating with the root and regulating flavonoid metabolism

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Introduction: Endophytes play a significant role in regulating plant root development and facilitating nutrient solubilization and transportation. This association could improve plant growth. The present study has uncovered a distinct phenotype, which we refer to as "white root", arising from the intricate interactions between endophytic fungi and bacteria with the roots in a sugarcane and bamboo fungus (*Dictyophora indusiata*) intercropping system.

Methods: We investigated the mechanisms underlying the formation of this “white root” phenotype and its impact on sugarcane yield and metabolism by metabarcoding and metabolome analysis.

Results and Discussion: Initial analysis revealed that intercropping with *D. indusiata* increased sugarcane yield by enhancing the number of viable tillers compared with bagasse and no input control. Metabarcoding based on second-generation and third-generation sequencing indicated that *D. indusiate* and *Bacillus aryabhattai* dominates the fungal and bacterial composition in the “white root” phenotype of sugarcane root. The coexistence of *D. indusiata* and *B. aryabhattai* as endophytes induced plant growth-promoting metabolites in the sugarcane root system, such as lysoPC 18:1 and dihydrobenzofuran, probably contributing to increased sugarcane yield. Furthermore, the association also enhanced the metabolism of compounds, such as naringenin-7-O-glucoside (Prunin), naringenin-7-O-neohesperidoside (Naringin)*, hesperetin-7-O-neohesperidoside (Neohesperidin), epicatechin, and aromadendrin (Dihydrokaempferol), involved in flavonoid metabolism during the formation of the endophytic phenotype in the sugarcane root system. These observations suggest that the “white root” phenotype promotes sugarcane growth by activating flavonoid metabolism. This study reports an interesting phenomenon where *D. indusiata*,

coordinate with the specific bacteria invade, forms a "white root" phenotype with sugarcane root. The study also provides new insights into using *D. indusiata* as a soil inoculant for promoting sugarcane growth and proposes a new approach for improve sugarcane cultivation.

KEYWORDS

***Bacillus*, full-length 16S rRNA, metabolome, metabarcoding, plant growth, sugarcane root**

1 Introduction

Plants live in nature alongside various microbes, including archaea, bacteria, fungi, and protists. These microorganisms, collectively known as the plant microbiota, form intricate communities and play a significant role in regulating the growth and productivity of plants (Hassani et al., 2018). Endophytes, which primarily reside within plant tissues, play a significant role in regulating plant root development and facilitating nutrient solubilization and transportation (Verma et al., 2021; Santoyo et al., 2016). Therefore, gaining a deeper understanding of the relationship of interaction between endophytes and plants is significant for optimizing agricultural production and ecological environments.

Sugarcane is the world's largest sugar crop and is extensively cultivated in the Guangxi Province of China. Utilizing sugarcane bagasse for field incorporation and growing *Dictyophora indusiata* (bamboo fungus) as intercrops have helped enhance the added value of the sugarcane industry. Therefore, we conducted an experiment intercropping sugarcane and *D. indusiata* from March to October 2022. The studies have reported that the intercropping system, *D. indusiata* and sugarcane, enhances soil chemical properties and activates soil metabolism (Duan et al., 2023a); Additionally, the same genomic and metabolomic study revealed that the fruiting body of *D. indusiata* could synthesize IAA (indole-3-acetic acid) (Duan et al., 2023b), which probably leads to improved soil fertility and sugarcane yield. These preliminary studies suggest that intercropping *D. indusiata* with sugarcane benefits both species.

We further investigated the interaction between the sugarcane root system and *D. indusiata* during intercropping to build upon the aforementioned experiment. This approach has led to the discovery of a fascinating phenotype we named as "white root" (Figure 1A), wherein the sugarcane root system forms a symbiotic relationship with the *D. indusiata* mycelium in the intercropping system. Notably, the mycelium was found to be enriched inside the root system (Figure 1D). Through this study, we have unveiled the unique interaction and mutualism between sugarcane and fungi. As a result, we have identified an interesting interacting relationship between *D. indusiata* and sugarcane roots, which has the potential to enhance sugarcane yield. Overall, DI has the potential to be developed as a novel microbial inoculant, and the development of microbial inoculants can enhance crop yield and resistance to diseases in an environmentally friendly manner (Kashyap et al., 2023). Therefore, elucidating the mechanism underlying this association will provide new insights for sugarcane cultivation and yield improvement.

Metabarcoding and metabolomics are the rapidly developing omics technologies that help reveal the mechanisms underlying plant-microbe interactions. A study based on metabarcoding and metabolome techniques revealed the mechanisms promoting plant growth by a fairy ring fungi (Duan et al., 2022b). Similar metabarcoding and metabolome techniques will unravel the interaction mechanisms between *D. indusiata* and sugarcane roots. Especially with the third-generation sequencing technology-based full-length sequencing of metabarcoding tags, it is possible to accurately obtain longer microbial barcode sequences as mentioned by Rubiola, full-length 16S rRNA sequencing offers several advantages in identifying the dominant bacteria within microbial communities (Rubiola et al., 2022).

Therefore, the present study aimed to unravel the interactions between endophytes and metabolism between *D. indusiata* and sugarcane roots and the impact of this interaction on sugarcane yield. We analyzed the effects of cultivating *D. indusiata* on sugarcane yield using a blank (CK) and bagasse (non-inoculated) as controls. Then, the relationship of endophytes and metabolism involved in the "white root" phenotype were analyzed using metabarcoding and metabolome techniques. Finally, through correlation analysis, we elucidated the regulatory potential of key metabolites of major fungi and bacteria during the interactions between endophytes and metabolism.

2 Materials and methods

2.1 Materials

The experiment took place in a cultivated land in Laibin City, Guangxi Province, China (23°16′N, 108°24′E) from February to August 2022. The following treatments were used: control (CK), standard management conditions for sugarcane growth; Bagasse, crushed and air-dried bagasse applied to the soil between rows of

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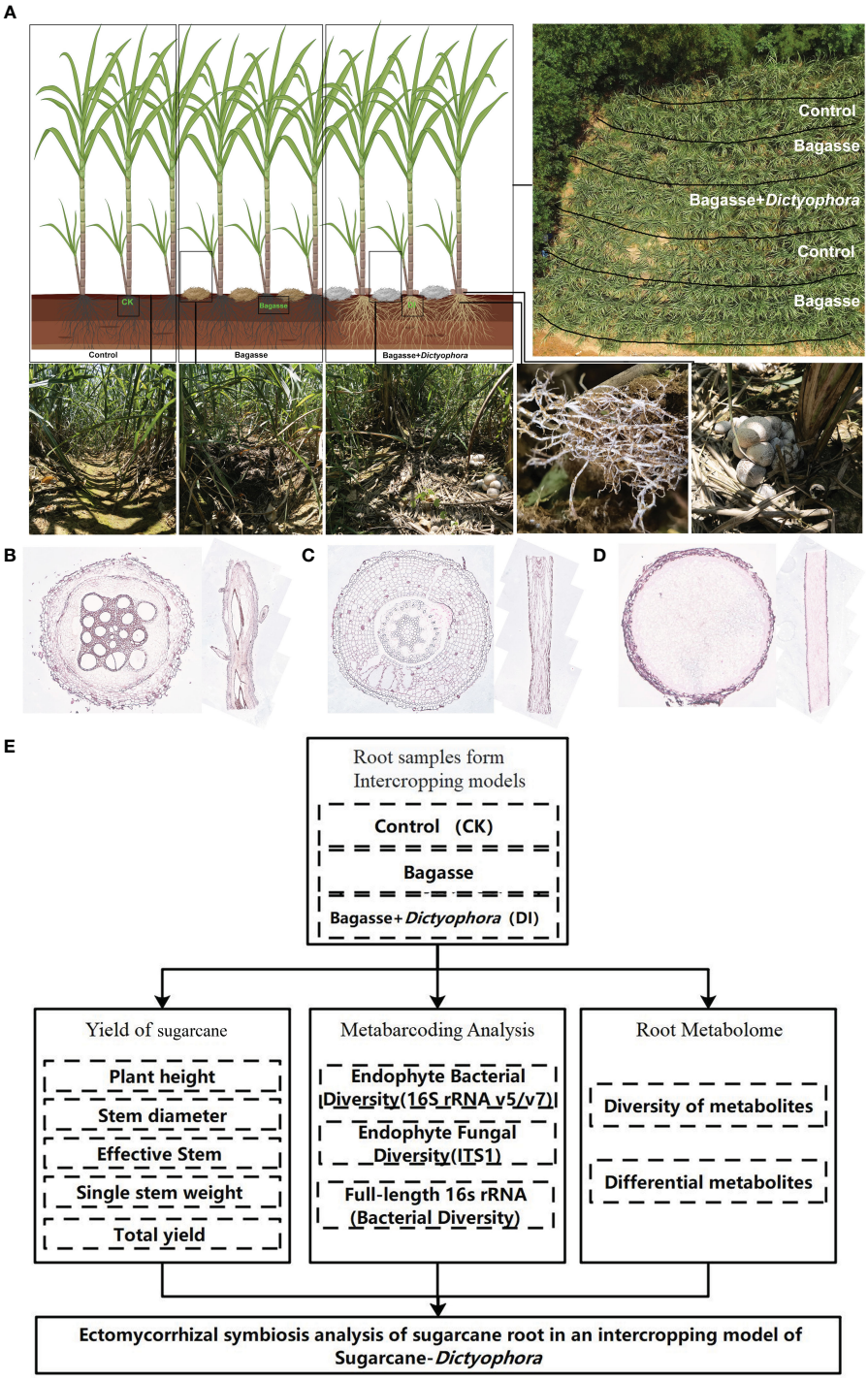

FIGURE 1
The experimental approach used in our study. **(A)** Sugarcane and *Dictyophora indusiata* intercropping. The image in the upper right shows an aerial view of the field and the division of the treatments within the field. The three images in the lower left show the sugarcane rows corresponding to the different treatments (CK, Bagasse and DI), and the two images in the lower right show the growth of mycelium under the effects of the DI on the sugarcane root system. **(B)** Microscopic anatomical figure of sugarcane root under CK treatments. **(C)** Microscopic anatomical figure of sugarcane root under Bagasse treatments. **(D)** Microscopic anatomical figure of sugarcane root under DI treatments. **(E)** The technical route of this study.

sugarcane plants; and DI, crushed and air-dried bagasse mixed with *D. indusiata* (DI) strains obtained via wheat grain fermentation, applied to the soil between rows of sugarcane plants (Figure 1A). The local sugarcane variety GT42 was utilized in this study. The sugarcane plants were initially grown with an interrow spacing of approximately 50 cm and a row depth of 30 cm. After 60 days of cultivation, when the sugarcane plants reached a height of approximately 50 cm, five semi-rectangular areas were marked for the three treatments (Figure 1A). Soil moisture levels were maintained at a high level throughout the experiment. After

approximately 90 days, the fruiting bodies of DI matured. Root samples were randomly collected from the middle three treatments in the rows, with three biological replicates per treatment. Using a microscope (Axio Imager M2m, ZEISS, Germany) and resorcinol staining method (Resorcinol staining solution, No. G1060, Servicebio, China), the internal microstructures of the root systems from three treatments were observed. It can be seen that there were no fungal mycelium observed in the cross-section and longitudinal section of the root systems from treatments CK (Figure 1B) and Bagasse (Figure 1C). However, the cross-section and longitudinal section figure of the root system from treatment DI (Figure 1D) were filled with fungal mycelium. In addition, it also shown individual roots are being killed and fully infected by the invading fungus of *D. indusiata*. Approximately 5 g of healthy (disease-free) root sample was collected from each plant, washed three times with sterile water, immediately frozen in liquid nitrogen, and stored at −80°C for further analysis.

2.2 Methods

The progression of this study follows the technical route shown in Figure 1E.

2.2.1 Method for calculating sugarcane yields

We chose an area of about 660 square meters to conduct sugarcane yield detection. Through statistics, we found a total of 4320 sugarcane plants were cultivated, with an average of 288 plants per row for 15 rows. The Plant height and Stem diameter were directly measured in meters. For plant height, the length from the first node above ground to the top node with an internode length of at least 2 cm was measured. Stem diameter was measured at the sixth node from the ground. The "Single stem weight" was obtained by removing the leaves from the sugarcane stem and then measuring the weight. For the Effective Stem of per-are calculation, 15 effective stems (over 1 meter in height) were randomly selected from each treatment in each row of sugarcane, and the average value was calculated and then totaled for all rows. The Yield of per-are (about 660 m^2) was obtained by multiplying the Effective Stem data by the Effective Stem of per-are data.

2.2.2 Metabarcoding

DNA was extracted from the leaf and root samples using the CTAB method (Cheng et al., 2003). Subsequently, the bacterial 16S rRNA V5–V7 region was amplified with the 799F and 1193R primers (Beckers et al., 2016), while the fungal ITS 1–2 region was amplified with the ITS1-F and ITS2 primers (Manzar et al., 2022). The amplification process involved a reaction mixture containing 1× Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and approximately 10 ng of template DNA. The thermal cycling conditions included an initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. Subsequently, sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) as per the manufacturer's recommendations, and index codes were added. The library quality was assessed using a Qubit@ 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. The purified amplicon libraries were then pooled in equimolar ratios and subjected to paired-end sequencing (PE250) on an Illumina platform (Novaseq 6000 sequencing) following the standard protocol (Second generation sequencing). For a detailed classification of the endophytic bacteria in the root system, the full-length 16S rDNA was amplified from root samples in the DI treatment using the 27F and 1492 R primers (Kesharwani et al., 2022). Subsequently, sequencing libraries were generated using the SMRTbell TM Template Prep Kit (PacBio, Menlo Park, CA, USA) as per the manufacturer's recommendation. These libraries were then sequenced on a PacBio Sequel platform (Third generation sequencing).

The data were analyzed using Kujawska's approach (Kujawska et al., 2021). Initially, the paired-end reads were assembled and subjected to quality control. High-quality reads were assigned to samples based on their unique barcode, and then truncated by removing the barcode and primer sequence. Subsequently, FLASH (V1.2.7) was used to merge the paired-end reads and generate raw tags (Magoč and Salzberg, 2011). These raw tags underwent quality filtering under specific conditions to obtain high-quality clean tags (Bokulich et al., 2013), following the QIIME (v1.9.1) quality control process (Caporaso et al., 2010). The resulting tags were compared with the SILVA database (v132) (16S rRNA metabarcoding data) (Pruesse et al., 2007) and the UNITE database (v8.0) (ITS metabarcoding data) (Nilsson et al., 2019) using the UCHIME algorithm (Edgar et al., 2011) to detect and remove chimera sequences (Haas et al., 2011), resulting in the acquisition of effective tags. The relative abundance of each operational taxonomic unit (OTU) and taxonomic grouping (e.g., Genus and Family levels) were calculated by counting the number of tags and expressing it as a percentage. For the full-length 16S rRNA sequencing reads, the DADA2 R package (Callahan et al., 2016) (v1.14) was utilized to implement a complete pipeline for denoising and removing chimeras, resulting in inferred sample sequences. Subsequently, the reads were clustered into OTUs using the UPARSE software (v7.0.1001) (Edgar, 2013), with sequences having ≥97% similarity being assigned to the same OTUs. The representative sequences from each OTU were then assigned to bacterial and fungal taxa based on the SILVA (v132) and the UNITE (v8.0) databases, using a confidence threshold value of 0.8.

Additionally, alpha diversity indices, including observed species richness (Sobs), Chao1, and Shannon indices, were utilized to evaluate the species complexity in the samples. These indices were calculated using QIIME software (v1.9.1) and visualized using R software (v2.15.3). Principal component analysis (PCA) based on the OTU number was performed to assess the similarity among the samples using the R project vegan package (v2.6-2; http://CRAN.R-project.org/package=vegan; 2022.4.20) and the ggplot2 package (v3.3.3; http://CRAN.R-project.org/package=ggplot2; 2022.3.1). Furthermore, Venn analysis was conducted to compare the OTUs of different groups using the VennDiagram package (v1.6.16) in R (Chen and Boutros, 2011).

These analyses were carried out using the OTU numbers without any model transformation.

For the full-length 16S rRNA sequences, taxonomic classification was performed using BLAST (v2.6.0) (Altschul et al., 1990). The representative OTU sequences or ASV sequences were searched against the NCBI 16S ribosomal RNA Database (Bacteria and archaea) (http://www.ncbi.nlm.nih.gov) (v202101) using the best hit criteria (E-value < e-5, query coverage ≥ 60%; sequence identity ≥92% was considered to belong to the same species; sequence identity ≥88% was considered to belong to the same genus; sequence identity ≥85% was considered to belong to the same family; sequence identity ≥80% was considered to belong to the same order; classes were inferred when sequence identity ≥75%; and phylum were inferred when sequence identity ≥70%).

2.2.3 Widely targeted metabolome analysis

A comprehensive metabolome analysis using ultra-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) was conducted at Metware Biotechnology Co., Ltd. (Wuhan, China) to identify differences in metabolites among the root samples, as previously described (Duan et al., 2022b). The root samples underwent freeze-drying for 48 hours and were ground into powder. Approximately 100 mg of the powder was then extracted with 70% aqueous methanol (0.6 mL) and analyzed using a UHPLC-ESI-MS/MS system (UHPLC, Shim-pack UFLC SHIMADZU CBM30A system, Kyoto, Japan; MS, Applied Biosystems 4500 Q TRAP, Framingham, MA, USA). Each root sample had three biological replicates, and their extracts were combined in equal amounts to create a quality control (QC) sample for assessing measurement accuracy after every six samples.

The qualitative analysis of the primary and secondary mass spectrometry data was performed using a self-built database, MWDB (v2.0; Metware Biotechnology Co., Ltd. Wuhan, China), as well as publicly available databases such as MassBank (http://www.massbank.jp), HMDB (Human Metabolome Database; http://www.hmdb.ca), and METLIN (http://metlin.scripps.edu/index.php). Additionally, the quantitative analysis of the metabolites was carried out using the multiple reaction monitoring mode (MRM) of triple quadrupole mass spectrometry. The MultiQuant software (v3.0.2) was employed to access the mass spectrometry files, integrate and correct the peaks, with the chromatographic peak area representing the relative content of the metabolite.

The raw metabolite data were processed using Analyst 1.6.3 software (AB Sciex, Framingham, MA, USA). The original metabolite abundance was log-transformed to reduce variance and normalize the data. Subsequently, PCA, cluster analysis, and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) were conducted with the metabolite data in R (http://www.r-project.org/) following previously described methods (Li et al., 2021). VIP values of all metabolites from the OPLS-DA were extracted using the first component. Finally, metabolites from various pairwise comparisons (CK vs. Bagasse, CK vs. DI, and Bagasse vs. DI) with VIP ≥ 1 (indicating high confidence in pairwise comparisons) and a fold change ≥ 2 and ≤ 0.5 were identified as differential metabolites. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation and metabolic pathway analysis were performed for the differential metabolites using a hypergeometric test to identify significantly enriched pathways ($p < 0.05$).

2.2.4 Correlation analysis

Finally, the correlation between the metabarcoding and metabolome data was analyzed using OmicShare tools, a free online platform for data analysis (https://www.omicshare.com/tools). The Z-score (zero-mean normalization; $z = (x - \mu)/\sigma$, where x is the original value, μ is the mean value, and σ is the standard deviation value) of the marker metabolites (peak area units) and the OTU numbers of the top ten fungal and bacterial genera with the highest relative abundance in the root samples were utilized to assess the correlation. Subsequently, a heat map was generated using the Pearson correlation coefficients with the correlation heat map tools in OmicShare.

3 Results

3.1 Sugarcane yield statistics

The study first analyzed the yield of sugarcane under CK, Bagasse and DI treatments. After 11 months of growth, the sugarcane from DI group showed a significant yield advantage (Table 1). We conducted statistical analysis on five agronomic traits of plant height (cm), stem diameter (cm), effective Stem of per-are, single stem weight (kg) and yield of per-are (kg). The DI group showed advantage in plant height (241.67 cm, 250.27 cm, and 254.94 cm in CK, Bagasse, and DI), effective stem of per-are (3973, 3672, and 4540), single stem weight (1.27 kg, 1.41 kg and 1.40 kg), and yield of per-are (5045.71 kg, 5177.52 kg, and 6356 kg) compared with CK and Bagasse. These observations suggest that DI could significantly improve the yield by increasing the tillering of sugarcane compared with Bagasse.

3.2 Microbial diversity

3.2.1 Metabarcoding

We performed metabarcoding sequencing using the root system from three treatments to reveal how the "white root" phenotype

TABLE 1 Sugarcane yield statistics.

	CK	Bagasse	DI
Plant height (cm)	241.67	250.27	254.94
Stem diameter (cm)	2.55	2.63	2.57
Effective Stem of per-are (~ 660 m^2= 1 are)	3973	3672	4540
Single stem weight (kg)	1.27	1.41	1.4
Yield of per-are (kg)	5045.71	5177.52	6356

Plant height (cm), stem diameter (cm), and single stem weight (kg) are based on 15 replicates; the effective stem of per-are and yield of per-are were determined based on the conversion estimate. Yield of per-are = Single stem weight * Effective Stem of per-are. All values represent the mean values.

induced by *D. indusiata* affects the endophytic ecology of sugarcane roots. A total of 3,876,163 effective metabarcoding tags were obtained from 15 samples via sequencing of the fungal ITS 1–2 region and the bacterial 16S rRNA V5–V7 region. Subsequent clustering identified an average of 226 fungal OTUs (ITS) and 598 bacterial OTUs (16S rDNA) per sample (Supplementary Table S1). The PCA scatter plots based on the fungal and bacterial OTU abundance in the roots showed clear separation of CK, Bagasse, and DI samples, which indicates that both the process of sugarcane bagasse (Bagasse treatment) and the cultivation of DI (DI treatment) have the ability to regulate the composition of the root endophytic microbial community; here, PC1 contributed to 85.97% and 96.93% variations in fungi and bacteria (Figures 2A, B). The Venn diagram showed that 119 and 311 fungal and bacterial OTUs were shared among all root samples. Meanwhile, DI had the least unique fungal OTUs (94, 147, and 4 in CK, Bagasse, and DI; Figure 2C and bacterial OTUs (252, 482, and 31; Figure 2D). These observations suggest that *D. indusiata* had a drastic synergistic effect on sugarcane root endophytic fungi and bacteria.

