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Plant-microbes interactions and resistance against abiotic stress

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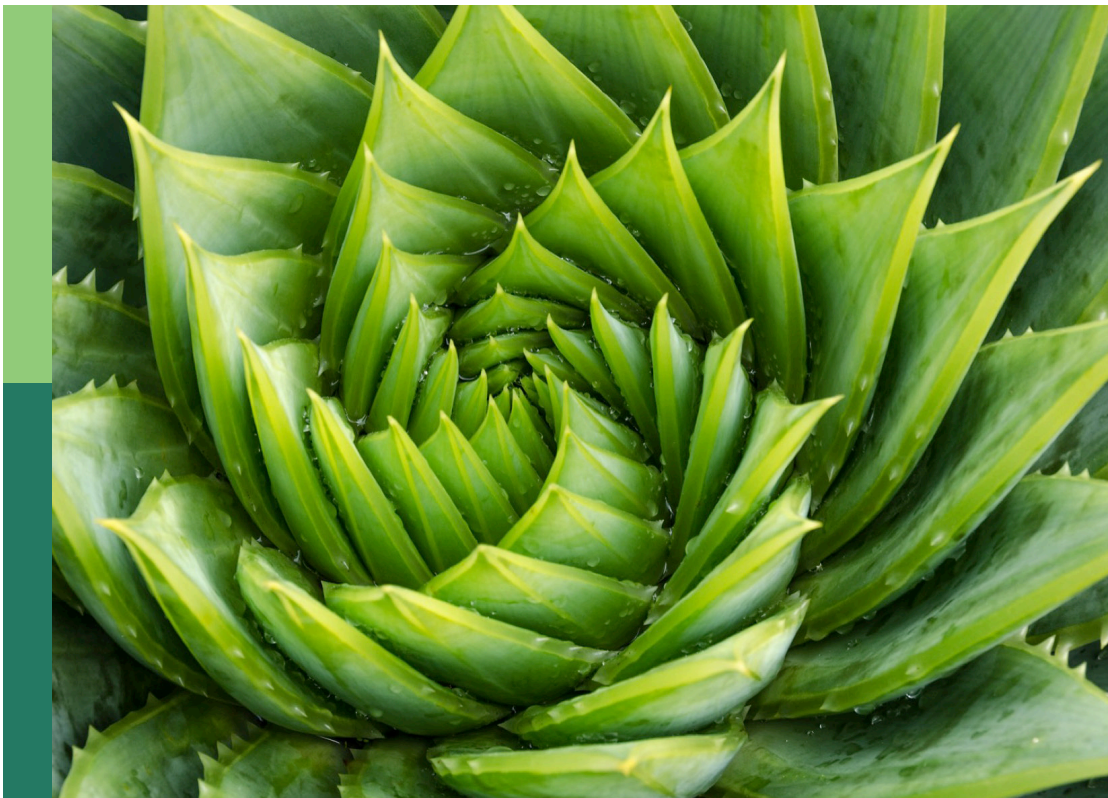

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Plant-microbes interactions and resistance against abiotic stress

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Editorial: Plant-microbes interactions and resistance against abiotic stress

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KEYWORDS

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Editorial on the Research Topic
Plant-microbes interactions and resistance against abiotic stress

This Research Topic was published in late 2023. It attracted 13 papers written by 92 authors, mainly achieving our goal of providing insight into the mechanisms of plant-microbe associations and the mediation of microbes in plant tolerance to abiotic stresses. This Research Topic includes a combination of Reviews and Original Research articles covering molecular, cellular, applied, and ecological aspects of plant interactions with symbiotic microbes (rhizobia and arbuscular mycorrhizal fungi), beneficial endophytes, or plant growth-promoting (PGP) bacteria and also the role of microbes in plant resistance to various abiotic stress conditions (nutrient deficiency, osmotic stress, drought, and cold) in both the rhizosphere and phyllosphere.

Plant-microbe interactions

Plants interact with a wide range of microbes that can modulate various aspects of plant growth and development. The review by Yang et al. provides insight into root colonization by microbes and the research gaps regarding the molecular mechanisms of plant-microbe interactions at different stages of root colonization. The Authors summarized and discussed chemotactic signals emitted by plant roots, signal reception, bacterial attachment to the root surface, bacterial immune evasion, biofilm formation and stable colonization.