3.2.2 Microbial alpha diversity and key microbial taxa

We further calculated the alpha diversity indices to determine the species diversity and used the *t*-test to compare the samples. Sobs for root samples of CK, Bagasse, and DI ranged from 127 to 303 for fungi (Figure 3A) and 365 to 931 for bacteria (Figure 3C). Meanwhile, the Shannon index ranged from 0.46 to 4.85 for fungi (Figure 3B) and 1.31 to 6.84 for bacteria (Figure 3D). Besides, both Sobs and Shannon index were significantly different among CK, Bagasse, and DI. Among them, the largest difference was detected in the CK vs. DI and Bagasse vs. DI comparisons ($P < 0.01$, Figure 3A–D), which DI shown the significantly lowest diversity, regardless of whether it was measured by Sobs and Shannon index (e.g., bacterial Shannon index CK vs. DI was 1.5 vs. 6.45; Figure 3D). In addition, other alpha indices, such as simpson, chao, and ace, have also exhibited similar trends to the Shannon index. (detailed information shown in Supplementary Table S2). These observations suggest that the diversity of root fungi and bacteria decreased dramatically under DI compared with CK and Bagasse.

Further taxonomic annotation based on the SILVA and UNITE databases revealed that the differences in the endophytic fungi and bacteria were responsible for the huge differences in alpha diversity indices between the treatments. As shown in Figure 3E, Phallaceae (2.36%, 8.82%, and 95.28% in CK, Bagasse, and DI groups), Chaetomiaceae (11.07%, 6.63%, and 0.47%), and Trichocomaceae (13.34%, 3.02% and 0.22%) were the top three most abundant fungi at the family level. Meanwhile, *Bacillus* (10.4%, 10.47%, and 91.32%), *Burkholderia-Caballeronia-Paraburkholderia* (15.2%, 10.31%, and 0.35%), and *Rhodanobacter* (12.99%, 9.36% and 1.35%) were the top three most abundant bacteria at the genus level (Figure 3F). Above shown in DI treatment, Phallaceae and *Bacillus* were high compared with CK and Bagasse; The abundance

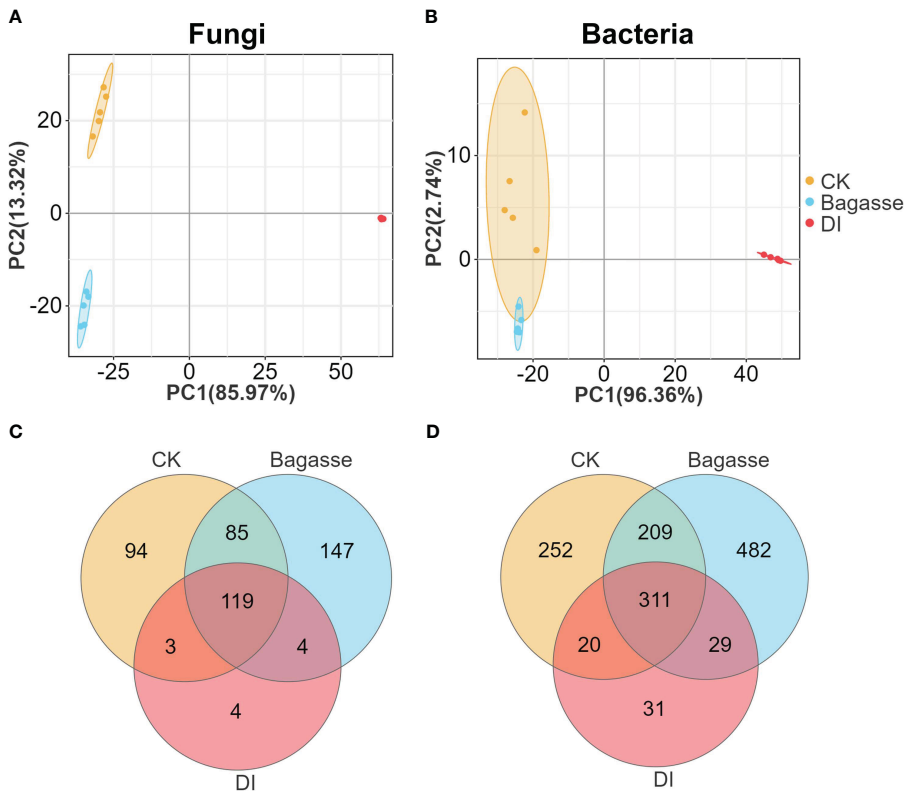

FIGURE 2
PCA and Venn analysis of the endophytic OTUs in sugarcane roots under CK, Bagasse, and DI. PCA of the **(A)** bacterial and **(B)** fungal OTUs. Venn analysis of the **(C)** fungal and **(D)** bacterial OTUs.

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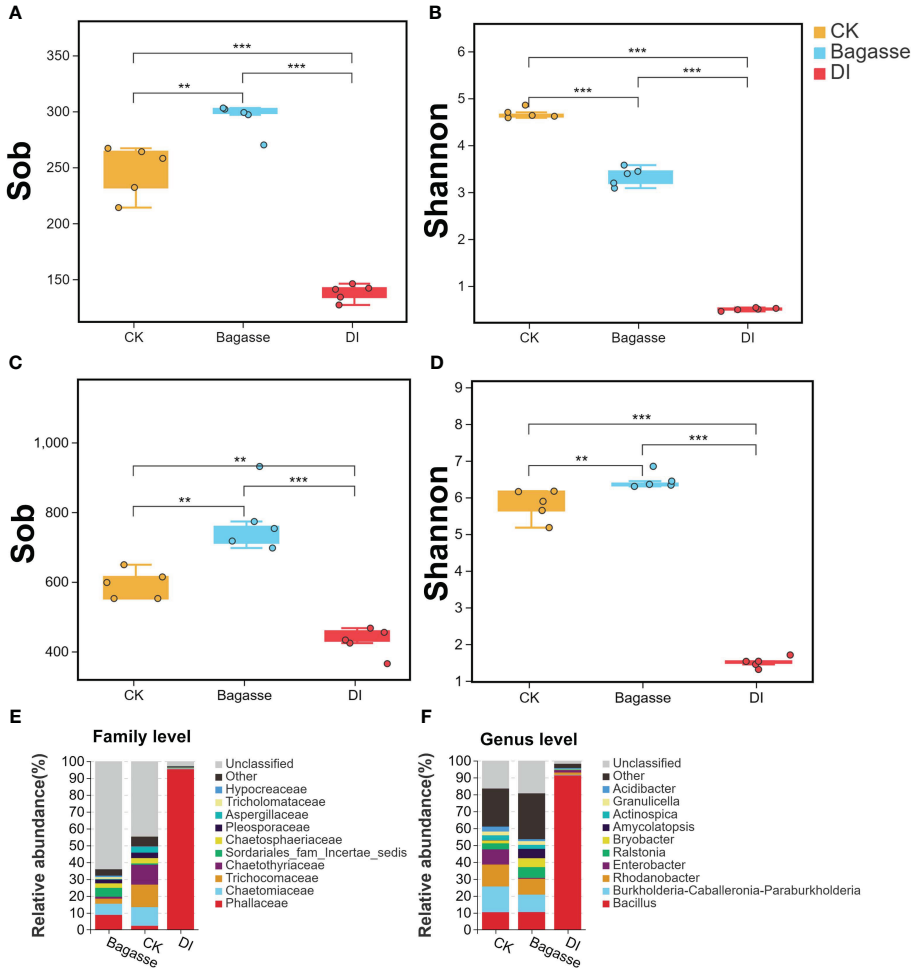

FIGURE 3
Alpha diversity and community composition of microbes in sugarcane root under CK, Bagasse, and DI. **(A–D)** Boxplot shows the alpha diversity indices of the endophytic fungi and bacteria in the sugarcane roots. Observed species index (Sob) of **(A)** fungi and **(C)** bacteria. Shannon index of **(B)** fungi and **(D)** bacteria. All indices were calculated based on OTU. The top and bottom whiskers of the boxes represent the maximum and minimum values; the line inside the box represents the median, the top margin of the box represents the upper quartile, and the lower margin of the box represents the lower quartile. The scatters represent repetition (N = 5). "**" and "***" indicate significance at $P < 0.01$ and $P < 0.001$, respectively. **(E, F)** The relative abundance of fungal community at the family level **(E)** and bacterial community at the genus level **(F)** in the sugarcane roots.

of Chaetomiaceae, Trichocomaceae, *Burkholderia-Caballeronia-Paraburkholderia* and *Rhodanobacter* decreased after Bagasse and DI treatment.

3.2.3 ITS-16S rRNA correlation analysis

To further reveal the consistency in diversity of root fungi and bacteria under CK, Bagasse, and DI, which to investigate whether there is any interaction between root-associated fungi and bacteria in terms of endophytic colonization, we performed ITS-16S rRNA association analysis (Including Pearson correlation analysis, sPLS (sparse partial least squares) association analysis). The Pearson correlation coefficients and Mantel test based on the Shannon index from alpha diversity (R-value = 0.9, Figure 4A) and genetic distance from beta diversity (R-value = 0.97, Figure 4B) showed significant correlations between fungi and bacteria. Further sPLS association analysis was conducted based on the OTU data of fungi and bacteria (family level) with R-values greater than 0.5 or less than -0.5. While Phallaceae showed a strong positive correlation with Bacillaceae (R = 0.98) and negative correlations with many other bacterial families, such as Burkholderiaceae (R = -0.98) and Rhodanobacteraceae (R = -0.93) (Figure 4C). Meanwhile, Bacillaceae showed the trend of passive regulation, including only a negative association with Trichocomaceae (R = -0.58). These observations indicate a specific pattern of fungi interacting with bacteria, that the root invasion of *D. indusiata* (Family: Phallaceae) leads to a large-scale colonization of Bacillaceae in sugarcane root system.

3.2.4 Full-length 16S rRNA sequencing of roots under DI

To further reveal which bacterial species of the Bacillaceae family were promoted by *D. indusiata* invasion to the root during DI, we performed third-generation sequencing (PACBIO) and generated the full-length 16S rRNA of roots. A total of 63656

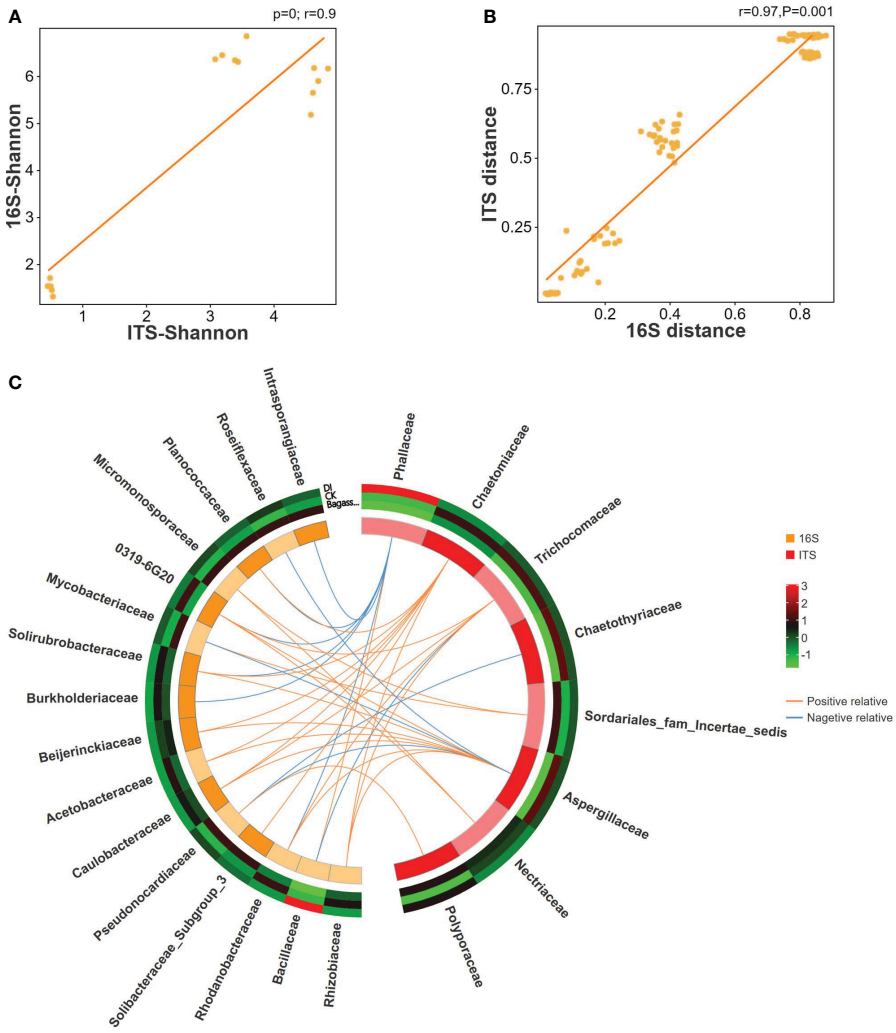

FIGURE 4
Correlation between ITS and 16S rRNA of sugarcane root under CK, Bagasse, and DI. **(A)** Pearson correlation analysis of Shannon index based on ITS and 16S rRNA OTUs. The vertical and horizontal axes show the coefficients from the Shannon index of the samples in ITS and 16S rRNA OTUs. The indicator line represents the linear correlation coefficient of the fit, and the dots represent samples. **(B)** Mantel test based on Bray-Curtis distance matrix. The horizontal axis represents the 16S rRNA distance matrix, and the vertical axis represents the ITS distance matrix. A dot in the figure represents a pair of samples. The indicator line represents the linear correlation coefficient of the fit. **(C)** Circos map of the sPLS model shows the correlation between fungi and bacteria. The outside circle external heat map shows the abundance of the microbial families under different treatments, and the color represents abundance while the red represents higher abundance and green represents lower abundance. In the internal Circos map, the colors represent fungi and bacteria, each cell represents a family, the lines represent correlations, and the orange line represents a positive correlation and the blue line represents a negative correlation.

clean CCS (Circular Consensus Sequence) reads were obtained as full-length reads, with an L50 size of 1528 bp and L90 size of 1512 bp. Then, 505 ASVs (amplicon sequence variants) were obtained from one DI root sample after clustering analysis. Further analysis of the top 10 ASVs with the highest relative abundance (Table 2) revealed that 9 out of 10 belonged to Bacillaceae, including *Bacillus aryabhattai* (top 1, 4, 7, and 10 with relative abundance at 27.39%, 3.63%, 2.92%, and 1.49%), *Priestia megaterium* (top 2, 3 and 5, with 15.93%, 4.52% and 3.51%), *Bacillus cereus* (top 6 with 3.27%), and *Bacillus altitudinis* (top 8 with 2.42%). These observations suggest that *D. indusiata* could cause the colonization of *B. aryabhattai* and *Priestia megaterium*, from the Bacillaceae family, in the roots of sugarcane; *Bacillus aryabhattai* was the main bacterial species (total relative abundance of 35.43%).

3.3 Study on the metabolome of sugarcane root system

3.3.1 Metabolite composition

The present study related the regulation of root endophytic diversity by the "white root" phenotype induced by *D. indusiata* to its yield enhancement. Therefore, we conducted a widely targeted metbaolome analysis (UPLC-ESIMS/MS) on the sugarcane roots from CK, Bagasse, and DI treatments. The heat map based on the abundance of metabolites (Figure 5A) showed large-scale variance for the DI group compared with CK and Bagasse, which indicated the "white root" greatly affected root metabolism. PCA based on metabolite peak area unit showed a clear separation of CK, Bagasse, and DI groups (Figure 5C), which indicates that both the

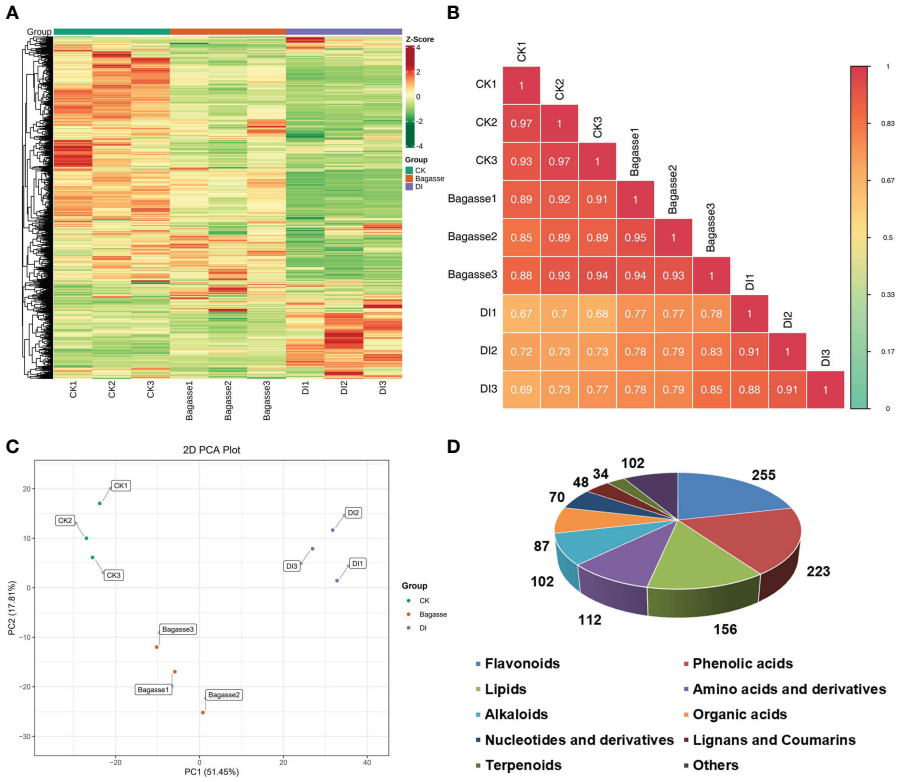

FIGURE 5
Metabolic composition of sugarcane roots under CK, Bagasse, and DI. **(A)** Clustering heat map of all metabolites. Each column represents a sample, and each row represents a metabolite. The color bar represents the metabolite abundance. Different shades of red and green represent the upregulated and downregulated metabolites, respectively. **(B)** Pearson's correlation heat map between CK, Bagasse, and DI. The longitudinal and diagonal lines show the sample names and different colors represent the Pearson correlation coefficients; the key is shown on the right side. **(C)** PCA plot based on metabolite peak area unit of root samples. PC1 represents the first principal component, and PC2 represents the second principal component. The percentage represents the interpretation rate of the principal component of the data set from peak area unit. A dot represents a sample, and samples in the same color are of the same group. **(D)** Pie figure shows the proportion classes of metabolites. The number near each metabolite class represents metabolite number.

process of Bagasse and DI treatment could regulate the metabolite composition of sugarcane roots. Then, the sample-to-sample correlation heat map based on metabolite further verified the above pattern (Figure 5B), and DI group showed a lower correlation than CK and Bagasse groups, which indicates that the cultivation of endophytic symbionts (DI treatment) has a significant impact on the metabolic processes and composition of metabolites in sugarcane roots. Specifically, based on public and commercial databases, 1189 metabolites (grouped into ten classes) were identified from these root samples under CK, Bagasse, and DI, including 255 flavonoids, 223 phenolic acids, 156 lipids, 112 amino acids and derivatives, 102 alkaloids, 97 organic acids, 70 nucleotides and derivatives, 48 lignans and coumarins, 34 terpenoids, and 102 others (Figure 5D). Detailed information on these metabolites is presented in Supplementary Table S3.