Yuan et al. investigated the dynamics of the phyllosphere microbiome of pomelo (*Citrus maxima*) under changing weather parameters. The authors used Hi-Seq analysis and showed that both bacterial and fungal communities exhibited annual cycle dynamics. In another paper, Zhao et al. demonstrated how different preceding crops enhance tobacco plant growth and influence the microbial diversity and nutrient content of the rhizosphere. Preceding crops such as canola, wheat, and maize significantly increased available phosphorus, potassium, boron, and zinc in the tobacco rhizosphere. Additionally, both canola and wheat enhanced soil bacterial diversity, while wheat cultivation had the most significant impact on rhizosphere metabolite content.

The review by Verma et al. summarized the functional and mechanistic basis for the interactive role of PGP bacteria and large-scale application of nanoparticles (NPs). NPs have typically been used to enhance soil quality, while PGP bacteria have been used to stimulate plant growth and increase resistance to biotic and abiotic stresses (Kumar et al., 2017; Upadhayay et al., 2023). The Authors summarized the synergistic interactions between NPs and PGP bacteria in enhancing the soil-plant system. They presented how the negative effects of environmental factors on soil health and plant growth can be reduced by the significant potential of cooperation between PGP bacteria and NPs.

In another study, Yang et al. investigated the role of the *GmWRKY33a* gene in soybean, which is associated with brassinosteroid (BR) signaling and nodulation during symbiosis with rhizobia. BRs are phytohormones that regulate a wide range of plant developmental processes and plant interactions with different microbes (Chen et al., 2023). Notably, the Authors showed that *GmWRKY33a* is a crucial transcription factor in BR signaling and plays a negative role in nodule formation and symbiosis establishment.

Increasing tolerance to abiotic stresses

Abiotic stress factors have unfavorable effects on plant growth and yield. Stress tolerance in plants can be enhanced by treating plants or seeds with selected microbes or synthetic compounds (Rai et al., 2021). Plant-associated microbes have the ability to alleviate stress by activating physiological, biochemical, and molecular pathways that coordinate ion uptake, nutrient metabolism, and the synthesis of compounds with osmotic or antioxidant activity (Liu et al., 2020).

Considerable interest has been shown to symbiotic fungi and PGP bacteria involved in increasing stress tolerance in plants exposed to different stressors (Kumar et al., 2017; Liu et al., 2020; Oleńska et al., 2020; Sujkowska-Rybkowska et al., 2023; Upadhayay et al., 2023). Five contributions addressed drought, which is one of the leading causes of crop losses worldwide. The mitigation of drought stress by fungi was explored in three articles. The symbiosis of desert plants (*Populus euphratica* and *Haloxylon ammodendron*) with arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) was analyzed in the article by Wang et al. DSE are a group of endophytic fungi that can facilitate plant growth and stress tolerance, especially in harsh ecosystems (Li et al., 2018). The Authors showed that DSE are more dominant in extreme drought environments. Moreover, they demonstrated that AMF are more susceptible to soil factors (such as soil moisture and nutrient content), whereas DSE are more affected by pH. The article by Xu et al. focused on the enhancement of drought resistance in *Pinus tabuliformis* through ectomycorrhizal fungi and DSE application. They showed that these symbiotic fungi were able to mitigate drought stress in plants through the activation of the antioxidant system and the regulation of the osmotic balance in plant seedlings. The study by Wu et al. evaluated the impact of AMF (*Claroideoglomus etunicatum*) on tea plants under drought conditions. They showed that AMF improved tea plant adaptation to drought by enhancing the expression of N assimilation-related genes and activating related enzymes. Two studies explored the mitigation of drought stress by PGP bacteria. In another study, Kim et al. examined a novel exopolysaccharide-producing PGP bacterium, *Pseudescherichia liriopis* sp. nov. isolated from *Liriope platyphylla*, in alleviating drought and salt stress in carrot. The authors also characterized the exopolysaccharide produced by *P. liriopis*, which was shown to be an excellent antioxidant with potential practical application. The study by Park et al. investigated the drought-mitigating ability of PGP *Bacillus velezensis* GH1-13 in rice. Bacillus spp. are known for their potential and usefulness in mitigating the effects of various stresses in plants (Kumar et al., 2017). They showed that *Bacillus* enhances drought stress tolerance in rice by activating the expression of antioxidant genes and suppressing reactive oxygen species levels.