3.3.2 Differential metabolites

Furthermore, we analyzed metabolites' abundance and distribution characteristics in the root samples under CK, Bagasse, and DI. The analysis identified l-phenylalanine, vidarabine, lysopc 18:1(2n isomer), adenosine, serotonin*, 7-methoxy-3-[1-(3-pyridyl) methylidene]-4-chromanone, crotonoside; 2-hydroxyadenosine, stearic acid, 1-methoxy-indole-3-acetamide, cyclo-(gly-phe), 16-methylheptadecanoic acid, l-arginine, guanosine, 2'-deoxyadenosine, methyl 5-caffeoylquinate, n-hydroxytryptamine*, 3,5-dimethyl-2,3-dihydrobenzofuran, choline, 2'-deoxyguanosine and 4-o-caffeoylquinic acid methyl ester*3 as the top 20 most abundant metabolites (Figure 6A). Further, the quanitifcation of the peak area unit showed that the abundance of metabolites in DI differed greatly compared with CK and Bagasse, while the abundance was similar in CK and Bagasse. Mainly, the abundance of Stearic Acid, 1-Methoxy-indole-3-acetamide, cyclo-(Gly-Phe), and 16-Methylheptadecanoic acid in DI was different from that in Bagasse and CK (Figure 6A). Next, we analyzed the differential metabolites of the CK vs. Bagasse and CK vs. DI comparation groups. As shown in Figures 6B, C, 31 and 81 metabolites were upregulated, and 86 and 377 metabolites were downregulated in CK vs. Bagasse and CK vs. DI, respectively, which suggests that the "white root" phenotype (DI) greatly changed the root metabolism compared with the CK. Venn analysis showed that CK vs. DI (118) and Bagasse vs. DI (62) comparisons had more unique

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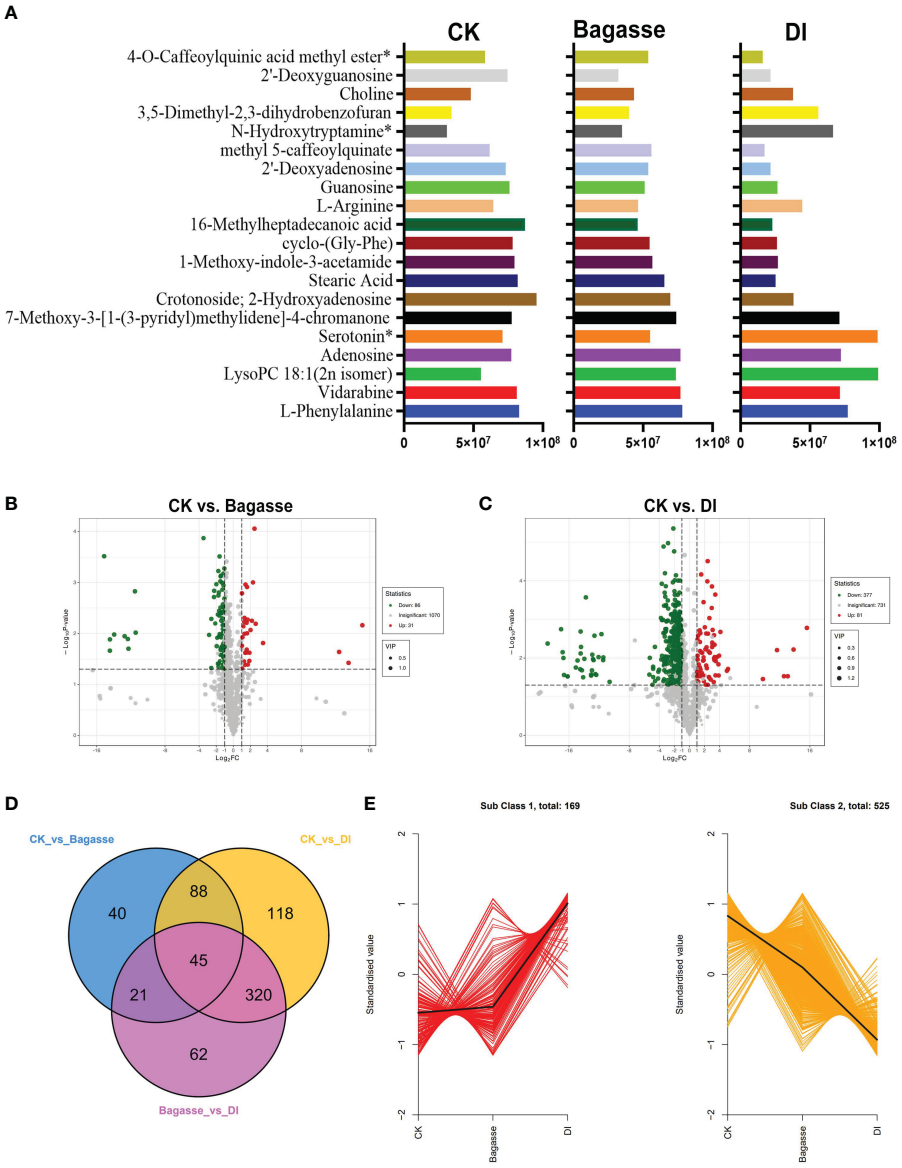

FIGURE 6
Differential metabolites of sugarcane root under CK, Bagasse, and DI. **(A)** Top 20 most abundant metabolites of sugarcane root under CK, Bagasse, and DI. The abscissa represents peak area units, and the ordinate represents the differential metabolites. **(B)** Volcano map of differential metabolites identified from the comparison groups CK vs. Bagasse, CK vs. DI. Each point in the volcano plot represents a metabolite; green represents the downregulated metabolites, red represents the upregulated metabolites, and gray represents the metabolites with no significant difference. The horizontal coordinate represents the log value of the metabolite difference between the comparison groups (log^2FC); the ordinate represents the significance level ($-log^{10}P$-value); the dot size represents the VIP value. **(C)** Venn diagram shows the differential metabolites of different comparison groups. **(D)** K-means clustering of metabolites of sugarcane root under CK, Bagasse, and DI. The abscissa represents the sample group, the ordinate represents the standardized relative metabolite content, and the class represents the metabolite category number with the trend in sugarcane root under CK, Bagasse, and DI. *Indicates the metabolite presence of isomer.

metabolites than CK vs. Bagasse (40) (Figure 6D). Then, we analyzed the distribution characteristics of all metabolites according to the K-means clustering method and identified two distinct metabolic clustering patterns. As shown in Figure 6E and Supplementary Table S4, the abundance of subclass 1, including 169 metabolites, was high in DI but similar in CK and Bagasse. Meanwhile, the abundance of subclass 2, including 525 metabolites, was the highest in CK and the lowest in DI. Our differential metabolite analysis thus indicated that the *D. indusiata*-induced "white root" phenotype greatly affected the metabolic process of sugarcane roots.

3.3.3 Kyoto Encyclopedia of Genes and Genomes analysis

Further, we performed a KEGG enrichment analysis to determine the function of the differential metabolites regulated by the *D. indusiata*-induced "white root" phenotype. The differential metabolites identified from the CK vs. DI group significantly enriched ($P < 0.05$) "Flavonoid biosynthesis (ko00941)", "Isoflavonoid biosynthesis (ko00943)", "Nucleotide metabolism (ko01232)", "Purine metabolism (ko00230)", and "Stilbenoid, diarylheptanoid and gingerol biosynthesis

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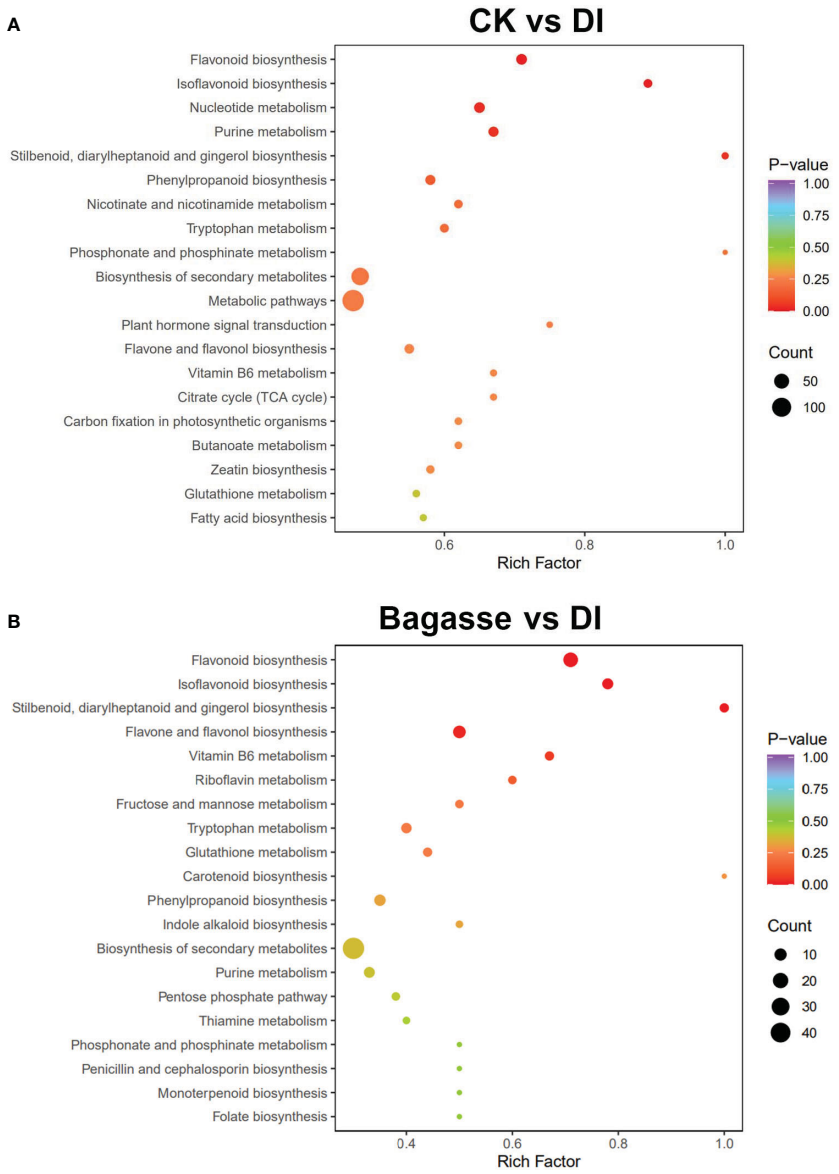

FIGURE 7
KEGG enrichment analysis of differential metabolites of sugarcane root under Bagasse and DI. **(A)** stands KEGG enrichment analysis of CK vs. DI, and **(B)** stands Bagasse vs. DI. Each bubble in the plot represents a metabolic pathway; the abscissa and the bubble size jointly indicate the magnitude of impact. A larger bubble indicates that more metabolites are enriched; the bubble color represents the *P*-value of the enrichment analysis, with darker colors indicating a higher degree of enrichment.

(ko00945)" pathways (Figure 7A); The differential metabolites identified from the CK vs. DI group significantly enriched ($P < 0.05$) Flavonoid biosynthesis (ko00941), Isoflavonoid biosynthesis (ko00943), Stilbenoid, diarylheptanoid and gingerol biosynthesis (ko00945) and Flavone and flavonol biosynthesis (ko00944) pathways (Figure 7B). Detailed information on the metabolite enrichment is provided in the "kegg_map" part of Supplementary Table S3. The KEGG enrichment analysis indicated that flavonoid metabolism is an important pathway regulated by the "white root" phenotype discovered in this study.

3.3.4 Association analysis of metabolites with fungi and bacteria

Since the regulatory trend of microbial diversity showed consistency with the root metabolome result, we analyzed root metabolites affected the DI-mediated structure and related *Bacillus association*. We used the top 10 fungal and bacterial OTUs with high relative abundance based on tag numbers to analyze the association with the significantly regulated metabolites (base peak area unit; the top 20 most abundant ones) in sugarcane roots under CK, Bagasse, and DI (Figure 6A) and metabolites associated with the "Flavonoid

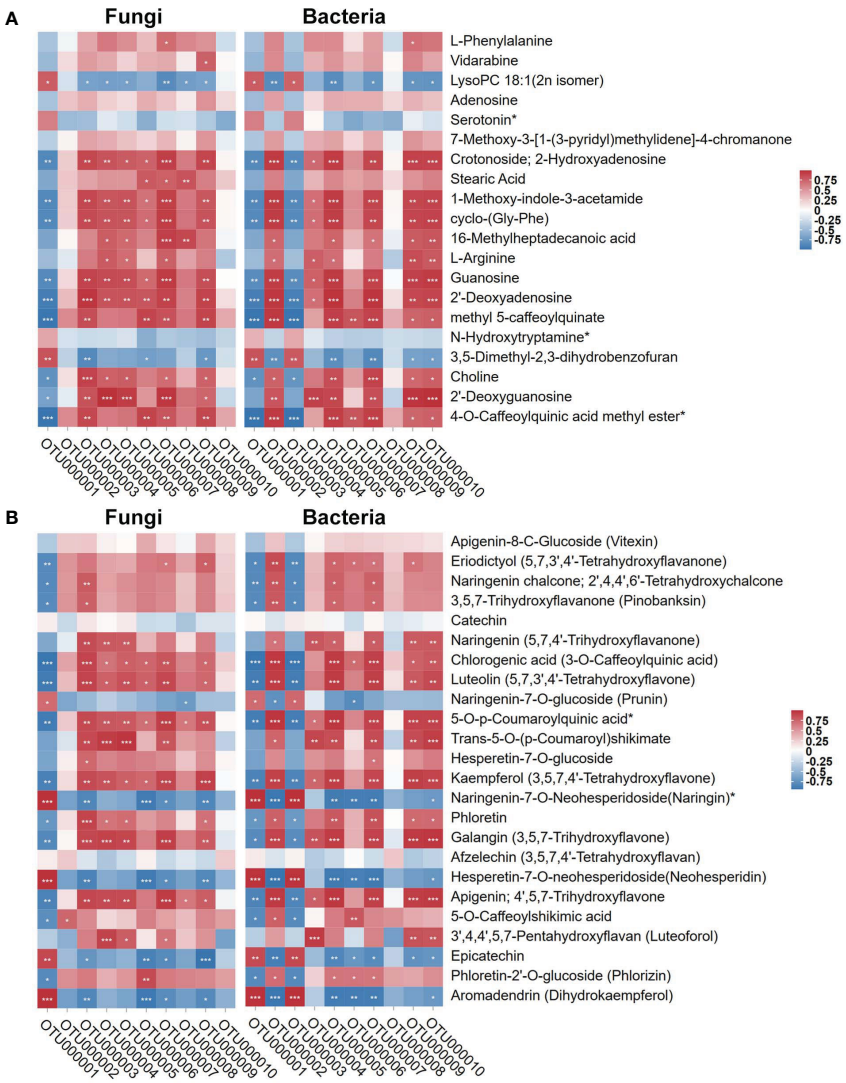

FIGURE 8
Pearson correlation of top 10 OTUs of endophytes (fungi and bacteria) with the top 20 most abundant metabolites and metabolites involved in "Flavonoid biosynthesis" KEGG pathway (ko00941). **(A)** stand the top 20 most abundant metabolites. **(B)** stand metabolites involved in "Flavonoid biosynthesis" KEGG pathway (ko00941). Red and blue represent Pearson correlation (*r*); the key is shown at the top right side. '*', '**', and '***' indicate significant correlation at $P \leq 0.001$, $0.001 < P < 0.01$, respectively; not marked: insignificant correlation ($0.01 < P < 0.05$; $P \geq 0.05$).

biosynthesis (ko00941)" pathway. As shown in Figure 8, according to the study's microbial diversity results, out000001 from fungi and bacteria could stand for *D. indusiata* (Because *D. indusiata* belongs to Phallaceae family, which is the dominance abundance family of DI group, Figure 3E) and *B. aryabhattai* (Table 2), respectively. The analysis using the top 20 most abundant metabolites revealed that *D. indusiata* (OTU000001 of fungi) and *B. aryabhattai* (OTU000001 of bacteria) were positively correlated ($P < 0.05$) with lysopc 18:1(2n isomer) and 3,5-dimethyl-2,3-dihydrobenzofuran ($r > 0.5$) and negatively correlated with crotonoside, 2-hydroxyadenosine, 1-methoxy-indole-3-acetamide, cyclo-(gly-phe), guanosine, 2'-deoxyadenosine, methyl 5-caffeoylquinate, choline and 2'-deoxyguanosine ($r < -0.5$) (Figure 8A). Meanwhile, the analysis using metabolites associated with the "Flavonoid biosynthesis" (ko00941) pathway revealed that *D. indusiata* (OTU000001 of fungi) and *B. aryabhattai* (OTU000001 of bacteria) were positively correlated ($P < 0.05$) with naringenin-7-o-glucoside (prunin), naringenin-7-o-neohesperidoside(naringin)*, hesperetin-7-o-neohesperidoside(neohesperidin), epicatechin and aromadendrin (dihydrokaempferol) ($r > 0.5$); and negatively correlated with eriodictyol (5,7,3',4'-tetrahydroxyflavanone), naringenin chalcone; 2',4,4',6'-tetrahydroxychalcone, 3,5,7-trihydroxyflavanone (pinobanksin), chlorogenic acid (3-o-caffeoylquinic acid), luteolin (5,7,3',4'-tetrahydroxyflavone), 5-o-p-coumaroylquinic acid*,

TABLE 2 Full-length 16S rRNA taxonomy in sugarcane roots under DI.

ASV ID	Family	Genus	Species	Number	Relative abundance(%)
ASV000001	Bacillaceae	Bacillus	Bacillus aryabhattai	14091	27.39
ASV000002	Bacillaceae	Priestia	Priestia megaterium	8198	15.93
ASV000003	Bacillaceae	Priestia	Priestia megaterium	2323	4.52
ASV000004	Bacillaceae	Bacillus	Bacillus aryabhattai	1869	3.63
ASV000005	Bacillaceae	Priestia	Priestia megaterium	1808	3.51
ASV000006	Bacillaceae	Bacillus	Bacillus cereus	1680	3.27
ASV000007	Bacillaceae	Bacillus	Bacillus aryabhattai	1500	2.92
ASV000008	Bacillaceae	Bacillus	Bacillus altitudinis	1243	2.42
ASV000009	Symphyonemataceae	–	–	1216	2.36
ASV000010	Bacillaceae	Bacillus	Bacillus aryabhattai	767	1.49

kaempferol (3,5,7,4'-tetrahydroxyflavone), phloretin, galangin (3,5,7-trihydroxyflavone), apigenin; 4',5,7-trihydroxyflavone, 5-o-caffeoylshikimic acid and phloretin-2'-o-glucoside (phlorizin) ($r < -0.5$) (Figure 8B).

4 Discussion

The sugarcane-*D. indusiata* intercropping system is a cultivation model used to increase the economic added value and yield. Interestingly, we have discovered a "white root" phenotype, where endogenetic fungi and bacteria interact together in the root system, when plant sugarcane intercropped with *D. indusiata*. Plant endophytes play a significant role in regulating root development and facilitating nutrient solubilization and transportation (Verma et al., 2021; Santoyo et al., 2016). This cultivation method which cased "white root" phenotype, utilizing DI treatment to activate endophytes, has increased sugarcane yield and represents a new cultivation approach with the potential to enhance sugarcane production.

Further analysis revealed that the diversity of root endophytic bacteria/fungi was significantly regulated under the DI treatment. Basically, the diversity of endophytes in the roots of sugarcane intercropped with *D. indusiata* was dominance in root, which was reflected in the large principal component differences (PC1 in Figure 2A) and dramatically shrinking alpha diversity index (Figures 3A, C). Interestingly, in the sugarcane root of DI, the *Bacillus* genus was associated with the same abundance as *D. indusiata* (Phallaceae) (Figures 3E, F). Interestingly, based on my preliminary research, it was also found that the DI treatment has the ability to reduce soil fungal diversity, which aligns with the observed decreasing trend in endophytic fungal diversity in this study (Duan et al., 2023a). This indicates that the cultivation of *D. indusiata* may invasive and symbiotic growth with sugarcane root, which in turn may regulate the metabolism of sugarcane roots and ultimately impact sugarcane growth. In the identified root-enriched fungal families in sugarcane root, the enrichment of Phallaceae in the root-associated fungi of the DI treatment suggests that the fungi belonging to this family originate from the mycelium of DI, as DI itself belongs to Phallaceae (Melanda et al., 2021); Chaetomiaceae family have the ability to regulate plant activity (Zhang et al., 2021); Trichocomaceae family is a common group of endophytic fungi, which can be found in such as *Bauhinia galpinii* and *Cycas bifida* (Feitosa et al., 2016; Zheng et al., 2018); The genus of *Bacillus* genus can produce a variety of compounds that participate in plant disease control and promote plant growth (Miljaković et al., 2020; Kashyap et al., 2022; Kashyap et al., 2021); The genus of *Burkholderia-Caballeronia-Paraburkholderia* group has been found to possess the ability to degrade herbicides (Zhao et al., 2022), suggesting that it may have the potential to assist sugarcane root in resisting herbicide damage; The genus of *Rhodanobacter* enhances plant tolerance to salt-induced osmotic stress, according to a recent study (Lee et al., 2019). The endophytic fungi and bacteria mentioned above all have the potential to regulate the growth of sugarcane.

Moreover, the ITS-16S rRNA correlation analysis confirmed a significant association (Figure 4C) between families of Phallaceae (DI) and Bacillaceae (Genus of *Bacillus*), indicating that *D. indusiata* mediates the intracellular colonization of *Bacillus* in the sugarcane root system. *Bacillus aryabhattai* was further identified as the dominant species from the specific genus based on our full-length sequencing. Thus, we propose that during the interacting association between *D. indusiata* and sugarcane roots, *B. aryabhattai* would be plays a prominent role, which may be an important factor regulating sugarcane growth. *Bacillus aryabhattai*, as a plant growth-promoting rhizobacteria (PGPR), has been isolated from the plant rhizosphere soil and reported to synthesize auxin and help the host to resist oxidation and heavy metal toxicity (Ghosh et al., 2018; Park et al., 2017; Bhattacharyya et al., 2017; Lee et al., 2012). Therefore, the study suggests that *B. aryabhattai* may be related to the yield increase in sugarcane under DI. Further investigation of the interaction between *D. indusiata* and *B. aryabhattai* in sugarcane roots will help promote sugarcane growth.