The article by Li et al. focused on cold stress and the effect of the endophytic fungus *Piriformospora indica* on the cold resistance of tobacco plants. In particular, they showed that at low temperatures, this fungus enhances the activity of the host plant's antioxidant systems, thereby reducing oxidative stress. Furthermore, the fungus stimulates the accumulation of protective osmolytes and activates the expression of cold-responsive genes.

Finally, the study by Abd El-Daim et al. demonstrated the ability of a novel halotolerant bacterial endophyte, *Bacillus velezensis* CBE, to enhance osmotic stress tolerance in *Brachypodium distachyon* under nitrogen-deprived conditions. Moreover, they identified the molecular factors in plants that contribute to the beneficial effects of *B. velezensis* CBE in *B. distachyon* plants.

The diverse topics of the papers published in this Research Topic reflect the complexity of the interactions between plants and microorganisms in changing environments. Knowledge of the determining factors and mechanisms that regulate plant-microorganism interactions can help develop new agrobiotechnological strategies to improve plant biomass production in sustainable agriculture, as well as in soil remediation processes.

Author contributions

MS-R: Writing – original draft, Writing – review & editing, Conceptualization. AR: Writing – review & editing.

Conflict of interest

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Synergistic interactions of nanoparticles and plant growth promoting rhizobacteria enhancing soil-plant systems: a multigenerational perspective

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Sustainable food security and safety are major concerns on a global scale, especially in developed nations. Adverse agroclimatic conditions affect the largest agricultural-producing areas, which reduces the production of crops. Achieving sustainable food safety is challenging because of several factors, such as soil flooding/waterlogging, ultraviolet (UV) rays, acidic/sodic soil, hazardous ions, low and high temperatures, and nutritional imbalances. Plant growth-promoting rhizobacteria (PGPR) are widely employed in *in-vitro* conditions because they are widely recognized as a more environmentally and sustainably friendly approach to increasing crop yield in contaminated and fertile soil. Conversely, the use of nanoparticles (NPs) as an amendment in the soil has recently been proposed as an economical way to enhance the texture of the soil and improving agricultural yields. Nowadays, various research experiments have combined or individually applied with the PGPR and NPs for balancing soil elements and crop yield in response to control and adverse situations, with the expectation that both additives might perform well together. According to several research findings, interactive applications significantly increase sustainable crop yields more than PGPR or NPs alone. The present review summarized the functional and mechanistic basis of the interactive role of PGPR and NPs. However, this article focused on the potential of the research direction to realize the possible interaction of PGPR and NPs at a large scale in the upcoming years.

KEYWORDS

agro-ecological responses, food security, plant-microbiome, soil amendment, NPs, PGPR

Introduction

Recently, soil amendments have been implemented in agroecosystems to promote plant growth and development, especially by adding organic and inorganic nutrients. Adding specific substances to the soil enhances its capacity to sustain plant life (Urra et al., 2019; Rubin et al., 2023; Kumari et al., 2023b). Plant growth-promoting rhizobacteria (PGPR) and nanomaterials (NMs) or nanoparticles (NPs) are under consideration as a novel approach to boost crop productivity and soil fertility (El-Ramady et al., 2022; Upadhayay et al., 2023; Rajput et al., 2023a; Kumari et al., 2024). Several research studies have demonstrated numerous benefits that soil organic amendments may provide, including better soil texture, higher soil fertility, restored soil health, and, ultimately, higher crop yield (Urra et al., 2019; Rani et al., 2023).