Among the metabolites identified with the highest peak areas in the Top 20, some have been reported to have the ability to affect plant growth. E.g., l-phenylalanine is a plant growth regulator capable of modulating phenolic compounds and enzymatic activity in sweet basil (Koca and Karaman, 2015); Vidarabine having antimicrobial actions, found in the *S. birrea* stem (bark) (Abdallah et al., 2021); Lysopc 18:1 has been reported to possess antipathogenic capabilities in tobacco plants (Wi et al., 2014); serotonin regulates plant growth functions, including roles in chronoregulation and the modulation of reproductive development (Erland et al., 2015). These high-abundance metabolites possess various functions in regulating plant growth and may be subject to regulation by the DI treatment, which in turn could influence the metabolic processes in the sugarcane root system.

The interaction between microorganisms and plant root systems typically triggers an immune response in the host plant (Berendsen et al., 2012), enhancing host resistance to stress (Castrillo et al., 2017), stimulating synthesis of beneficial metabolites, such as auxin, and thereby promoting host plant growth (Spaepen et al., 2007). In this study's intercropping system, *D. indusiata* significantly improved the metabolism of sugarcane roots through root symbiosis. Studies have found that the *D. indusiata* fruiting bodies synthesize IAA (Duan et al., 2023b), and *D. indusiata* improves soil metabolism, accumulating carbohydrate metabolites (Duan et al., 2023a). These earlier reports suggested that *D. indusiata* promotes plant growth metabolism. In addition, the present study found that the interaction between *D. indusiata* and *B. aryabhattai* activates flavonoid metabolism in sugarcane roots. Flavonoids are natural polyphenols abundant in plants and play essential roles in biological processes within the plant and in response to environmental factors (Shen et al., 2022; Anjali et al., 2023). This change may be one of the important factors contributing to the increased tillering and yield in sugarcane under DI treatment. Earlier studies demonstrated that the wild mushroom *Leucocalocybe mongolica* similarly enhanced the flavonoid metabolism in *Leymus chinensis*, through soil transformation in a fairy ring ecosystem (Duan et al., 2022b; Duan et al., 2022a). Therefore, it is worth mentioning that the enrichment of certain metabolites is induced by the interaction between *D. indusiata* and *B. aryabhattai* to root. Among the various metabolites detected, lysoPC 18:1 is a naturally occurring bioactive lipid that plays a key role in the defense response of plants against pathogens (Wi et al., 2014). Besides, a study has reported that dihydrobenzofuran promotes plant growth by inhibiting IAA oxidase (Lee, 1976). Moreover, in the sugarcane root, various flavonoids were found enriched in response to the symbiotic stimulation of *D. indusiata* and *B. aryabhattai*, such as Naringenin-7-O-glucoside (Prunin), Naringenin-7-O-Neohesperidoside (Naringin)*, Hesperetin-7-O-neohesperidoside (Neohesperidin), Epicatechin, and Aromadendrin (Dihydrokaempferol), which are involved in the flavonoid biosynthesis (ko00941) pathway. These metabolites may be part of the mechanism by which *D. indusiata* stimulates flavonoid metabolism in sugarcane root and promotes growth and yield. However, further validation is required to provide a deeper understanding of the mechanisms by which *D. indusiata* interacts with sugarcane.

5 Conclusion

This study reports an interesting phenomenon where *D. indusiata* forms a "white root" phenotype with sugarcane and positively impacts sugarcane growth and yield in an intercropping system. Detailed analysis revealed that *D. indusiata* promotes flavonoid synthesis in sugarcane roots. The study provides new insights into using *D. indusiata* as a soil inoculant for plant growth promotion and a new approach for sugarcane growth study. However, further studies are required to identify the formation mechanism of this "white root" phenotype and determine the role of increased flavonoid synthesis in promoting sugarcane growth.

Data availability statement

The datasets generated for this study upload in the NCBI Sequence Read Archive with submission of PRJNA1064011.

Author contributions

MD: Conceptualization, Investigation, Software, Writing – original draft, Writing – review & editing. XL: Investigation, Software, Writing – review & editing. XW: Investigation, Writing – review & editing. SL: Investigation, Writing – review & editing. HH: Investigation, Validation, Writing – review & editing. YL: Investigation, Writing – review & editing. QL: Investigation, Writing – review & editing. GZ: Investigation, Writing – review & editing. BF: Investigation, Writing – review & editing. SQ: Investigation, Writing – review & editing. CL: Investigation, Writing – review & editing. HY: Investigation, Writing – review & editing. JQ: Investigation, Writing – review & editing. ZC: Conceptualization, Writing – review & editing. ZW: Conceptualization, Writing – review & editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1326917/full#supplementary-material

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Characterization of arbuscular mycorrhizal fungal species associating with *Zea mays*

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Taxonomic identification of arbuscular mycorrhizal (AM) fungal spores extracted directly from the field is sometimes difficult because spores are often degraded or parasitized by other organisms. Single-spore inoculation of a suitable host plant allows for establishing monosporic cultures of AM fungi. This study aimed to propagate AM fungal spores isolated from maize soil using single spores for morphological characterization. First, trap cultures were established to trigger the sporulation of AM fungal species. Second, trap cultures were established with individual morphotypes by picking up only one spore under a dissecting microscope and transferring it to a small triangle of sterilized filter paper, which was then carefully inoculated below a root from germinated sorghum seeds in each pot and covered with a sterile substrate. All pots were placed in sunbags and maintained in a plant growth room for 120 days. Spores obtained from single spore trap cultures from each treatment, maize after oats (MO), maize after maize (MM), maize after peas (MP), and maize after soybean (MS), were extracted using the sieving method. Healthy spores were selected for morphological analysis. Direct PCR was conducted by crushing spores in RNAlater and applying three sets of primer pairs: ITS1 × ITS4, NS31 × AML2, and SSUmcf and LSUmBr. Nucleotide sequences obtained from Sanger sequencing were aligned on MEGA X. The phylogenetic tree showed that the closest neighbors of the propagated AM fungal species belonged to the genera *Claroideoglomus*, *Funneliformis*, *Gigaspora*, *Paraglomus*, and *Rhizophagus*. The morphological characteristics were compared to the descriptive features of described species posted on the INVAM website, and they included *Acaulospora cavernata*, *Diversispora spurca*, *Funneliformis geosporus*, *Funneliformis mosseae*, *Gigaspora clarus*, *Gigaspora margarita*, *Glomus macrosporum*, *Paraglomus occultum*, and *Rhizophagus intraradices*. These findings can provide a great contribution to crop productivity and sustainable management of the agricultural ecosystem. Also, the isolate analyzed could be grouped into efficient promoters of growth and mycorrhization of maize independent of their geographical location.

KEYWORDS

AM fungi, maize crop, morphological characterization, single spore, trap culture

1 Introduction

Mycorrhizal fungi are soil microorganisms distributed worldwide. They form symbiotic and mutualistic associations with more than 80% of vascular land plants (Smith and Read, 2008; Brundrett, 2009). Different types of mycorrhizal associations involving other groups of fungi and host plants have been identified. Among them, arbuscular mycorrhizal (AM) fungi form symbiotic associations with plants (Hata et al., 2010) in almost all habitats and climates (Azcón-Aguilar and Barea, 1997; Chen et al., 2018), including disturbed agricultural soils (Enkhtuya et al., 2000) and those derived from mining activities (Bi et al., 2003). These are considered Earth's most essential and ubiquitous symbionts (Dodd et al., 1996; Bever et al., 2001). AM fungi are obligate biotrophs that require the roots of a living host to grow and complete their life stages (Kehri et al., 2018). They colonize plant roots in exchange for carbohydrates, up to 20% of photosynthetically fixed, organic carbon (C)-based compounds (C) (Jakobsen and Rosendahl, 1990; Smith and Read, 2008), and provide their plant hosts with mineral nutrients required for plant growth (Jakobsen et al., 1992; Luginbuehl et al., 2017).

The importance of AM fungi is being increasingly considered in agriculture, horticulture, forestry, and environmental reclamation (IJdo et al., 2011; Sheteiwy et al., 2023). The global use of agriculturally beneficial microorganisms tends to contribute directly or indirectly to crop improvement and increases nutrient uptake efficiency (Bargaz et al., 2018; Plett, 2018; El-Sawah et al., 2023). Potentially, AM fungi could replace inorganic fertilizers or at least reduce the use of inorganic fertilizers (Begum et al., 2019; Sheteiwy et al., 2022).

AM fungi form symbiotic relationships with most vascular plants, and identifying AM fungal species is essential. Maize (*Zea mays* L.) is one of the most important crops grown globally for livestock feed, food, and industrial materials (Lv et al., 2016). Maize plants produce high dry matter yields and therefore have a high requirement for nutrients, especially three macro-nutrients: nitrogen (N), phosphorous (P), and potassium (K) (Pettigrew, 2008; Zörb et al., 2014). The use of artificial chemical fertilizers has been an effective way to improve maize production worldwide (Gao et al., 2017; Dai et al., 2018). Although many nutrients required to grow maize are abundant in soil, some may occur at low levels (Mtambanengwe and Mapfumo, 2009; Gao et al., 2020). The majority of plant species form symbiotic relationships with AM fungi to augment N and P uptake from soil (Begum et al., 2019; Yang et al., 2022). AM fungi transfer N (~20%) and P (~90%) to plants in exchange for C from photosynthates (Smith and Read, 2002; Whiteside et al., 2009). It can also boost plant growth by an average of 80% under unfertilized conditions (Hoeksema et al., 2010). In many parts of the world, maize production occurs in semi-arid environments and thus often faces high temperatures and water scarcity (Cairns et al., 2012; Zhao et al., 2016; He et al., 2017). These climate change-induced stresses have significantly threatened maize yields and decreased world maize production by 15%–20% annually (Lobell et al., 2011; Chen et al., 2012; Abdoulaye et al., 2019).

Traditionally, microscopic examination of extracted spores was used to identify AM fungal species before molecular techniques were established (Sanders, 2004; Young, 2012). Conventional morphological observation is still essential and should be addressed for identification, although there is a trend that the sequence data of AM fungi are over-emphasized for identification. The recommendation is that molecular and morphological characterization should be combined where practical because of the production of unique and crucial information, such as the impact of land management on AM fungal spore abundance and richness from each of the methods (Overby et al., 2015; Säle et al., 2015).

Taxonomic identification of AM fungal spores extracted directly from the field is sometimes difficult because spores are often degraded or parasitized by other organisms (Clapp et al., 1995), and it is recommended that morphological spore characteristics are best observed in trap cultures. The Glomeromycota classification based on morphological and molecular data was revised in 2013 by systematists with expertise in the biology and taxonomy of AM fungi (Redecker et al., 2013). The taxonomic classification of AM fungi was constructed by grouping the fungal strains based on similarities and differences in their morphological characteristics (spore morphology, spore formation, and spore wall structure) (Gerdemann and Trappe, 1974; Walker and Sanders, 1986; Morton and Benny, 1990; Schenck and Perez, 1990). Most of the 214 currently described species (https://www.amf-phylogeny.com) are characterized only by spore morphology, and the majority of older species have not been cultured *in vitro* (trap cultures) (Tisserant et al., 1998; Clapp et al., 2001). There are approximately 160 species of AM fungi described by spore morphology according to the International Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM; http://invam.caf.wvu.edu/). The variation in morphological characteristics in the spores of AM fungi is limited, thus creating difficulties in identification and morphotyping (Tisserant et al., 1998; Clapp et al., 2001). Walker and Vestberg (1998) reported that in nature there can be significant variation in spore morphology even within an AM fungal species. Still, many AM fungi may reproduce only vegetatively without producing spores (Helgason et al., 2002). Also, fungal spore diversity differs seasonally, with some fungi sporulating in late spring and others at the end of summer (Douds and Millner, 1999; Oehl et al., 2003). Using successive trap cultures and subsequent extraction and study of spores is time-consuming but may reveal significantly greater diversity (Oehl et al., 2004).

Single-spore inoculation of a suitable host plant allows for establishing monosporic cultures of AM fungi. It can also assist in understanding the individual effects of AM fungi on plant growth and the combined effects of other stresses on different crops. Few studies have been conducted on the propagation of AM fungi from single spores as starter inocula using substrate-based methods (Jansa et al., 2002; Panwar et al., 2007; Selvakumar et al., 2018). We hypothesize that inoculation with AM fungal single spores may help to elucidate the efficiency in preventing the contamination of spores (degraded or parasitized by other organisms) extracted directly from the field. The present study aimed to morphologically characterize AM fungal species associating with maize using single-spore propagation.

2 Materials and methods

2.1 Soil sampling and treatments

The soils in this study originated from fields (in the Free State and KwaZulu-Natal provinces, South Africa) of different farming practices, including conventional and conservational production of maize and four different treatments: maize after oats (MO), maize after soybean (MS), maize after maize (MM), and maize after peas (MP). Soils were sampled three times, first pre-planting (PP), 2 weeks before planting (October 14, 2019); second germination (GN), 2 weeks after germination (January 9, 2020); and third germination, at harvest (AH) (July 2020). Samples were collected from the bulk (0–10-cm depth) and rhizosphere (0–30-cm depth) soil profiles. From each of the sites [Free State (C, conventional; V, Zunckel farms) and KwaZulu-Natal (Z, Van Rooyenswoning farm)], the upper layer of soil organic matter (SOM) and debris was removed, and the bulk soil was then taken for pre-planting. At the same time, for germination and at harvest, the plant was first removed, and roots with rhizosphere soil were then placed in a sampling bag before the above-ground plant material was removed. Then, an additional bulk soil sample was taken from the vicinity where the plant was removed. Five samples were taken per treatment using an auger, with the aim to spread the intra-field samples as far apart as possible while still being accessible. The samples were then thoroughly mixed to make one composite (a single representative sample, created to reduce some of the massive variability) as described by Okalebo et al. (2002). In the Free State farms (large commercial fields), one soil sample was collected in a center sample, and then four soil samples were collected in a clockwise direction, starting at 9 o'clock, around the center sample when facing away from the farm road. In the KwaZulu-Natal (KZN) fields (under pivot irrigation), soil samples were collected trying to obtain a sample from every side and within every ring left by the irrigation system. Approximately 1 kg of the composite (soil adhering to the roots and next to plants) soil samples were placed in plastic bags (Ziploc freezer bags), labeled, and transported to the Mycorrhizal Laboratory at Rhodes University, Makhanda, Eastern Cape, South Africa, for further processing.

2.2 Trap culture

AM fungi are obligate biotrophs and cannot be grown in an artificial medium. They must be associated with a host plant. Trap cultures were established to trigger the sporulation of AM fungal species. These cultures aim to maintain a living collection of organisms to study and obtain fresh spores to set up monosporic cultures for identification. *Sorghum bicolor* was chosen as the symbiotic partner because of its high mycorrhizal dependency, wide adaptability, and high resistance to abiotic stresses, including drought, salinity, waterlogging, and heavy metals (Dar et al., 2018). A non-soil clay substrate was sterilized at 121°C for 15 min (Getenga et al., 2004; Kariman et al., 2022) and, when cooled, was placed in 9.5 × 6.5 × 9.5 cm plastic pots (until they were two-thirds filled). A soil sample from the field (5 g each; from four different treatments, MO, MS, MM, MP, and under two different agriculture management practices) was used as inoculum and placed (2 cm deep) in the pots below the seeds (approximately three disinfected seeds of sorghum per pot). More non-soil clay substrate was added until the pot was full. The seeds started to germinate after 3 days. The pots were placed in sunbags (Sigma-Aldrich, St. Louis, MO, USA; B7026) and watered with distilled water (Figure 1). Sunbags were sealed and monitored regularly, and water was supplied once a week, if needed, to ensure the soil moisture level was maintained, promote normal growth, and allow for AM fungal infection. Pots were placed in a growth room with external lighting on a 12-h day/night cycle, and the temperature was maintained at 25°C–28°C (Katta, 2016). Pot cultures were grown for 4 months (Oehl et al., 2003), after which the host plant was allowed to dry. After harvesting, the trap contents were thoroughly mixed, substrate and root samples were collected, and spores were extracted as described by Schenck (1982) and Smith and Dickson (1997) and grouped into different morphotypes according to their morphological characteristics observed under a dissecting microscope (Goswami et al., 2018).

2.2.1 Single spore trap culture

Sorghum seeds were surface-disinfected by soaking them in 10% sodium hypochlorite for 30 min and rinsing them three times with sterile distilled water. Using plastic pots (9.5 × 6.5 × 9.5 cm) filled with sterile non-soil clay substrate (autoclaved at 121°C for 15 min), some disinfected sorghum seeds (3 seeds/pot) were sown and grown with regular watering with distilled water in a plant growth room with 12 h of light (25°C) and 12 h of darkness (18°C) (Sreedasyam et al., 2023). The seeds started germinating after 3 days. Following seed germination, a trap culture was established with individual morphotypes by picking up only one spore (that appeared healthy based on shape, color, and surface condition) under a dissecting microscope and transferring it to a small triangular sterilized filter paper (Figure 1). Additionally, intact spores of each type were mounted in polyvinyl alcohol/lactic acid/glycerol (PVLG) with (to obtain the most intense staining reaction) and without Melzer's reagent (to observe diagnostic features with a compound microscope for identification using the keys of Schenck and Perez (1990) and INVAM (http://www.invam.caf.wvu.edu) based on the wall structure and, if possible, to classify the genus and species rank using the current taxonomy (Schüßler and Walker, 2010; Redecker et al., 2013). The tools used here are important in morphology-based diagnoses; they are effective in detecting amyloid A substance present in the spores of some species of fungi that appear as a blue-black stain under the microscope (Josserand 1983; Vizzini et al., 2020). The small filter paper (carrying a single spore) was then carefully inoculated below a root from germinated sorghum seeds (Figure 1) in each pot (Brundrett and Juniper 1995; Selvakumar et al., 2018). The spores were then covered with sterile substrate. Cultures with one spore from an existing culture

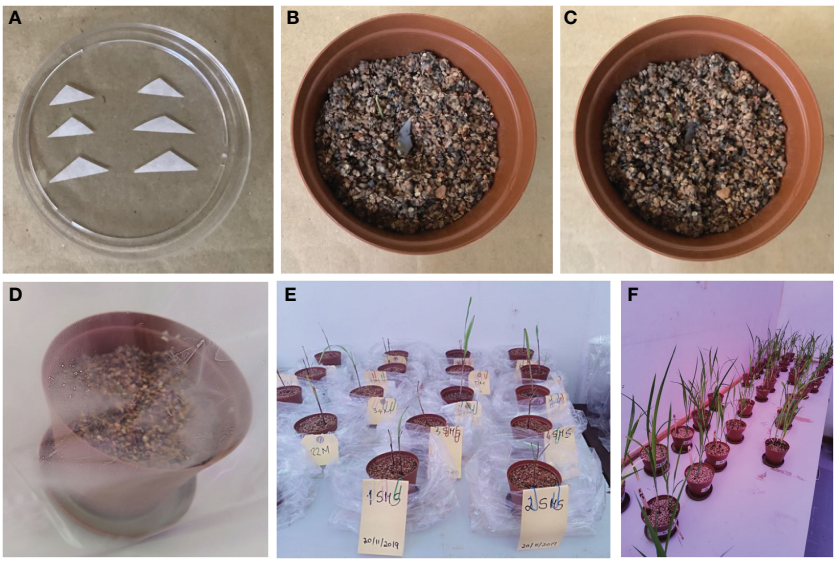


FIGURE 1
Single spore inoculation method in the pot. **(A)** Triangle filter paper with single spore prepared for inoculation. **(B)** Pre-germinated sorghum inoculated with single spore. **(C)** Inoculated seed covered with the non-clay substrate. **(D)** Single spore inoculated in pre-germinated seedlings. **(E)** pregerminated seedlings. **(F)** Four-month-old seedlings.

ensure that only one species of fungus is present and can significantly reduce the possibility of transferring parasitic organisms to healthy spores. All pots were placed in sunbags, maintained in a plant growth room (120 days) with regular watering with distilled water, and periodically assessed for AM fungal development. After 4 months, plants were harvested along with complete roots, and spores were extracted for molecular identification of the AM fungal species.

2.2.2 Direct PCR amplification of single spore

Spores obtained from single spore trap cultures from each treatment (MO, MM, MP, and MS) were extracted following the sieving method described by Schenck (1982) and Smith and Dickson (1997). Under a dissecting microscope (Leica L2), a single spore (from each treatment) was picked and transferred to a 1.5-mL centrifuge tube (a different tube for each treatment), and 10 µL of RNAlater™ (Sigma, Lot# MKCP0642) was added to the tube. The tube was left overnight in a refrigerator (4°C), followed by overnight incubation in a freezer (−20°C). The spores were gently crushed in a centrifuge tube using a pipette tip under a stereoscope to ensure the spore wall ruptured. Three pairs of primers were used for PCR amplification: ITS1 × ITS4 (White et al., 1990), SSmAf × LSUmAr/SSUmcf × LSUmBr (Krüger et al., 2009), and NS31 × AML2 (Lee et al., 2008). These primers are known to be suitable for specifically amplifying AM fungi rDNA, characterizing the diversity of AM fungal species, and allowing phylogenetic analysis with species-level resolution. The internal transcribed spacer (ITS) region is the common barcoding region used to identify and distinguish different fungal species (Rajaratnam and Thiagarajan, 2012; Raja et al., 2017). This region includes the ITS1 and ITS2 regions separated by the 5.8S gene, situated between the 18S SSU and 28S LSU genes in the rDNA repeat unit (Wipf et al., 1999). The ITS region is the most used for fungal analysis because it has a higher degree of variation between species that are closely related; this is evident by the sequences on the barcoding gap that show a higher difference in sequences among species than those within species (Toledo et al., 2013; Aslam et al., 2017). The SSU, ITS, and LSU regions have different rates of evolution, which resulted in different levels of genetic variation; e.g., SSU evolves the slowest, resulting in it having the lowest amount of variation among taxa, is highly conserved, and has a high specificity of the primer combination (Lee et al., 2008; Lekberg et al., 2018; Perez-Lamarque et al., 2022). ITS evolves the fastest and shows the highest variation. LSU is generally considered less variable than the ITS region (White et al., 1990; Bruns et al., 1991; Begerow et al., 2010). The greater sequence variation in the ITS (ITS1/ITS2) makes them more suited for species and strain identification than the 18S region, the 5.8S region, and the 28S region (Iwen et al., 2002). PCR was conducted in a total volume of 20 µL consisting of 10 µL of 2x Phire Plant direct PCR Master Mix (Thermo Scientific™, Waltham, MA, USA; Lot# 01098165), 1 µL Primer A, 1 µL Primer B, 6 µL nuclease-free H_2O, and 2 µL crushed spores in RNAlater. The thermal cycling parameters are presented in Table 1.