Because of the serious risk factors to human health caused by antibiotic-resistant bacteria, antibiotic residues, and antibiotic-resistant genes that exist in agricultural organic amendments, such contaminants in emerging pollutants are still a major problem (Upadhayay et al., 2023). Soil supplements should have attributes including ecological safeguards and negatively affect the fertility of the soil, soil composition, or the enviro-ecosystem (Garbowski et al., 2022; Rajput et al., 2023a). Owing to their unique properties, PGPRs, and NPs have attracted more interest in recent years as potential soil amendments that can mitigate the risk associated with other soil additions under normal and adverse conditions (Chen et al., 2021; Tolisano and Del Buono, 2023; Kumari et al., 2024).

Most PGPRs have been reported and confirmed to improve plant productivity by reducing environmental challenges (Singh et al., 2021; Kumari et al., 2023a). Through both direct and indirect strategies, it may enhance the overall quality of soil and plant yield (Figure 1) (Sharma et al., 2023; Rajput et al., 2023a). The functions of PGPR occur through direct mechanisms such as nitrogen fixation, phosphate, and potassium solubilization, and the production of growth-promoting phytohormones like indole acetic acid (IAA) and siderophores. However, indirect mechanisms are associated with the production of lytic enzymes and antibiotics, dropping the soil pH and producing exopolysaccharides. Several studies have assessed the efficacy of PGPR for maintaining a sustainable agroecosystem in normal and stressful conditions (Oleńska et al., 2020; Magnabosco et al., 2023; Timofeeva et al., 2023). Numerous articles and meta-analyses have observed the beneficial impacts of NPs on soil health and agronomic productivity as well as the variables that facilitate the ameliorative role of NPs (Urra et al., 2019; Sharma et al., 2020; Verma et al., 2022a, b). The NPs have also alleviated various environmental stresses during plant development (Dilnawaz et al., 2023; Pramanik et al., 2023; Verma et al., 2023a).

Sustainable food security and safety have become extremely challenging in the 21st century, particularly in developing nations with limited resources. The teeming millions in the developing world associated with the era of climate change threaten agricultural crop production and management, a serious challenge to sustainable agriculture (Fanzo et al., 2017; Watts et al., 2018). According to the Food and Agriculture Organization of the United Nations, over 2 billion people do not have enough food to eat due to the COVID-19 pandemic. Agriculture systems and food have already experienced significant modifications, but additional research has to be done in light of the transforming global landscape (Frona and Szenderák 2018; Kakaei et al., 2022). Land reforms, modified water management, stress-tolerant cultivars, increased fertilizer use, better seed, pesticide use, genetically modified crops, plant growth regulators, and soil amendments are some of the approaches used to improve soil quality and crop yields (Urra et al., 2019; Abdul Aziz et al., 2022; Verma et al., 2022c; Akanmu et al., 2023).

Crop yield downregulated because of various environmental stresses (Verma et al., 2020a; Kumari et al., 2022; Patni et al., 2022;

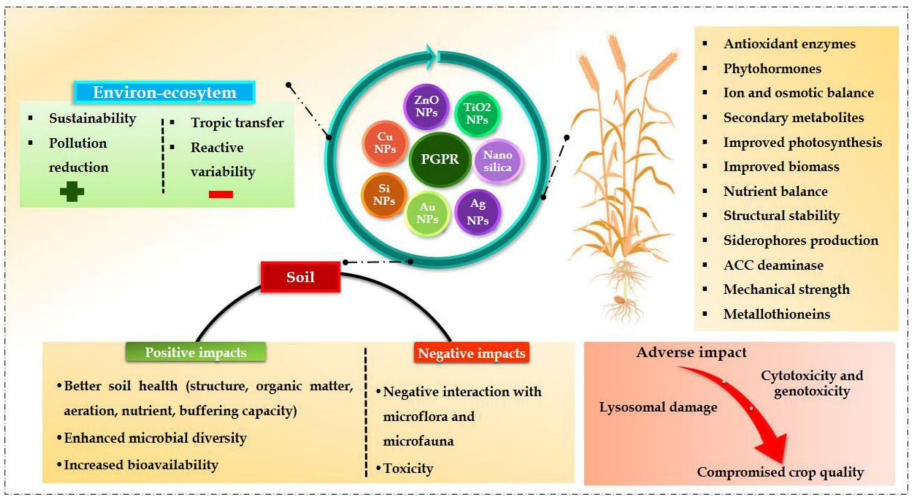

FIGURE 1
An overview of the interactive effects of NPs and PGPRs on plant, soil and enviro-ecosystems.

Verma et al., 2023b). Nearly 6% of the world's total surface area (1125 mha), impacted by salinity; it includes 20% of agricultural land and 33% of irrigated land. The loss of productivity from saline soil can exceed 46 mha annually (Hossain, 2019; Negacz et al., 2022). Annually, 1.5 mha of cropland is lost to saltwater due to soil erosion. Production of crops and animals requires more water as agriculture worldwide, provides 70% of total water returns. Water deficit is a major abiotic stressor prevalent in sub-tropical and tropical regions around the world (Kumari et al., 2022). Furthermore, droughts are becoming more severe due to the era of climate change. Water for agriculture has the potential to become more in requirement globally by 60% upto 2025. The development of plants and production is regularly reduced during drought stress because of insufficient nutrient availability, lower leaf photosynthetic CO_2 assimilation rate and inadequate water availability (Figure 2). Moreover, dehydration increases plants' biological ethylene synthesis, inhibiting the length and development of their roots (Hanjra and Qureshi, 2010; Mancosu et al., 2015; Boretti and Rosa, 2019; Verma et al., 2023b).

Heavy metals in soils are a major abiotic stressor that reduces agricultural output. Around the world, substantial amounts of heavy metals are frequently present in the soil because of several natural and human activities. Globally, over 10 million locations of polluted soil have been monitored, with a majority of 50% polluted areas with toxic ions. The toxic ions come into the agricultural land from different types of industries, coal burning, wastewater irrigation systems, petrochemical/hydrocarbon spillage, coal combustion, animal waste, and sewage sludge (Boretti and Rosa, 2019; Urra et al., 2019; Rashid et al., 2023; Verma et al., 2023b).

Currently, co-applying PGPRs and NPs has been used in several studies to enhance agronomic productivity and soil quality in different scenarios (Figures 1, 2). The explicit assumption in these studies has been that the NPs would make more nutrients available and generate a conducive habitat for the PGPRs to establish. In response, the latter would carry out their specific work (production of plant hormones, solubilization of nutrients, etc.) at maximum levels. Both stressed and non-stressed soils were used for these analyses. In the meantime, these demonstrations have yet to undergo an in-depth synthesis and critical evaluation. This review article aims to address the knowledge gap. Additionally, highlights the research avenues that will be explored soon to fully utilize the combined potential of PGPR and NPs for sustainable agroecosystems in the years to come.

Enhancing Soil Quality through Nanoparticle and PGPR Interactions

Because the soils carry out a wide range of ecological functions, it is characterized from the viewpoint of those functions. From the perspectives of concurrent agriculture and the environment, it is described as "the capacity of a soil to function within ecosystem and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health" (Verma et al., 2022d; Anikwe and Ife, 2023; Sharma et al., 2023). The main chemical elements of soil quality are soil organic matter, pH, and accessible macronutrients (nitrogen, phosphorus, and potassium). Comparably, the most widely utilized biological indicators are soil respiration, microbial biomass, nitrogen mineralization, and extracellular enzymatic activities, whereas the most used physical indicators are bulk density, structural stability, and the retention of water (Nielsen and Winding, 2002; Bunemann et al., 2018). This review will evaluate the contribution of co-applying NPs and PGPR to enhance soil quality based on these parameters. Co-applying NPs and various PGPRs has been suggested as an effective approach to improving soil quality. Because NPs provide PGPR with a substrate with a high surface area and increased nutrition for their survival, their presence may enhance PGPR efficiency (Nayana et al., 2020; Akhtar et al., 2021; Alharbi et al., 2023; Rajput et al., 2023b; Verma et al., 2023b). The impact of co-applying different PGPRs and NPs on crop productivity and soil quality has been explored in the subsequent sections (Figures 1, 2; Table 1).