Repeated PCR was performed using the same set of primers for ITS1 × ITS4 and NS31 × AML2; however, for SSU and LSU, a new set of primers was used: SSUmcf and LSUmBr (Krüger et al., 2009). PCR amplicons were electrophoresed on a 1% agarose gel and visualized using UV light in a Bio-Rad Molecular Imager® ChemiDoc™ XRS+ with Image Lab™ Software (USA). PCR amplicons were cleaned using a kit from WizardR SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA; ZR-96 DNA

TABLE 1 Thermal cycle parameters used in the direct PCR amplification.

Parameters	Temperature (°C)	Time (s)	Cycles
Initial denaturation	98	300	1
Denaturation	98	5	35
Annealing (ITS1/ITS4)	49	30	35
Annealing (SSU/LSU)	58	30	35
Annealing (NS31/AML2)	58	30	35
Elongation	72	20	35
Final elongation	72	60	1

Clean-up Kit™). Samples were then sent for Sanger sequencing to Inqaba Biotechnical Industries (Pty) Ltd. in Pretoria.

2.3 Microscopy survey

Healthy spores (showing numerous lipid globules inside and neither a turbid content nor an air bubble) obtained from single spore trap cultures and collected by the wet sieving method described by Schenck (1982) and Smith and Dickson (1997) were selected for identification using the morphological approach. The morphological characteristics of the spores and details of the wall structure were determined by examining several slides of intact spores mounted in PVLG (Omar et al., 1979) and a mixture of PVLG and Melzer's reagent (1:1, v/v). On each labeled microscope slide, two drops of PVLG were added to one set of spores, and one drop of PVLG and one drop of Melzer's reagent were added to the second set of spores (Koske and Tessier, 1983). A cover slip was placed on each group, and localized light pressure was applied to break the cell walls of some spores. The slides were incubated at room temperature for at least 3–5 days (to clear their contents from the oil droplets) before being examined under a light microscope. After that, they were examined under an Olympus BX50 DIC compound microscope. Microphotographs were recorded using a Sony 3CDD color video camera coupled to a microscope. The terminology for spore structure has been suggested by Stürmer and Morton (1997) and Walker (1983). The spore size and color of fresh specimens immersed in distilled water were examined under a dissecting microscope. Color names were obtained from Kornerup and Wanscher (1983). The number of cell wall layers, the reaction of individual layers with the stain, and the flexibility of the coatings were observed and recorded. The subtending hyphae, continuity of the spore cell walls with those of subtending hyphae, and the existence of a septum or occlusion were also considered. These characteristics were compared to the descriptive features of described species posted on the INVAM website (http://fungi.invam.wvu.edu/the-fungi/species-descriptions.html), and morphotype determination of the genus was made based on the classifications described by Morton and Benny (1990). Each morphotype was assigned the name of the species with the closest match. The nomenclature for fungi is that of Schüßler and Walker (2010) and Redecker et al. (2013)

2.4 Data analysis

Nucleotide sequences obtained from Sanger sequencing were aligned on MEGA X version 10.2.4 (Kumar et al., 2018; Stecher et al., 2020) using MUSCLE (Edgar, 2004a, 2004b). MEGA X and MUSCLE settings were by default for gap penalties and memory/iterations, while advanced options were by the neighbor-joining method. The fasta file containing the nucleotide sequences was subjected to a Basic Local Alignment Search Tool (BLAST$^+$) program available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) for comparison against the GenBank database (Altschul et al., 1997) to determine the closest sequence matches that enabled taxonomic identification. The most comparable sequence matches with at least 97% similarity (this threshold is considered given rather than being a tunable parameter, following the conventional wisdom that 97% corresponds approximately to species; Schloss and Handelsman, 2005; Westcott and Schloss, 2017) with the reference sequence, and meant taxonomic identification to the genus level could be selected. The nomenclature of AM fungal genera was assigned according to the Index Fungorum website (https://www.indexfungorum.org) to determine the currently accepted name. Additional taxonomic assignment was based on phylogenetic relationships. A phylogenetic tree was constructed based on multiple sequence alignments from sequences of AM fungal isolates (in the four treatments: MM, MO, MP, and MS) and GenBank (NCBI) data sequences and was estimated by the neighbor-joining method (Saitou and Nei, 1987) using unbiased estimates of evolutionary distances. The bootstrap tests assessed the reliability for the maximum parsimony (Kumar et al., 2018). The branches corresponding to partitions reproduced in less than 50% of bootstrap replicates collapsed (Felsenstein, 1985). *Chrysoporthe austroafricana* (JN942337 and JN942338) sequences were taken from the NCBI database and used as an outgroup.

3 Results

3.1 Single spore identification

After 4 months of observation of 32 inoculants (trap culture pots inoculated with single spores from each treatment) in the plant growth room, only 29 inoculants showed propagations with more than 100 spores (Figure 1). Out of the 29 propagations, only 13 samples had good sequence (Sanger) quality, and one sample (good chromatogram with high-quality peaks) from each treatment was chosen for further analysis. BLAST$^+$ on NCBI was performed for ID identification. The analysis involved 39 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1,259 positions (ITS1 × ITS4) in the final dataset (Kumar et al., 2018; Stecher et al., 2020). The percentage of replicate trees in which the associated taxa clustered

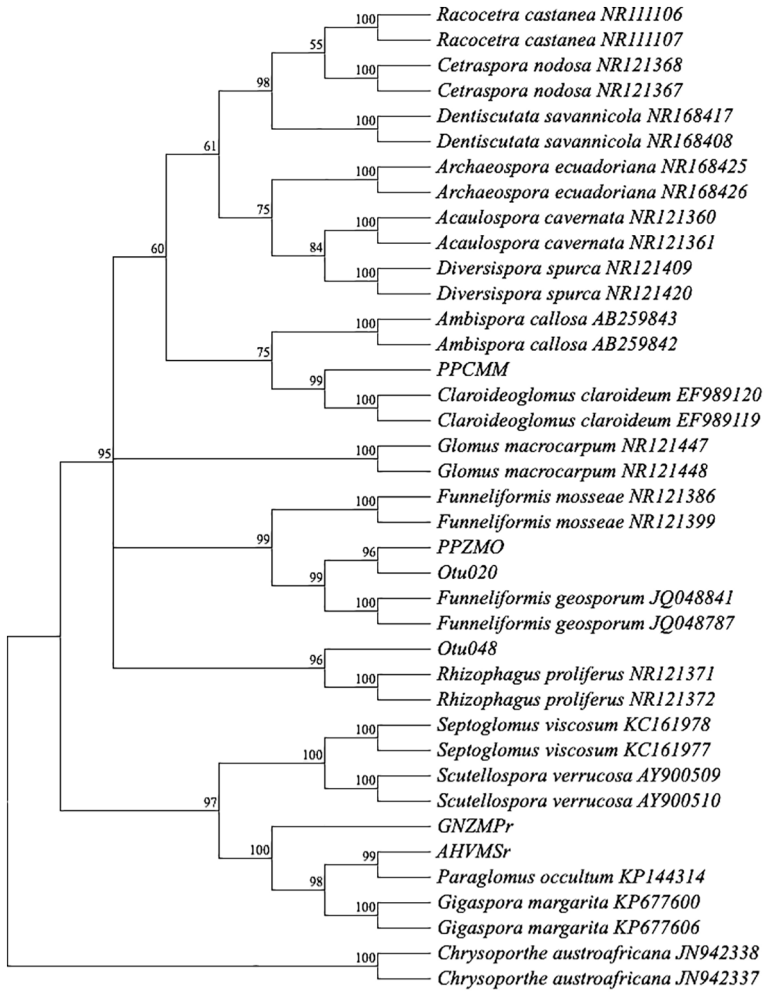


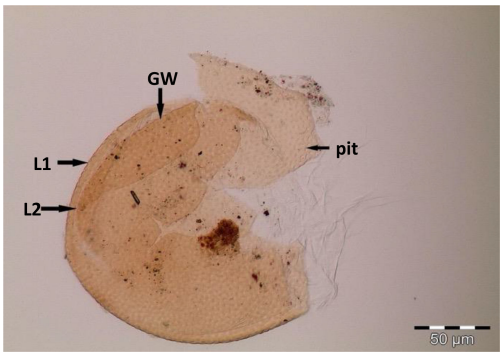
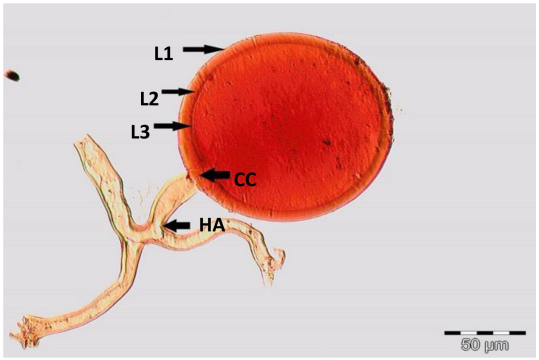
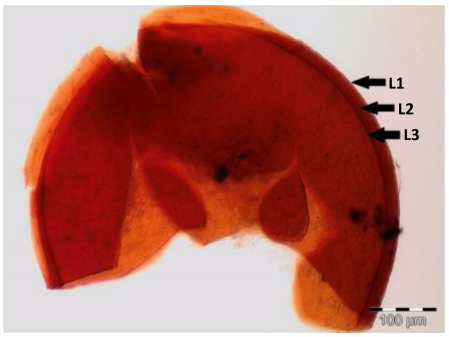
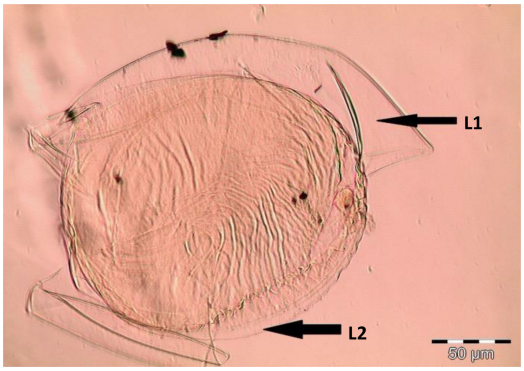
FIGURE 2
Bootstrap consensus tree. A neighbor-joining phylogram of selected sequences of AM fungi based on distance analysis of the 18S rDNA. For comparison, identified sequence types from GenBank were included in the analysis. Bootstrap supports values greater than 50%, given on the branches (1,000 resembling branches). Samples from this study do not show an accession number. The tree was rooted in *Chrysoporthe austroafricana*. PP, pre-planting; GN, germination; AH, at harvest; r, rhizosphere; MO, maize after oats; MM, maize after maize; MP, maize after peas; MS, maize after soybean; Z, Zunckel farm; C, conventional farm; V, Van Rooyenswoning farm; AM, arbuscular mycorrhiza.

together in the bootstrap test (1,000 replicates) is shown next to the branches (Figure 2) (Felsenstein, 1985). The constructed phylogenetic tree separated the four AM fungal isolates from their outgroup, *C. austroafricana*. This phylogenetic tree (Figure 2) showed that the PPCMM isolates formed the same cluster as *Claroideoglomus claroideum* and were in a different clade from the *Ambispora* genus. The AHVMSr isolates formed the same cluster with the *Paraglomus* genus and on a distinct clade with *Gigaspora margarita*. The phylogenetic tree also showed that the GNZMPr isolate was in the same clade as *G. margarita* and *Paraglomus occultum*. The PPZMO isolate was in the same clade as *Funneliformis geosporus*. In the phylogenetic tree, the closest neighbors of the propagated AM fungal species revealed that the AM fungal spores had characteristics belonging to the genera *Claroideoglomus* (PPCMM), *Gigaspora* (GNZMPr), *Paraglomus* (AHVMSr), and *Funneliformis* (PPZMO) (Figure 2) and were present under both agriculture practices. Also, described species such as OTU024 and OTU021 could be seen in the tree close to the genera *Funneliformis* and *Rhizophagus*.

3.2 Morphological characterization of AM fungi

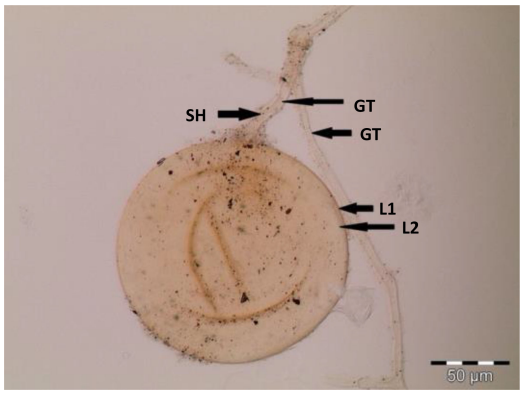
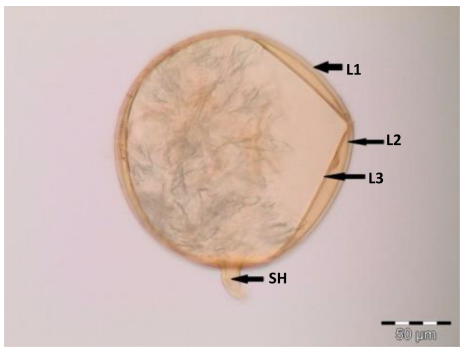
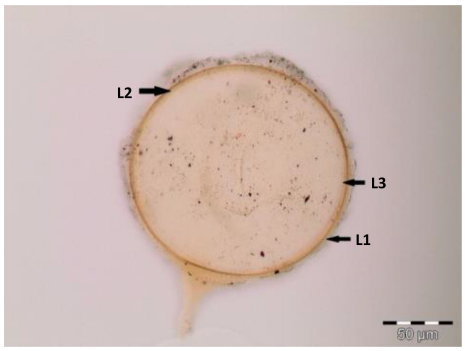
Spores (some) obtained from the single spore trap cultures were used for morphological characterization. The macro-characteristics considered for description included the spore color, size, and shape; shape, width, pore occlusion of the subtending hypha; and the size of the auxiliary cells. The micro-characteristics for characterizing the spore wall structure included color, dimension, number, type, and ornamentation (Table 2). Yellowish, reddish brown, dark red-brown, yellow-brown, and hyaline to pale yellow spores were observed in this study, with sizes ranging from 128- to 220-µm diameters. Overall, eight species of AM fungi belonging to five

TABLE 2 Morphological characteristics of some AM fungal species.

AM fungal species	Description
Acaulospora cavernata Blaszk (1989) spores in PVLG 	Spore pale yellowish in color to light brown with two spore wall layers (L1 and L2), namely, outer (L1) membranous light yellow colored layer. Sub-globose, 130–150 µm in diameter, sessile on the neck of a sporiferous saccule. Mature spore showing one terminal germinal wall (GW) layer and pitted ornamentation on the wall
Funneliformis geosporus C. Walker & A. Schüßler (2010) spores in PVLG + Melzer's reagent 	Reddish brown walled spore, with lengthy tube-like subtending hyphae. Spore shows three wall layers (L1, L2, and L3), which are tightly adherent, laminated, and membranous. L1, a hyaline sloughing granular layer; L2, a rigid layer consisting of adherent sublayers appeared orange-brown in color; L3, a semi-rigid resolved by slightly darker color (yellow to orange-brown). A germ tube emerged from the lumen of the subtending hypha and originated from the recurved septum. Globose, 128–135 µm in diameter with loosely sleeve-like hyphal attachment at right angle to spore wall. Cross channel (CC) in wall layers, common hyphal attachment (HA)
Gigaspora margarita W.N. Becker & I.R. Hall (1976) spores in PVLG + Melzer's reagent 	Crushed spore of *Gigaspora margarita*, globose in shape, dark red-brown in color, and consisted of three layers: L1, an outer permanent rigid layer, smooth, adherent to inner laminae, pale brownish; L2, hyaline sublayer, rigid, dark red-brown; and L3, germinal layer that is concolorous and adherent with the laminate layer, 215–220 µm in diameter
Diversispora spurca C. Walker & A. Schüßler (2004) spores in PVLG 	Crushed spore of *Diversispora spurca*, subhyaline, globose in shape, and consisted of two layers: L1, hyaline to pale yellow-brown, separating from the L2; and L2, thin hyaline to subhyaline sublayer. L2 of the spore wall stops abruptly in the region of attachment and thus is not part of the more distant hyphal wall structure; 155–182 µm in diameter

(Continued)

TABLE 2 Continued

AM fungal species	Description
Rhizophagus aggregatus in PVLG + Melzer's reagent 	Globose spores of *R. aggregatus*, 143–145 µm in diameter, hyaline to pale yellow in color, with two yellow layers (L1 and L2). Single subtending hyphae, which stop abruptly in the region of attachment and thus are not part of the more distant hyphal wall structure. The hypha is closed by a thin septum, thickening of the spore wall. Germ tube (GT), subtending hyphae (SH)
Paraglomus occultum (C. Walker) J.B. Morton & D. Redecker (2001) spores in PVLG 	Globose spore of *Paraglomus occultum*, 152–159 µm in diameter, hyaline to pale yellow in color, with three layers (L1, L2, and L3). L1, a sloughing layer; L2, a permanent layer, continuing into the wall of subtending hypha; and L3, a permanent layer, SH subtending hyphae
Funneliformis mosseae C. Walker & A. Schüßler (2010) spores in PVLG + Melzer's reagent 	Globose spore of *Funneliformis mosseae*, 152–154 µm in diameter, hyaline to straw to yellow in color, with three layers (L1, L2, and L3). L1 hyaline, mucilaginous; L2 hyaline, rigid, attached firmly to the underlying laminae; and L3 pale sublayer

AM, arbuscular mycorrhiza; PVLG, polyvinyl alcohol/lactic acid/glycerol.

families in the Glomeromycota phylum were found in this study: Acaulosporaceae (one species), Diversisporaceae (one species), Gigasporaceae (one species), Glomeraceae (four species), and Paraglomeraceae (one species) (Figure 3; Table 2). Furthermore, from a total of 10 morphotypes of AM fungal generated and classified on Mothur using the UNITE database, the results were then blasted on NCBI for comparison. Sequences were deposited into GenBank under accession numbers OR822200–OR822205. Species such as *Acaulospora lacunosa*, *Archaeospora leptoticha*, *G. margarita*, *Gigaspora rosea*, *Funneliformis mosseae*, *F. geosporus*, *Glomus monosporum*, *Rhizophagus aggregatus*, *Rhizophagus intraradices*, and *P. occultum* showed more than 97% similarity with NCBI comparison (Supplementary Table 1).

4 Discussion

Trap culture and monosporic culture techniques of spore multiplication are the most commonly employed cultivation strategies in the substrate-based method (Douds et al., 2006; Panwar et al., 2007). These two AM fungal propagation techniques provide an environment that closely mimics the field conditions. Since AM fungi

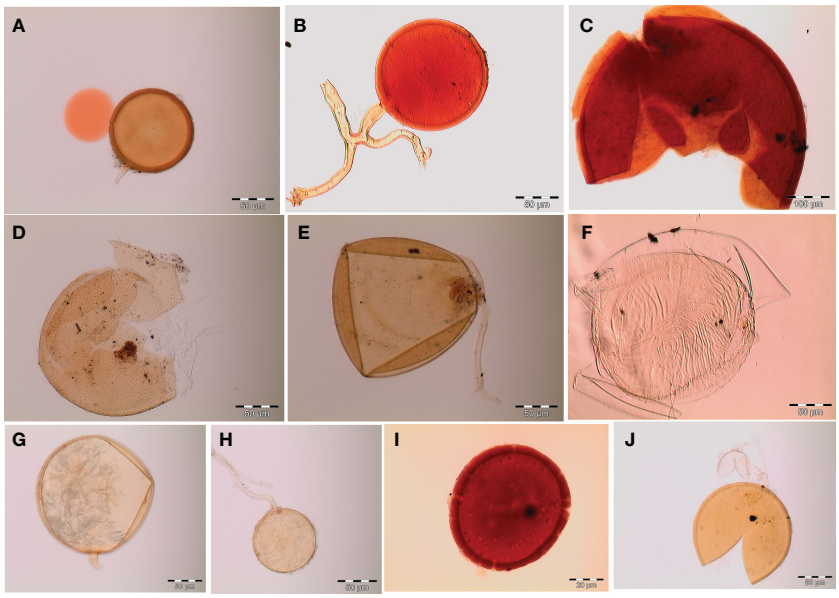


FIGURE 3
Spore identification using morphological characteristics: **(A, B)** *Funneliformis mosseae*, **(C)** *Gigaspora margarita*, **(D)** *Acaulospora cavernata*, **(E)** *Rhizophagus intraradices*, **(F)** *Diversispora spurca*, **(G, H)** *Glomus macrosporum*, **(I)** *Funneliformis geosporus*, and **(J)** *Paraglomus occultum*.

have been reported to be able to provide their hosts with access to numerous soil resources such as P, N, and water, the present study was involved in the propagation of AM fungal single spores isolated from maize soil samples from four different treatments (PPZMO, AHVMSr, PPCMM, and GNZMPr) under two different agriculture management practices. The initial inoculum should start with a single spore to obtain a pure culture. Only 29 samples out of the initial 32 pots showed some colonization in this study. These could be because of the source of the initial AM fungal spores or the quality of the spores, as the spores were isolated from soil samples under different crop rotations, which could have influenced the quality of the spores. Maize is an obligatory mycorrhizal species readily colonized by many non-host-specific AM fungi (Daisog et al., 2012). Maize growth phases strongly affect the abundance, diversity, and community composition of AM fungi (Lü et al., 2020). In the current study, the selected single spores were picked from soil samples collected in different farms and different maize growth phases (PP, AH, and GN), which could explain the occurrence of different AM fungal species at a particular stage of maize development, underlying the fact that AM fungi have contrasting seasonal sporulation dynamics and their different phenologies can cause disparate community composition across plant growing seasons (Pringle and Bever, 2002; Bainard et al., 2012; Varela-Cervero et al., 2016). Also, Mathimaran et al. (2007) and Nord and Lynch (2009) demonstrated that plant phenologies and the dynamics of soil processes could likely differ in different cropped soils.