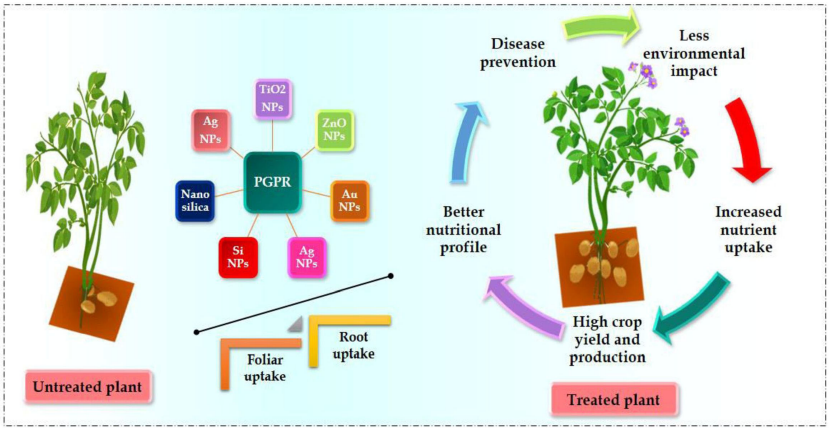

FIGURE 2
Advantages of NPs and PGPRs applications in food security and safety.

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TABLE 1 Influence of co-applied PGPRs and nanoparticles on plants in response to normal and stressed conditions.