Identification of AM fungi both morphologically and molecularly is essential to determine with some certainty the identity of an organism (Schenck and Perez, 1990; Lee et al., 2006; Schüßler and Walker, 2010). Brundrett (1991) suggested a variation of AM fungal species in a habitat, which is related to their capability of growing under certain conditions and the ability of fungi to form a symbiosis with various types of plants in the vicinity. Identification of the four AM fungal isolates based on morphology complemented the identification based on molecular (species level) analysis. Therefore, there are more detailed and valid data, high confidence, and strong evidence that the morphological characterization technique is also important for AM fungal identification. Nonomura et al. (2011) stated that combining the results from morphological characterization and molecular methods is the best approach to identifying AM fungal taxonomy. Also, spores are viewed as among the most important and convenient characteristic features, and they can help researchers rapidly identify mycorrhizae faster than sequence techniques (Fall et al., 2022). In this study, AM fungal species belonging to the genera *Glomus*, *Funneliformis*, *Gigaspora*, *Acaulospora*, *Diversispora*, *Rhizophagus*, and *Paraglomus* were associated with maize and described morphologically. Similar results were expected in the work of Tobolbai et al. (2018); Baltruschat et al. (2019), and Fall et al. (2022) where they studied the morphological diversity of native AM fungal species associated with the rhizosphere of maize in different agroecosystems and found similar AM fungal species. Also, studies by Sanders (2003); Hijri et al. (2006); Alguacil et al. (2008), and Sasvári et al. (2011) described the seven genera (similar to the ones encountered in the current study) as the most dominant genera associated with maize crops under different management practices.

The applications of the findings of this research, particularly for the maize crop, show that all AM fungal spores obtained are good candidates for mass-producing inocula or material for identification in other studies and, also, can guide the management of these important symbioses as part of integrated conservation of land management plans.

5 Conclusion

The results of the current study demonstrated that the production of pure cultures of AM fungal species could be achieved using single-spore inoculum through the trap culture method. Selected AM fungal isolates (from the most predominant spore morphotypes) exhibited more spores produced, and all belonged to the phylum Glomeromycota. Future studies are needed to further identify more different genera in the Glomeromycota to explore the diversity of AM fungal species associated with maize and, if possible, authenticate the method applied in this study. This applied method can also be used to initiate starter cultures for bioinoculants in large-scale agricultural applications with the advantage of being more readily adopted by farmers due to the lack of requirement of a skilled technique in spore propagation.

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

SM-S: Conceptualization, Formal analysis, Methodology, Writing – original draft. JD: Conceptualization, Methodology, Supervision, Writing – review & editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1345229/full#supplementary-material

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Inoculation with *Jeotgalicoccus* sp. improves nutritional quality and biological value of Eruca sativa by enhancing amino acid and phenolic metabolism and increasing mineral uptake, unsaturated fatty acids, vitamins, and antioxidants

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Plant growth-promoting bacteria (PGPB) are considered a promising tool for triggering the synthesis of bioactive compounds in plants and to produce healthy foods. This study aimed to demonstrate the impact of PGPB on the growth, accumulation of primary and secondary metabolites, biological activities, and nutritional qualities of Eruca sativa (arugula), a key leafy vegetable worldwide. To this end, Jeotgalicoccus sp. (JW0823), was isolated and identified by using partial 16S rDNA-based identification and phylogenetic analysis. The findings revealed that JW0823 significantly boosted plant biomass production by about 45% ($P<0.05$) and enhanced pigment contents by 47.5% to 83.8%. JW0823-treated plants showed remarkable improvements in their proximate composition and vitamin contents, with vitamin E levels increasing by 161.5%. JW0823 induced the accumulation of bioactive metabolites including antioxidants, vitamins, unsaturated fatty acids, and essential amino acids, thereby improving the nutritional qualities of treated plants. An increase in the amounts of amino acids was recorded, with isoleucine showing the highest increase of 270.2%. This was accompanied by increased activity of the key enzymes involved in amino acid biosynthesis, including glutamine synthase, dihydrodipicolinate

synthase, cystathionine γ-synthase, and phenylalanine ammonia-lyase enzymes. Consequently, the total antioxidant and antidiabetic activities of the inoculated plants were enhanced. Additionally, JW0823 improved antimicrobial activity against several pathogenic microorganisms. Overall, the JW0823 treatment is a highly promising method for enhancing the health-promoting properties and biological characteristics of E. sativa, making it a valuable tool for improving the quality of this important leafy vegetable.

KEYWORDS

arugula, biosynthetic enzymes, antioxidant, fatty acids, flavonoids, plant growth promoting bacteria

1 Introduction

Beneficial bacteria, including endophytes and rhizobacteria, form symbiotic relationships with plants and are known as plant-growth-promoting bacteria (PGPB). PGPB stimulate plant growth and are used in agriculture to improve productivity and quality as an alternative to traditional fertilizers, promoting sustainability (Bakhshandeh et al., 2020; Saberi Riseh et al., 2021; Crecchio, 2020; Manoj et al., 2020). They maintain soil fertility (Harris, 1997), solubilize minerals like phosphate and zinc, and fix nitrogen (Pii et al., 2016; Yaghoubi Khanghahi et al., 2021b). Furthermore, they produce growth regulators (Khan, 2021), enhancing plant growth, metabolism, and stress resistance (AbdElgawad et al., 2021a, 2021b; Ghorbel et al., 2023), and improve agronomic and physiological traits in various plants (Yaghoubi Khanghahi et al., 2021a) and induce hormone production (Harris, 1997). Overall, PGPB support ecosystem survival and function (Schimel et al., 2007; Paz-Ferreiro and Fu, 2016). Among PGPB, *Jeotgalicoccus* sp. can effectively promote plant growth, colonization and show great potential in plant tolerance to abiotic stresses (Gong et al., 2016; Li et al., 2022; Misra et al., 2019; Liu et al., 2011). The genus *Jeotgalicoccus* was introduced by Yoon et al. (2003), initially encompassing two species (*J. halotolerans* and *J. psychrophilus*). This new genus was classified within the *Staphylococcaceae* family of the Firmicutes phylum. *J. huakuii* improved maize growth in alkaline soil and increased the production of bioactive compounds such as antioxidants, chlorophyll, and soluble sugars (Misra et al., 2019). Mukasheva et al. (2016) reported that *J. halotolerans* strain can produce growth-promoting hormones (IAA) and ACC deaminase enzyme, reducing ethylene levels in plants. This genus (BAK1) demonstrated growth-promoting and phosphorus-solubilizing properties as well as antagonistic potential against the causative agents of fungal diseases (Mukasheva et al., 2016). *J. huakuii* NBRI 13E enhanced plant growth under salt stress (Misra et al., 2019). In this context, *J. huakuii* NBRI 13E boosted defense enzyme production and osmo-protectant (e.g., proline) accumulation, mitigating salinity stress. *J. nanhaiensis* is also known as a heavy metal tolerant bacterial strain enhancing phytoremediation potential to remediate arsenic from contaminated sites (Singh et al., 2019). Recent research has documented the heavy metal tolerance of *Jeotgalicoccus* strains (Ahamed et al., 2024; Alves et al., 2022; Kumari et al., 2022; Sharma et al., 2024). Additionally, James et al. (2024) highlighted the role of *Jeotgalicoccus*-associated plants in mitigating air pollution.

Eruca sativa Mill. (Arugula) is an annual herb found abundantly worldwide (Warwick, 1994) and is regarded as one of the most significant leaf vegetables, originating from the Mediterranean region (Zeven and de Wet, 1982). Valued for its health benefits, it is rich in fiber and contains antioxidants such as carotenoids, polyphenols, and vitamin C. Taramira oil, a flavorful oil, is traditionally extracted from its seeds. The plant's aerial parts are commonly eaten raw in salads, like rocket salad. For almost two centuries, the genera *Eruca* and *Diplotaxis* have been recognized for their various health benefits and medicinal properties, including depurative, anti-inflammatory, digestive, aphrodisiac, diuretic, and rubefacient effects (Yaniv et al., 1998). Thus, enhancing the growth and tissue chemical composition of *Eruca* plants is crucial for meeting food needs. PGPB play a crucial role in this process of meeting food and population requirements.

Given the biological potential of *Jeotgalicoccus* sp. (JW0823), we hypothesized that symbiotic interactions of JW0823 with *Eruca* plants could enhance their tissues' chemical composition, leading to improved plant growth and quality. Consequently, this study aimed to explore the effects of JW0823 on *E. sativa* by investigating its impact on plant growth, tissue chemical composition, and bioactive properties. Overall, JW0823 is introducing as a valuable tool for improving the quality of this important leafy vegetable, with the goal of improving agricultural practices and sustainability.

2 Materials and methods

2.1 Isolating, purification and identifying bacteria from the rhizosphere

Bacteria were isolated using the filtration method described by Brock (1983). Membrane filtration methods were utilized (Manaia

and da Costa, 1991). The filters are placed on bacterial media plates. Bacteria were incubated on the plates for 2-7 days at 30-37°C after inverting. Bacterial biodiversity was observed, and purification of bacterial colonies was done by streaking several times on the isolation medium using the streaking plate method and then subculturing on slants of the same medium. The ability of bacteria to produce indole acetic acid (IAA) was assessed by the method described by Patten and Glick (2002), using Salkowski's reagent [$FeCl_3$ (0.5 M) solution in perchloric acid (35%)] and orthophosphoric acid.

Extracting genomic DNA and identifying PCR products were performed to determine the culture species. DNA from the isolates was extracted using the Pure Link Genomic DNA Kit (K182001), a bacterial DNA extraction kit, following the manufacturer's procedure. The concentration of extracted DNA was also measured spectrophotometrically using a Nano Drop ND 1000 (Thermo Scientific, USA). The isolated DNA was validated using a standard agarose gel (1% w/v). PCR and Sequencing Work Purification as well as standard sequencing for PCR products were carried out by Macrogen Company (Seoul, Korea). The PCR reaction was conducted using 100 ng of genomic DNA in a total volume of 50 µl, with a reaction buffer at 1x concentration, 30 pmole of each primer, and 2 units of Taq polymerase. The thermal cycling conditions (denaturation step at 94°C for 5.5 minutes, followed by 30 cycles of denaturation at 93°C for 1 minute, primer annealing at 53°C for 1 minute, and extension at 72°C for 1.5 minutes). The PCR products were then purified using QIAquick PCR purification reagents. The gel was stained with ethidium bromide and visualized using an ultraviolet transilluminator.

Sequencing reactions were carried out in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the manufacturer's instructions. Each template was sequenced in a single pass using the universal primer 27F (5'-AGAGTTTGATC (AC)TGCCTCAG-3'). The fluorescent-labeled fragments were isolated from unincorporated terminators using the Big Dye®X Terminator™ purification process. The samples were resuspended in distilled water before electrophoresis using an ABI 3730xl sequencer (Applied Biosystems). The sequences were examined for sequence similarity using BLAST (www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997), and compared to reference sequences found in BLAST and downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/).

2.2 Experimental setup, plant materials, and growth conditions

The arugula seeds (*E. sativa*) (Agricultural Research Centre, Giza, Egypt). After that, for six hours at room temperature, sterile arugula seeds were immersed in a liquid suspension of the isolated strain, JW0823 inoculum (cultured at 30°C, pH 7.8 and 0% NaCl for 48 hours) at 25% concentrations (2.5×10^7 CFU mL^{-1}), while the control group was submerged in distilled water. The treated and controlled arugula seeds were sown into sterile soil and three biological replicates for each treatment are represented by the three pots. The clay soil initially contained 14.5 mg organic carbon (C), 13.7 mg nitrate-nitrogen (N), 1.7 mg ammonium-N, 9.3 mg phosphorus (P)/g air dry soil at a humidity of 0.41 g water/g dry soil. The soil was watered twice a day and maintained at 58%. The arugula was cultivated in pots (20 cm high and 15 cm width) and grown in growth-controlled chambers with the following conditions: 24°C, 290 ± 12 µmol PAR m^{-2} s^{-1}, 16 hours of light and 8 hours of darkness, and 58% relative humidity. All pots were arranged in a randomized complete block design with five replicates per treatment. After 5 weeks of growth, the fresh weight (FW) and dry weight (DW) of the shoots were measured and kept at -80°C pending biochemical studies. Ultimately, the arugula plants were preserved for additional examination by freezing them in liquid nitrogen at -196°C.

2.3 Determination of photosynthetic rate

An EGM-4 infrared gas analyzer connected to an Environmental Monitor Sensor Probe Type 3 (PP Systems, Hitchin, UK) was used to determine the photosynthetic rate (Lichtenthaler, 1987).

A net CO_2 exchange (NE) measurement was conducted under ambient light, followed by a dark respiration measurement with the enclosure covered with a dark cloth for180 s measurement.

2.4 Pigment analysis

A MagNALyser (Roche, Vilvoorde, Belgium) was employed to homogenize 200 mg of plant in acetone at 7000 rpm for one minute. Subsequently, they underwent centrifugation for 20 minutes at 14,000 × g and 4°C. Following the method described by Almuhayawi et al. (2020), the supernatant was filtered and subjected to HPLC analysis using a Shimadzu SIL10-ADvp system equipped with a reversed-phase column at 4°C. Carotenoids were isolated using a silica-based C18 column with acetonitrile/methanol/water (81:9:10) and methanol/ethyl acetate (68:32) as the solvents. A diode-array detector (Shimadzu SPDM10Avp) was employed to extract and identify β-carotene, chlorophyll a, and chlorophyll b at wavelengths of 420, 440, 462, and 660 nm.

2.5 Determination of the nutritional quality

Data about the proximate composition, amounts of amino, organic, and fatty acids, as well as minerals, vitamins, and phenolics were obtained according to the following methods, in order to provide insight into the nutritional quality of ***E. sativa*** plants.

2.5.1 Proximate composition analysis

Following Wong et al. (2000)'s procedure, the carbohydrate content of each arugula plant group, whether treated or untreated with PGPB, was determined. Additionally, protein concentration

(0.2 g FW) in each plant sample was extracted in 0.1 mM KPO_4 buffer at pH 7. Then it was measured according to the method described by Lowry et al. (1951). After that, it was measured using the Lowry et al. (1951) method. The total lipid content of the plants was assessed after homogenizing the samples in a 1:2 (v/v) mixture of methanol and chloroform, as outlined by Shiva et al. (2018). Subsequently, the plants were centrifuged at 3000× g for 15 minutes, and the resulting pellets were dissolved in a 4:1 (v/v) mixture of ethanol and toluene. To determine the total lipid content, the lipids were first concentrated and then quantified using a gravimetric method. The results were expressed as milligrams of lipid per gram of fresh plant weight. The crude fibers were isolated from the plant material (Lee, 2002). The enzymatic digestion was performed using protease at pH 7.6 and 55°C for 24 minutes, followed by treatment with amyloglucosidase at pH 6 and 0°C for 30 minutes to eliminate proteins and starches.

2.5.2 Elemental analysis

Following the methodology outlined by AbdElgawad et al. (2014), 200 mg of plants, treated with bacterial endophytic inoculation and control plants, were subjected to digestion in a 5:1 (v:v) HNO_3/H_2O solution to determine their mineral composition. Subsequently, both major and trace elements were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) on a Finnigan Element XR instrument from Scientific, Bremen, Germany.

2.5.3 Amino acids metabolism

Following the protocol outlined in Sinha et al. (2013), 100 mg of every plant sample were dissolved in five mL ethanol (80%), while being spun at 5000 rpm for one minute. Subsequently, a 25-minute centrifugation at 14,000 x g was conducted, and the supernatant that resulted was reconstituted in 5 milliliters of chloroform. Then, one milliliter of water was used to remove any residue. After resuspension in chloroform, the pellet and supernatant were centrifuged for 10 minutes at 8000× g. Then filtration through Millipore microfilters with 0.2-µm pore size was done. Elution (A, containing 10% acetonitrile, 84% ammonium formate and 6% formic acid, v/v) and quantification of amino acids (B, containing 2% formic acid, v/v and acetonitrile) were carried out using a Waters Acquity UPLC TQD apparatus connected to a BEH amide column. A set of amino acid standards was utilized as the reference.

Glutamyl synthase (GS) activity was measured using the methodology described by Almuhayawi et al. (2021), with extraction conducted in 100 mg mL^{-1} Tris-HCl (50 mM), pH 7.4, containing 2% polyvinylpyrrolidone, 4 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 2 mM PMSF. Subsequently, γ-glutamyl hydroxamate synthesis was assessed, indicating the presence of GS activity in a Tris-acetate reaction buffer (200 mM, pH 6.4). Dihydrodipicolinate synthase (DHDPS) activity was carried out as per Kumpaisal et al. (1987). Plants not exposed to L-aspartate-b-semialdehyde were used as a negative control. The reaction was conducted at 36.5°C to facilitate adduct formation between the reaction product and o-ABA. Trichloroacetic acid (TCA) at a concentration of 12% was added to stop the process, and samples were analyzed at 550 nm following a 60-minute dark incubation period.

Cystathionine γ-synthase (CGS) was extracted in 20 mM MOPS for 15 minutes at 4°C. The supernatants were combined with a reaction buffer containing O-phospho-homoserine (5 mM), L-cysteine (2 mM), PLP (100 µM), and AVG (200 µM). L-cystathionine formation was isolated using a phenomenex Hyperclone C18 BDS column on a Dionex HPLC system, following the method described by Ravanel et al. (1998).

2.5.4 Organic acid analysis

Organic acids in 200 mg of plant samples were detected using HPLC (0.001 N sulfuric acid, at 210 nm, flow rate of 0.6 mL min^{-1}), following the method outlined by Hamad et al. (2015). The detection system comprised an LED model detector (Ultimate 3000) and a liquid chromatographer (Dionex, Sunnyvale, CA, USA), equipped with an LPG-3400A pump, a TCC-3000SD column thermostat, and an EWPS-3000SI autosampler. The separation was conducted at 65°C using an Aminex HPH-87 H (300 × 7.8 mm) column with an IG Cation H (30 × 4.6) precolumn from Bio-Red company. UV detection system operating at 210 nm was used to estimate the concentrations of citric, succinic, fumaric, and malic acids (LaChromL-7455 diode array, LaChrom, Tokyo, Japan). Data analysis was performed using the Chromeleon v.6.8 computer program.

2.5.5 Fatty acid analysis

200 mg of the plant samples were extracted in 50% aqueous methanol at 25°C. The plant material was thoroughly homogenized in 50% aqueous methanol to ensure efficient extraction. The mixture was then subjected to shaking, and then the extract was centrifuged at 4°C for 20 minutes. The GC/MS (Hewlett Packard, Palo Alto, CA, USA) was used to sperate and identify fatty acid detection method, which was equipped with an HP-5 MS column (30 m × 0.25 mm × 0.25 mm). NIST 05 database and Golm Metabolome (http://gmd.mpimp-golm.mpg.de, accessed on February 23, 2024) were utilized. A set of amino acid standards was utilized as the reference.

2.5.6 Vitamin content analysis

To determine the levels of vitamins in plant samples, approximately 200 mg of fresh plant material was analyzed using UV and/or fluorescence detectors. For this analysis, thiamine and riboflavin contents were measured. A reverse-phase C18 column was utilized for the separation process in high-performance liquid chromatography (HPLC), with a methanol/water solvent system. Vitamin C (ascorbate) levels were quantified using HPLC with Shimadzu equipment (Hertogenbosch, The Netherlands). Antioxidants were isolated from plant tissues that had been extracted in 1 mL of ice-cold 6% (w/v) meta-phosphoric acid. The antioxidants were subsequently separated on a reversed-phase HPLC column (Farfan-Vignolo and Asard, 2012). The thiamine and riboflavin contents were again determined using UV and/or fluorescence detection, with a reverse-phase C18 column used for separation.

2.5.7 Analysis of phenolics and their biosynthetic enzymes

To assess the total flavonoid and phenolic concentrations, plant material (120 mg) was homogenized in 80% ethanol. After centrifugation at 4°C for 20 minutes, the phenolic content was determined using the Folin–Ciocalteu reagent, with gallic acid as the standard reference. Flavonoid content was measured using a modified colorimetric method with aluminum chloride, with quercetin serving as the calibration standard. For evaluating phenylalanine ammonia-lyase (PAL) activity, 0.25 g of frozen plant material was homogenized in a Tris-HCl buffer (100 mM, pH 8.8) containing L-phenylalanine (40 mM). The enzyme activity was assessed by measuring the absorbance of transcinnamic acid produced at 290 nm, following the protocol by AbdElgawad et al. (2014). Water was used as a negative control in place of the plant samples to ensure accuracy in the enzyme assay.

2.6 Biological activities

2.6.1 Antioxidant activities

Several tests were used to assess the plants' antioxidant potential (Almuhayawi et al., 2020). About 0.1 g was extracted in 80% ethanol to determine the ferric reducing antioxidant power (FRAP). Centrifugation was then carried out (14,000 rpm, 20 min). Next, 0.1 mL of the extract was combined with 20 mM $FeCl_3$ in 0.25 M acetate buffer, which is known as the FRAP reagent. 2.4 mM potassium persulphate was combined with 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to determine its concentration. The absorbance was measured at 734 nm, and 0.1 mL of the extract and 0.25 mL of the DPPH reagent were used to detect the DPPH activity. At 517 nm, the absorption was found.

2.6.2 Antimicrobial activity

2.6.2.1 Assessment of antibacterial activity in plants

The antibacterial activity of the plant extracts was evaluated using the standard dilution method as outlined by Almuhayawi et al. (2021). Initially, 100 mg of the plant material was extracted in dimethyl sulfoxide (DMSO). The resulting extract was then used to prepare the test medium, which was supplemented with 0.1 mL of a 1:10,000 dilution of a liquid culture of the reference strain *Staphylococcus aureus* ATCC 6538 P. This dilution contained approximately 10^4–10^5 bacterial cells per mL. The inoculated media was incubated at 37°C for 18 hours.

To determine the antimicrobial efficacy of the plant extracts, the Minimum Inhibitory Concentration (MIC) was measured. The extracts were tested against a range of bacterial and fungal species, including *Candida glabrata* (ATCC 90030), *Pseudomonas aeruginosa* (ATCC 10145), *Enterobacter aerogenes* (ATCC 13048), *Proteus vulgaris* (ATCC 8427), *Staphylococcus saprophyticus* (ATCC 19701), *Escherichia coli* (ATCC 29998), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus epidermidis* (ATCC 12228), *Candida albicans* (ATCC 90028), *Streptococcus salivarius* (ATCC 25975), *Aspergillus flavus* (ATCC 9170), *Enterococcus faecalis* (ATCC 10541), and *Serratia marcescens* (ATCC 99006).

For comparative purposes, ciprofloxacin (25 mg/mL) was used as a positive control, while 100% DMSO served as a negative control. This setup allowed for assessment of the antibacterial effectiveness of the plant extracts against various pathogens.

2.6.3 Antidiabetic activity

2.6.3.1 Inhibition of α-amylase assay

The α-amylase inhibitory activity was assessed following a modified method from Dada et al. (2017). To begin, 500 µL of the plant extract was mixed with 500 µL of 0.02 M sodium phosphate buffer (pH 6.9, containing 0.006 M NaCl) and 1.0 U/mL of α-amylase solution. This mixture was incubated at 25°C for 10 minutes. Following pre-incubation, 500 µL of 1% starch solution, also prepared in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl), was added to the mixture. The reaction was allowed to proceed at 25°C for another 10 minutes. To halt the reaction, 1.0 mL of DNS (dinitrosalicylic acid) color reagent was added. The test tubes were then heated in boiling water for five minutes, cooled to room temperature, and subsequently diluted with 10 mL of distilled water. The absorbance of the final solution was measured at 540 nm. The inhibitory activity of the plant extract against α-amylase was determined by comparing its effect to that of a control.

2.6.3.2 α-glucosidase inhibition assay

For the α-glucosidase inhibition test, a modified version of the protocol described by Dada et al. (2017) was used. Plant extract (500 µL) was diluted with 100 µL of 0.1 M potassium phosphate buffer (pH 6.9) containing 1.0 U/mL of α-glucosidase solution and incubated at 25°C for 10 minutes in a 96-well plate. After the pre-incubation, 50 µL of a 5 mM solution of p-nitrophenyl-α-D-glucopyranoside in 0.1 M potassium phosphate buffer (pH 6.9) was added to each well. The reaction was conducted at 25°C for five minutes. The inhibition % of α-glucosidase activity was calculated for the plant extract and compared to the control.

2.6.3.3 Estimation of glycemic index

The glycemic index of the plant extract was determined following the procedure outlined by Brouns et al. (2005). For this analysis, the samples were extracted using 80% ethanol, and the glycemic index was evaluated according to the specified method.

2.7 Statistical analyses

The SPSS program was employed to determine the statistical analyses (SPSS Inc., Chicago, IL, USA). A T-test was performed to determine the differences between means. Each experiment was conducted in triplicate (n = 3). All parameters were subjected to cluster analysis using the MultiExperiment Viewer (MeV) TM4 software (Dana-Farber Cancer Institute, Boston, MA, USA), based on Pearson's distance metric.

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TABLE 1 Biochemical activities of JW0823 bacteria.

Biochemical activities	JW0823 bacteria
Nitrate reduction	–
Decomposition of:	
Hypoxanthine	-
Starch	-
Tween 80	-
Tyrosine	+
Xanthine	-
Acid production from:	
Arabinose	+
Fructose	-
D-Galactose	-
D-Glucose	-
Glycerol	-
Maltose	-
D-Mannitol	+
Sucrose	+
Growth at:	
pH	
Range	5-9
Optimum	8
0% NaCl	+
15% NaCl	+
20% NaCl	-
Temperature (°C):	
Range	15-55
Optimum	28-40

3 Results

3.1 Bacterial identification and its characterizations

The isolate was identified using biochemical and molecular methods, while 16S rDNA sequencing analysis revealed that strain JW0823 is 96.13% linked to the genus *Jeotgalicoccus*. The biochemical analysis demonstrated that the bacterial isolate JW0823 decomposed tyrosine and produced acid from arabinose, D-mannitol, and sucrose. The isolate has a broad growth range. It showed growth at pH levels ranging from 5 to 9, with an optimal pH of 8. The isolate could grow at 0% and 15% NaCl, with an optimal NaCl of 7-8%, and no growth above 20% NaCl. Furthermore, the isolate exhibited a growth temperature range of 15-55°C, with an optimal temperature of 28-40°C. The isolated JW0823 can produce IAA (Table 1). The phylogenetic tree (Figure 1) showed that strain MD36 is closely related to JW0823.

3.2 Bacterial colonization improved the biomass and photosynthesis of *E. sativa*

The effect of JW0823 colonization on the biomass, photosynthesis and pigment contents of *E. sativa* was studied (Figure 2). JW0823 -inoculated plants showed a significant increase in biomass production by about 78.2% in comparison with untreated control plants ($p<0.05$). Generally, plant growth is closely related to the efficiency of photosynthesis, so the pigment contents were estimated. It was noticeable that chlorophyll a, chlorophyll b, and chlorophyll a+b contents were enhanced in the treated plants by about 67.5%, 15%, and 47.4% respectively, and these elevations were significant ($p < 0.05$) in the case of chlorophyll a and chlorophyll a+b only. In addition, β-carotene and lycopene pigments exhibited significant increases of about 83.3% and 150% respectively ($p < 0.01$).

3.2 JW0823 induced the proximate composition, minerals, and vitamins of *E. sativa*

To assess the effect of mycorrhizal colonization on the proximate composition of *E. sativa*, we determined total protein, fat, crude fiber, ash, and carbohydrate contents in treated versus untreated plants (Figure 3). The proximate composition of the JW0823-treated plants showed significantly higher contents of

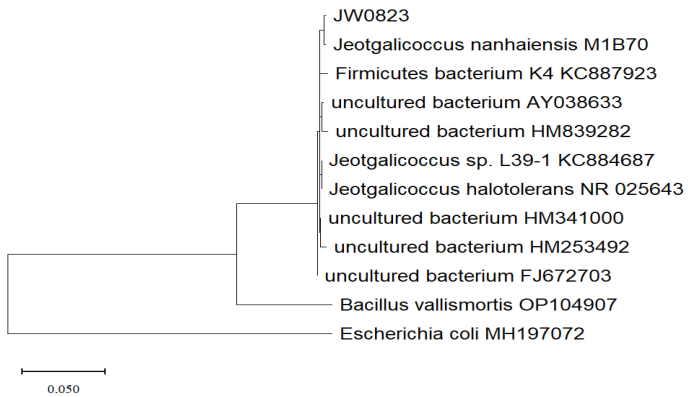

FIGURE 1
Phylogenetic tree based on 16S rRNA gene sequences, constructed by the Maximum of likelihood method using MegaX application, showing the position of strain JW0823 among closest species available at GenBank.

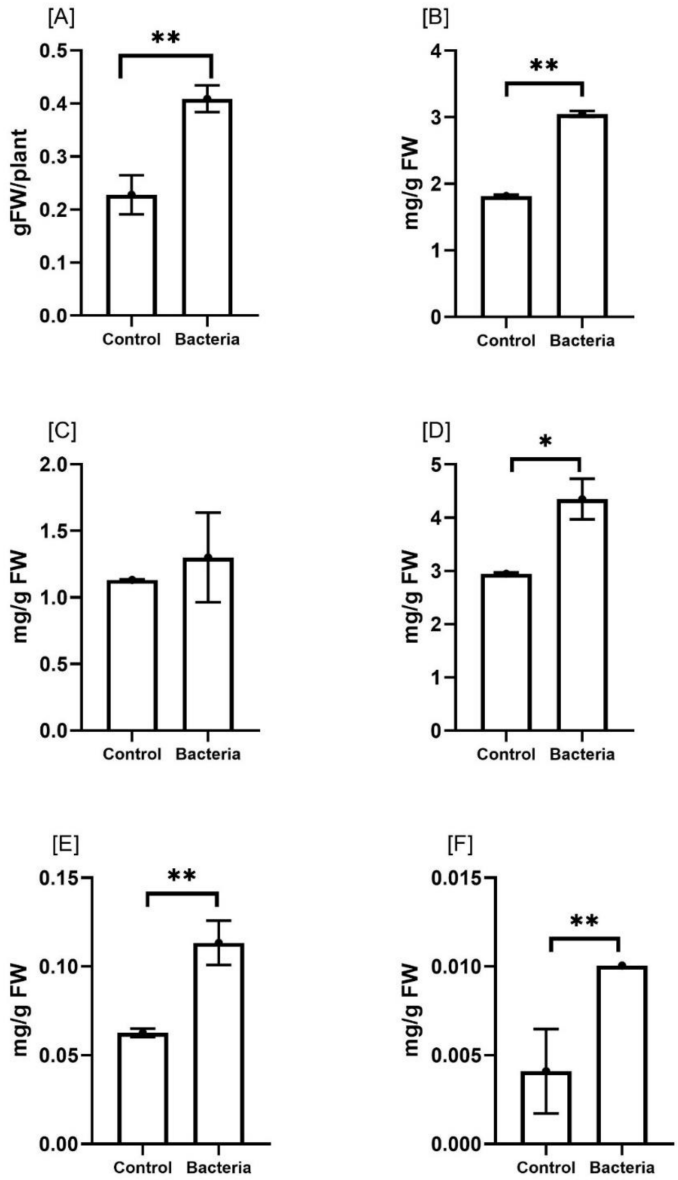

FIGURE 2
Effect of JW0823 bacteria on the biomass and pigment contents of *Eruca sativa* represented by **(A)** the biomass **(B)** chlorophyll a, **(C)** chlorophyll b, **(D)** chlorophyll a+b, **(E)** Beta carotene, and **(F)** lycopene. Data are represented by means ± standard errors. Bars flagged with 1, or 2 asterisks indicate significant differences between JW0823 bacteria-treated and control groups at p<0.05, or p<0.01 respectively.

total protein, crude fiber, ash, and carbohydrate by about 59.9%, 52.7%, 37.9% and 48%, respectively, compared to control samples ($p < 0.05$). On the other hand, moisture and fat percentages showed a slight decline in treated plants compared to untreated ones. Concerning mineral contents (Table 2), bacterial-treated plants displayed significantly higher contents ($p < 0.05$) in all measured minerals except Cu, Fe, and Mn (Table 3). The highest increases were recorded for K and Na by 119.1% and 100%, respectively. Both Zn and Mn contents exhibited an increase of 50% in treated plants compared to the untreated plants. About a 59.8% increase in Mg and 45.9% in phosphorus content was observed, whereas only a 5% increase in Fe content was recorded by the inoculated plants.

Regarding the impact of JW0823 on vitamin contents, the treated plants exhibited significantly higher levels of each examined vitamin (Figure 4A). Inoculated plants exhibited significantly higher levels of vitamin C, vitamin E, D-mannose, and L-galactose than untreated plants, with increases of approximately 23.9%, 46.11%, 161.5%, and 140.7%, respectively ($p < 0.05$).

3.3 JW0823 enhanced the functional food potentiality of *E. sativa*

To investigate the impact of JW0823 treatment on the functional food potential, nutritional quality, and bioactive metabolites of *E.*

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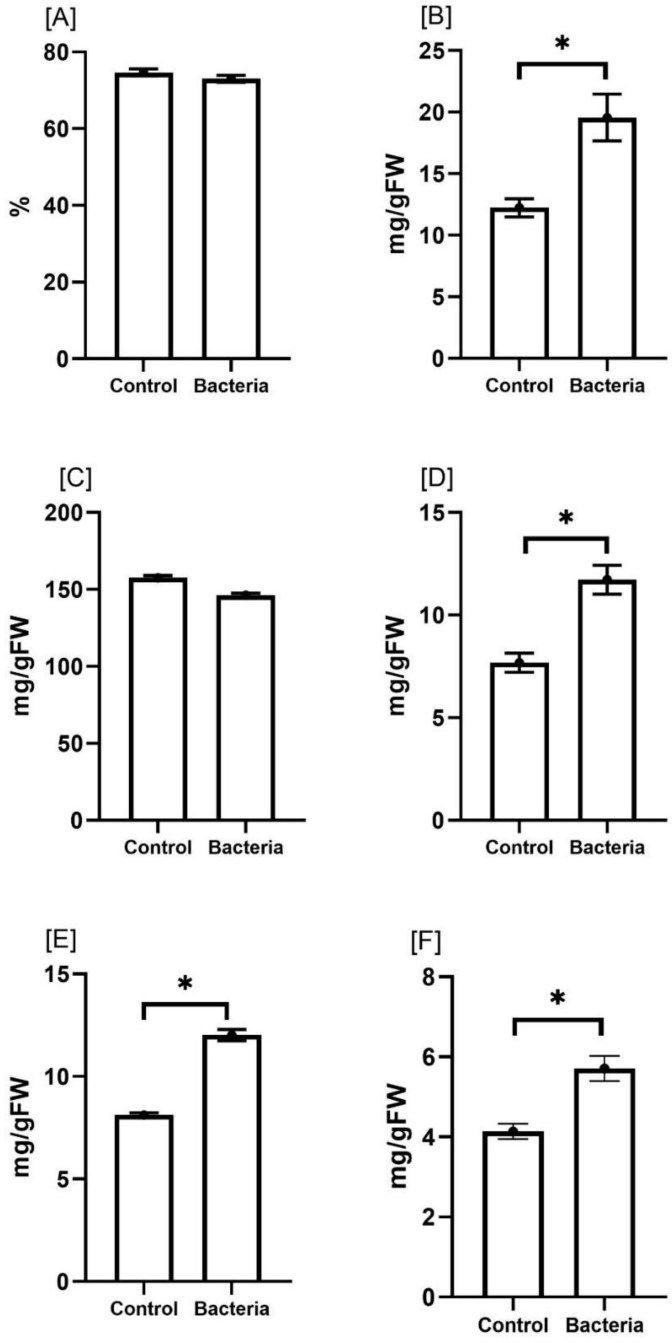

FIGURE 3
Effect of JW0823 bacteria on the proximate composition of *Eruca sativa* represented by **(A)** moisture, **(B)** total proteins, **(C)** total lipids, **(D)** crude fibers, **(E)** carbohydrates and **(F)** ash. Data are represented by means ± standard errors. Bars flagged with an asterisk indicate significant differences between JW0823 bacteria-treated and control groups at $p<0.05$.

sativa, we measured the levels of amino acids (Table 3), fatty acids (Table 4), organic acids (Figure 4B), and phenolics (Table 5) in treated and untreated plants. Nine out of twenty-two examined amino acids in the treated plants were significantly increased when inoculated with JW0823 compared to control ones ($p<0.05$). However, some amino acids decreased by JW0823 treatment, such as aspartic acid, glycine, alanine, histidine, valine, and threonine. Isoleucine, cystathionine γ-synthase (CGS), and methionine showed the highest increments of 270.2%, 200%, and 153.5%, respectively ($p<0.05$).

The five estimated organic acids recorded increases in the treated plants, with malic acid exhibiting the highest increase of 287.2% and oxalic acid the lowest increase of 2.3% (Figure 4B). Similarly, five out of sixteen detected fatty acids in the target plant were significantly increased when inoculated with JW0823 compared to control plants, whereas some fatty acids showed a decrease. Pentacosanoic acid (C24:0), octadecanoic acid (C18:1), and octadecanoic acid (C18:0) recorded increases in treated plants by 87.5%, 54.5%, and 43.4%, respectively ($p<0.05$).

TABLE 2 Effect of JW0823 bacteria on the levels of minerals in *Eruca sativa*.

Minerals (mg/g)	Control	JW0823 bacteria
K	11.474 ± 0.047	25.228 ± 0.55*
Na	1.561 ± 0.021	3.213 ± 0.02*
Ca	1.485 ± 0.177	1.872 ± 0.258*
Cu	0.008 ± 0	0.009 ± 0.002*
Fe	0.18 ± 0.015	0.172 ± 0.029
P	1.108 ± 0.088	1.625 ± 0.02*
Zn	0.1 ± 0.007	0.147 ± 0.006*
Mn	0.024 ± 0.001	0.034 ± 0
Mg	2.588 ± 0.263	4.143 ± 0.385*

Data are represented by means ± standard errors. Means with an asterisk indicate significant differences between treated and control groups at p<0.05.

Additionally, all detected phenolic metabolites exhibited significant increases in JW0823-treated plants except protocatechuic acid, which showed a decrease of 14.8%. Genistein and luteolin recorded the highest increases by 50% and 121.7%, respectively, followed by fisetin and o-hydroxydaidzein, which exhibited an increase of 50% each in the inoculated plants compared to untreated plants (p<0.05).

3.4 JW0823 boosted the biological activities of *E. sativa*

3.4.1 Antioxidant activity

To evaluate how JW0823 treatment affects the overall antioxidant activity of *E. sativa*, we analyzed the levels of total phenolics, total flavonoids, FRAP, ABTS, and DPPH in both treated and untreated plants (Figure 5). The plants treated with JW0823 exhibited significant increases in antioxidant activity, with total phenolics, total flavonoids, FRAP, ABTS, and DPPH levels increasing by approximately 48.2%, 56.9%, 46.5%, 13.2%, and 50.5%, respectively, compared to the untreated controls (p<0.05). These improvements in antioxidant activity were associated with higher phenolic content increased by JW0823 inoculation.

3.4.2 Antimicrobial activity

Furthermore, both inoculated and non-inoculated plant extracts demonstrated antimicrobial activity against various bacterial and fungal species. The most pronounced effects were observed in extracts from inoculated plants, particularly against *Aspergillus flavus*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*, as indicated by the inhibition zone diameters (Figure 6). The results showed that JW0823 inoculation led to significant enhancements in antimicrobial activity of *E. sativa* against *Staphylococcus saprophyticus, Staphylococcus epidermidis, Streptococcus salivarius, Salmonella typhimurium, Enterobacter aerogenes, Serratia marcescens*, and *Aspergillus flavus*, with increases of approximately 27.1%, 19.9%, 18.1%, 13.5%, 31.7%, 165%, and 37.3%, respectively (p<0.05) (Figure 6).

TABLE 3 Effect of JW0823 bacteria on amino acid contents in *Eruca sativa*.

Amino acids (mg/g)	Control	JW0823 bacteria
Aspartic acid	13.55 ± 0.5	6.54 ± 1.38*
Glutamic acid	12.75 ± 0.81	16.57 ± 0.41*
Glutamine	11.79 ± 0.6	14.53 ± 0.46*
Serine	6.01 ± 0.09	8.78 ± 0.86*
Glycine	11.44 ± 1.08	8.01 ± 0.23*
Arginine	21.68 ± 3.4	31.61 ± 9.47*
Alanine	3.49 ± 0.18	2.97 ± 0.22
Histidine	5.79 ± 0.15	2.03 ± 1.33*
Valine	9.07 ± 0.21	2.09 ± 1.47*
Methionine	1.14 ± 0.48	2.89 ± 0.04*
Cystine	1.53 ± 0.02	1.59 ± 0.15
Isoleucine	2.42 ± 1.08	8.96 ± 2.16*
Leucine	14.78 ± 1.05	10.27 ± 1.39*
Tyrosine	6.48 ± 0.15	4.69 ± 0.33*
Lysine	17.32 ± 1.23	14.28 ± 0.15*
Threonine	6.58 ± 0.57	3.01 ± 0.33*
Tryptophan	0.55 ± 0.02	0.39 ± 0
Glutamine synthase	4.91 ± 0.28	6.22 ± 0.01
Dihydrodipicolinate synthase (DHDPS) (lysine biosynthase)	1.94 ± 0.11	3.29 ± 0.1*
Cystathionine γ-synthase (CGS) (methionine biosynthase)	0.01 ± 0	0.03 ± 0
Phenylalanine	22.87 ± 2.47	33.87 ± 3.77*
Phenylalanine aminolyase	4.2 ± 0.37	7.01 ± 0.92*

Data are represented by means ± standard errors. Means with an asterisk indicate significant differences between treated and control groups at p<0.05.

3.4.3 Antidiabetic activity

Additionally, plants treated with the bacteria showed notable increases in antidiabetic activities, with α-amylase inhibition and α-glucosidase inhibition rising by approximately 24.7% and 49.4%, respectively (p<0.05). In contrast, there was a modest reduction in the glycemic index, decreasing by about 6.9% (Figure 7).

4 Discussion

4.1 JW0823 could improve growth and tissue chemical composition of *E. sativa*

PGPB can colonize plant roots, improving plant growth (Beneduzi et al., 2012). Our research findings revealed that *E. sativa* plants inoculated with JW0823 showed improved growth compared to non-inoculated plants. In line with our results, previous studies

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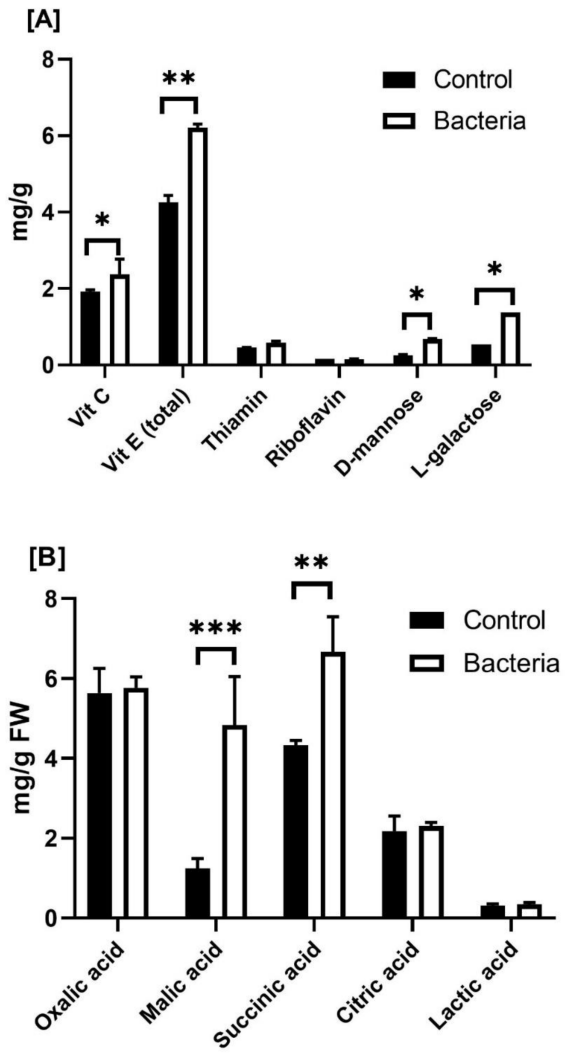

FIGURE 4
Effect of JW0823 bacteria on the levels of **(A)** vitamins and **(B)** organic acids in *Eruca sativa*. Data are represented by means ± standard errors. Bars flagged with 1, 2, or 3 asterisks indicate significant differences between JW0823 bacteria-treated and control groups at p<0.05, p<0.01, or p<0.001, respectively.

TABLE 4 Effect of JW0823 bacteria on fatty acids (%) in *Eruca sativa*.

Fatty acids (%)	Control	JW0823 bacteria
Tetradecanoic (C14:0)	1.29 ± 0.14	0.78 ± 0.2
Pentadecanoic (C16:0)	13.7 ± 2.53	16.87 ± 1.71*
Eicosanoic (C20:0)	1.93 ± 0.1	1.81 ± 0.33
Docosanoic (C22:0)	1.66 ± 0.25	1.43 ± 0.06
Octadecanoic (C18:0)	7.98 ± 0.68	11.45 ± 1.83*
Pentacosanoic (C24:0)	0.08 ± 0.03	0.15 ± 0.03
Total saturated fatty acids	26.64 ± 2.49	32.48 ± 2.34*
Pentadecanoic (C16:1)	2.26 ± 0.23	2.98 ± 0.62
Pentadecanoic (C16:1)	0.6 ± 0.15	1.29 ± 0.16*
Pentadecanoic (C16:3)	2.45 ± 0.27	1.36 ± 0.11*
Octadecanoic (C18:1)	3.85 ± 0.59	5.95 ± 0.46*
Octadecanoic (C18:2)	31.67 ± 6.57	23.82 ± 0.81*
Heptadecanoic (C18:3)	34.75 ± 8.76	34.4 ± 7.56
Heptadecanoic (C18:4)	2 ± 0.46	1.29 ± 0.16*
Tetracosanoic (C20:3)	0.45 ± 0.18	0.36 ± 0.11
Total fatty acids	78.03 ± 15.49	71.47 ± 7

(Continued)

TABLE 4 Continued

Data are represented by means ± standard errors. Means with an asterisk indicate significant differences between treated and control groups at p<0.05.

TABLE 5 Effect of JW0823 bacteria on the concentrations of phenolics in *Eruca sativa*.

Phenolics (mg/g FW)	Control	JW0823 bacteria
Total Phenolics	569.536 ± 5.283	1065.631 ± 29.584*
Total Flavonoids	60.662 ± 3.444	116.995 ± 2.954*
Caffeic acid	0.015 ± 0.001	0.026 ± 0
Ferulic acid	1.583 ± 0.025	2.429 ± 0.033*
Protocatechuic acid	0.384 ± 0.137	0.327 ± 0.003
Catechin	0.473 ± 0.019	0.77 ± 0.019
Galic acid	11.959 ± 0.537	18.911 ± 0.432*
p-Coumaric acid	2.244 ± 0.142	4.249 ± 0.072*
Cinnamic acid	2.564 ± 0.123	4.18 ± 0.088*
Resorcinol	0.052 ± 0.008	0.071 ± 0.001
Chlorogenic acid	0.153 ± 0.007	0.241 ± 0.005*
Syringic acid	0.693 ± 0.016	1.523 ± 0.033*
Quercetin	1.406 ± 0.132	2.499 ± 0.052*
Quercetrin	0.135 ± 0	0.268 ± 0.011*
Luteolin	0.046 ± 0.004	0.102 ± 0.003*
Apigenin	0.293 ± 0.03	0.577 ± 0.018*
Isoquercetrin	0.63 ± 0.085	1.264 ± 0.022*
Rutin	1.005 ± 0.102	2.008 ± 0.059*
Ellagic acid	0.258 ± 0.023	0.403 ± 0.009*
Velutin	0.272 ± 0.013	0.454 ± 0.008*
Naringenin	0.004 ± 0	0.007 ± 0
Genistein	0.002 ± 0	0.003 ± 0
Daidzein	0.001 ± 0	0.003 ± 0
Fisetin	0.002 ± 0	0.003 ± 0
O-hydroxydaidzein	0.002 ± 0	0.003 ± 0

Data are represented by means ± standard errors. Means with an asterisk indicate significant differences between treated and control groups at p<0.05.

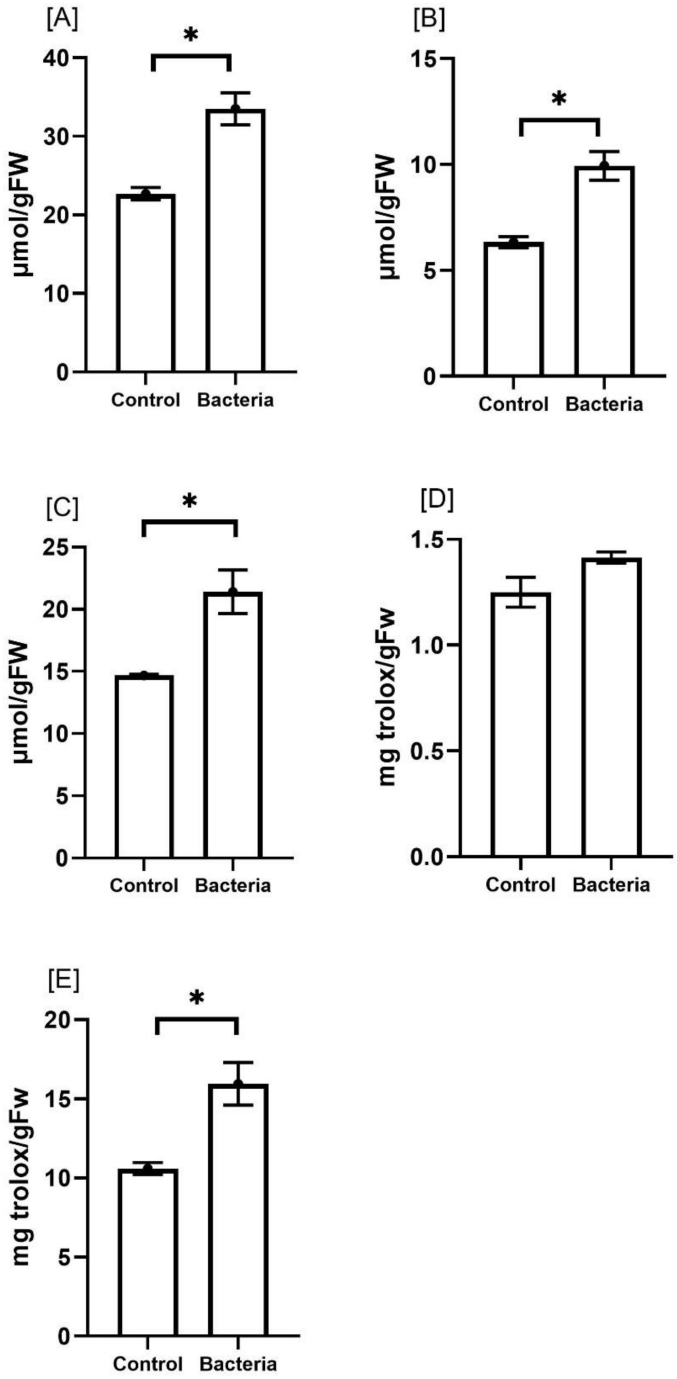

FIGURE 5
Effect of JW0823 bacteria on the total antioxidant activities of *Eruca sativa*: **(A)** total phenolics, **(B)** total flavonoids, **(C)** FRAP, **(D)** ABTS and **(E)** DPPH. Data are represented by means ± standard errors. Bars flagged with an asterisk indicate significant differences between JW0823 bacteria-treated and control groups at $p<0.05$.

highlighting positive effect of PGPB (e.g., *Jeotgalicoccus* sp.) such as enhanced yield and nutrient content (Saleh et al., 2010; Chavoshi et al., 2018; Misra et al., 2019). *Jeotgalicoccus* sp. can stimulate plant growth and yield (Gong et al., 2016; Misra et al., 2019; Singh et al., 2019; Li et al., 2022). In this context, *J. huakuii* NBRI 13E has been used in bioinoculant formulations to boost crop yield and quality (Misra et al., 2019). This positive effect of PGBP can be explain by their ability to induce the production of growth hormones (e.g., IAA) (Egamberdiyeva and Höflich, 2004), solubilization of phosphate and mineralization of nutrients (Jeon et al., 2003; Glick, 1995). In this regard, *J. halotolerans* was capable of producing growth-promoting hormones auxin (Mukasheva et al., 2016). IAA is well-documented for their role in enhancing plant growth by promoting cell elongation and division (Mishra et al., 2022).

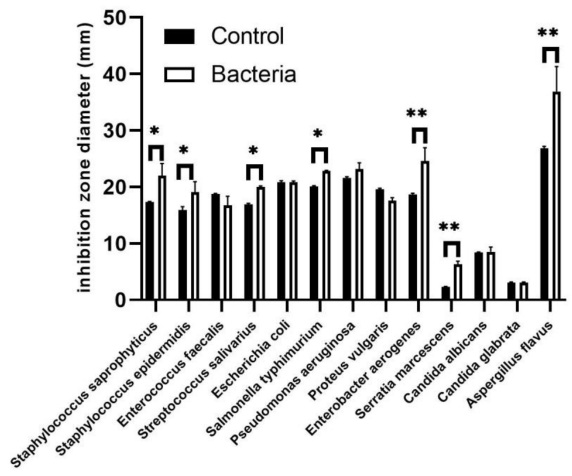

FIGURE 6
Effect of JW0823 bacteria on the antimicrobial activities of *Eruca sativa*. Data are represented by means ± standard errors. Bars flagged with 1 or 2 asterisks indicate significant differences between JW0823 bacteria-treated and control groups at $p<0.05$ or $p<0.01$, respectively.

The growth improvement can also be explained by the bacteria's ability to enhance nutrient uptake. The potential of bioinoculants as valuable tools in sustainable agriculture, offering benefits through enhanced nutrient uptake. The application of JW0823 enhanced nutritional accumulation in *E. sativa*. Previous studies highlight the role of PGBP in enriching plants with macro- and micronutrients (Yaghoubi Khanghahi et al., 2018; Meena et al., 2017; Ipek, 2019; Kumar et al., 2022; Helaly, 2017) and increasing soil mineralization (Shen et al., 2004; Esitken et al., 2010). For instance, *Jeotgalicoccus* sp. *BAK1* exhibits both growth-promoting and phosphorus-solubilizing properties (Mukasheva et al., 2016). Similar to *J. halotolerans*, other PGBP such as *Pseudomonas* and *Bacilli* are effective microorganisms in the solubilization of phosphate. In this context, Goswami et al. (2016) demonstrated increases in organic acid secretion, including mineral solubilization in soil (Tahjib-Ul-Arif et al., 2021).

Pervious research also demonstrated that PGBP can stabilize CO_2 levels, regulate stomatal conductance, and boost photosystem II efficiency (Esitken et al., 2010; Orhan et al., 2006; Chen et al., 2016). For example, *J. huakuii* improved maize growth through increasing chlorophyll content (Misra et al., 2019). Applying JW0823 boosts photosynthesis and soluble sugar production, which is essential for synthesizing primary (essential oils, unsaturated fatty acids) and secondary metabolites (polyphenolics) in plants (Misra et al., 2019). Our results also should that inoculated plants had higher protein, carbohydrate, and lipid contents compared to non-inoculated plants. In line with our results, PGBP boosted carbohydrate metabolism in *E. sativa* (Wang et al., 2022b; Ilyas et al., 2020, and Upadhyay and Singh, 2015) and protein and carbohydrate content in bean seeds (Stefan et al., 2013). It is noteworthy that carbohydrates not only support photosystem II but also serve as energy sources for maintain growth (Sami et al., 2016). The observed increase in protein and amino acid levels may be attributed to the ability of PGPB to enhance the uptake of NH_4 and NO_3, which are then converted into free amino acids (Johansen et al., 1996; Kumar et al., 2015).

PGPB-induced production of bioactive metabolites contributes to the synthesis of secondary plant metabolites (Zhou et al., 2021). For example, high phenolic and flavonoid contents have been reported in buckwheat plants inoculated with PGBP (Briatia et al., 2018). *J. huakuii* has also been shown to boost maize growth by producing bioactive compounds such as antioxidant phenolics and proline (Misra et al., 2019). When compared to the control, *Jeotgalicoccus* sp. increased the phenol and flavonoid content in cluster beans (Upadhyay and Singh, 2015), suggesting improved antioxidant power (Sarkar et al., 2021). On the other hand, phenolic compounds accelerate the symbiotic relationship between plants and microorganisms (Mandal et al., 2010). They can also facilitate oxygenation reactions, and thus, beneficial bacteria may help inhibit oxidizing enzyme activity (Notununu et al., 2022).

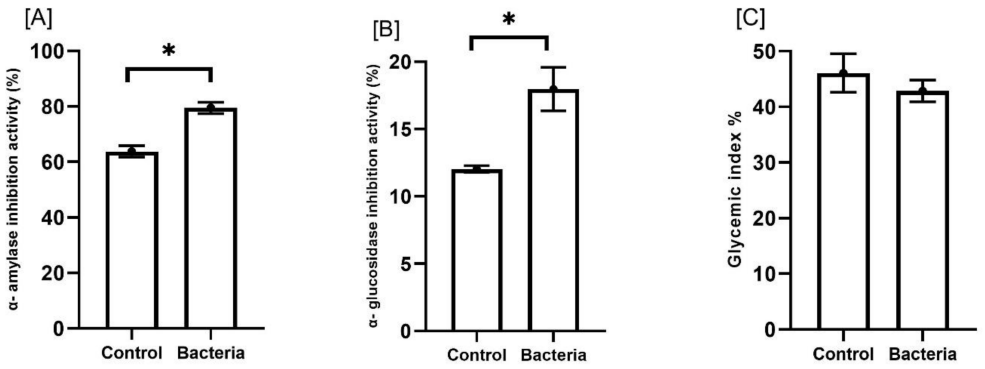

FIGURE 7
Effect of JW0823 bacteria on the antidiabetic activities of *Eruca sativa* sprouts represented by **(A)** α- amylase inhibition activity (%), **(B)** α- glucosidase inhibition activity (%), and **(C)** glycemic index %. Data are represented by means ± standard errors. Bars flagged with an asterisk indicate significant differences between JW0823 bacteria-treated and control groups at $p<0.05$.

4.2 JW0823 improved the biological activity of *E. sativa*

The higher bioactive metabolite content in *E. sativa* boosts its biological value, as it is rich in essential amino acids and polyunsaturated fatty acids, which are crucial nutrients (De Carvalho and Caramujo, 2018). JW0823 has been observed to significantly enhance the biological activity of plants and facilitate the exchange of nutrients, leading to the accumulation of bioactive metabolites. For instance, increased antioxidant content in *E. sativa* was accompanied by substantial enhancements in antioxidant activities (FRAP). *E. sativa* been reported to exhibit high total antioxidant capacities, as well as significant scavenging activity against ROS (Sarikurkcu et al., 2017). In agreement, plants exhibited high antioxidant capacities when treated with PGPB (Chen et al., 2014). Khoobchandani et al. (2010) also investigated the antimicrobial potential of various solvent extracts of *E. sativa* and seed oil against gram-positive (*Staphylococcus aureus* ATCC 6538 and *B. subtilis* MTCC 441) and gram-negative (*E. coli* ATCC 14169, *P. aeruginosa* MTCC 424, and *S. fexneri* MTCC 1457) bacteria that are resistant to antibiotics. They have also been connected to antifungal (Manici et al., 1997) and antinematode (Lazzeri et al., 1993) properties (Warton et al., 2001). However, the research on their antiseptic qualities is limited (Abdou et al., 1972; Hashem and Saleh, 1999). Overall, anti-diabetic, antibacterial and antioxidant properties of *E. sativa*, coupled with its nutritional value, make it a beneficial component of a balanced diet aimed at promoting wellness and preventing chronic diseases.

5 Conclusions

Inoculation with JW0823 notably boosted the growth and nutritional quality of *E. sativa*, enhancing its proximate composition, vitamin content, and bioactive metabolites. The treated plants also exhibited increased antioxidant, antidiabetic, and antimicrobial activities. Building on these promising results, future research should explore the broader application of JW0823 in various crop species and agricultural settings to confirm its versatility and efficacy. Further studies could also investigate the long-term effects of JW0823 on plant health and soil sustainability. Additionally, understanding the underlying mechanisms by which JW0823 enhances metabolite production and stress resilience could lead to optimized bioinoculant formulations and targeted application strategies. assessing the practical benefits and scalability of this approach in sustainable agriculture.

Data availability statement

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

Author contributions

EA, HA, AH: Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. AA, SK, NB: Investigation, Data curation, Software, Methodology, Resources, Writing – review & editing. AA, SK: Data curation, Formal analysis, Project administration, Visualization, Validation and Supervision, Writing – review & editing. AR, NB, MA, MM: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Funding acquisition, Validation, Writing – review & editing.

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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

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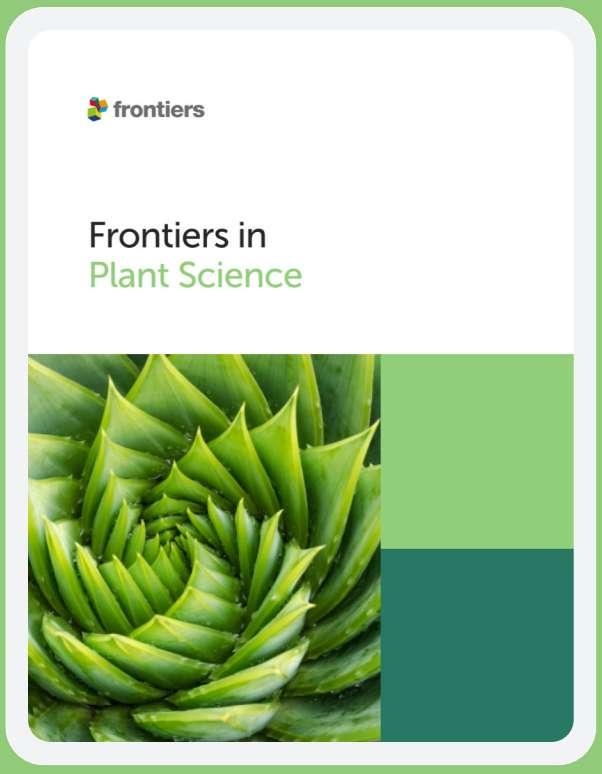

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