Stress	Crop	PGPR strains and NPs	Impact on crop and soil properties	Source
Salinity	Wheat (*Triticum aestivum* L.)	*Azospirillum lipoferum* SP2, *Bacillus coagulans* NCAIM B.01123, *Bacillus circulance* NCAIM B.02324, and *Bacillus subtilis* MF497446 with foliar spray of ZnO-NPs (950 g ha^{-a} and 500 mg L^{-g})	The interactive functions of PGPR and ZnO-NPs safe wheat plants during salinity stress via upregulating antioxidative enzymatic responses, such as, CAT, POD, and SOD (47, 102 and 106%), and uptake of K^+ (27%) as relative to control growing plants. However, alleviation of excess stress via combined application was illustrated by the considerable reduction in membrane stability index (EC), proline, MDA, and H_2O_2 content. Enhanced N uptake in treating plants (57%) with application of PGPR+ZnO-NPs. Upregulated the soil urease (80%) and dehydrogenase (232%) activity during saline stress with combined application of PGPR and ZnO-NPs.	Alharbi et al. (2023)
Salinity	Sugar beet (*Beta vulgaris* L.)	*Pseudomonas koreensis* and *Bacillus coagulans* ($1x10^8$ CFU/ml) with Si-NPs (12.5 ppm)	The application of PGPR, Si-NPs, and their interaction in upgrading agronomic responses, and productivity of sugar beet exposed to normal and saline water irrigation in salty soil. High saline soil and salty water irrigation enhanced imbalance of ions (K^+/Na^+ ratio) and reduced the RWC, relative membrane stability index (RMSI), stomatal conductance, and photosynthetic pigments. The combined application reduced oxidative stress indicators (H_2O_2 and MDA) and Na^+ ions while upregulating the enzymatic activities like SOD (1.9-folds), CAT (1.4-folds), and POD (2.5-folds) as compared normal and excess soil salinity and irrigation of saline water.	Alharbi et al. (2022)
Water deficit andheat	Wheat (*Triticum aestivum* L.)	Single and combined PGPR (*Pseudomonas* sp.) and ZnO-NPs (10 ppm)	Enhanced plant performance, and stress resistance efficiency. Interaction of NPs and *Pseudomonas* sp. protect from stress conditions by producing more proline, SOD, POD, CAT, APX, GR, DHAR and ABA levels. The highest recovery of stress was monitored by the leaf membrane stability reduction, H_2O_2 and MDA content. Overall, combined treatment may protect plants mortality from heat and water deficit or both conditions.	Azmat et al. (2022)
Salinity	Onion (*Allium cepa* L.)	*Bacillus pumilus* and *Pseudomonas moraviensis* with Ag-NPs (5ppm)	*Bacillus pumilus* associated with Ag-NPs better performed for the stimulation of plant growth. The higher soil moisture content was observed in saline stressed plants but the inoculated (PGPR) plants and Ag-NPs single and combined with PGPR exhibited loss in the salt induced retention in the moisture of soil. Interaction of Ag-NPs and PGPR and single enhanced the chlorophyll a+b and carotenoids contents during saline stress conditions. The Ag-NPs enhanced the content of sugar and proline.	Jahangir et al. (2020)
Waste/ contaminated water irrigation	Maize (*Zea mays* L.)	*Pseudomonas* sp., *Pseudomonas fluorescence* and *Bacillus cereus* with Ag-NPs	The colony forming unit of the PGPR was inhibited by Ag-NPs, but regulated by contaminated water. The Ag-NPs augmented the PGPR induced enhancement in root morphology. The application of Ag-NPs, root-shoot ratio was varied. The enzymatic activities (POD and CAT) were found higher by Ag-NPs and contaminated waste irrigation application. The Ag-NPs regulated phytohormones, such as ABA (~35%), IAA (56%), and GA (83%), enhanced proline level (71%).	Khan and Bano (2016)
No stress	Maize (*Zea mays* L.)	*Bacillus* sp. with Ag-NPs	The significant increment was observed in seed germination (87.5%). The highest plant and root growth was found in applied *Bacillus cereus* with Ag-NPs.	Kumar et al. (2020)
No stress	Cabbage (*Brassica oleracea* L.)	*Nocardiopsis* sp. individually or combined applied with Se-NPs	Enhanced fresh-dry mass and glucosinolate uptake. The myrosinase activity significantly upregulated via sprouts of seeds and consequently increased the amino-acid-derived glucosinolate induction. However, the antibacterial activities were upgraded.	AbdElgawad et al. (2023)
Galaxolide-contaminated soil	Soybean (*Glycine max* L.)	*Actinobacterium* sp. with Se-NPs (25 ppm)	The excess uptake of H_2O_2 (+180%), MDA (+163%), and oxidation of protein (+125%), indicating oxidative stress in galaxolide-toxic plants. However, excess uptake of detoxification activity markers, such as phytochelatins (+33%) and metallothioneins (+80%) were observed in mixed applications during contamination of galaxolide. The interactive application of PGPB and Se mitigated the Chl *a* (+58%), gs (+57%) and Fv/Fm (+36%), which resulted in maximum photosynthetic CO_2 assimilation rate (+36%) and production of biomass (+74%) under galaxolide contamination as relative to normal plants.	Halawani and Aloufi (2023)
No stress	Cucumber (*Cucumis sativus* L.)	*Pseudomonas putida* and *P. stutzeri* (@ 106 cells/ml) with Ag-NPs (5-ppm), foliar spray	Ag-NPs upregulated length of roots but reduced plant length of biomass. Leaf protein, proline, phenolics, flavonoids, Chl b, a+b, sugar and Phenylalanine Ammonia-Lyase (PAL) activities were enhanced as compare to control plants. Ag-NPs also suppressed the PGPR effect for the length of root and shoot but augmented the contents of protein and phenolics. Ag-NPs and PGPR increased flavonoids and PAL, SOD and CAT activities in plant leaves. Ag-NPs enhanced the PAL, CAT and SOD responses in both varieties. The application of *Pseudomonas putida* can be applied either single or in mixed with Ag-NPs to upregulate the antioxidantive and defense enzymatic responses.	Nawaz and Bano (2020)

(Continued)

TABLE 1 Continued

Stress	Crop	PGPR strains and NPs	Impact on crop and soil properties	Source
Excess Fe and Mn	Bitter gourd (Momordicacharantia L.)	*Pseudomonas stutzeri* (10^8 cells/ml) with Ag-NPs	Carotenoids, protein, and proline activity were found higher as 366, 450, and 678% in bore well water with PGPR and Ag-NPs treatments. *Pseudomonas stutzeri* was more significant than Ag-NPs to minimize oxidative stress with highest carotenoids, flavonoids, proline contents, and enzymatic activities, such as SOD and CAT.	Tariq and Bano (2023)
Chromium	Rice (*Oryza sativa* L.)	Chromium-resistant bacterium *Staphylococcus aureus* and Fe-NPs (20 ppm)	Fe-NPs significantly upgraded plant performance, production, and leaf gas exchange responses by upregulating the Chl content and mitigating the damage of oxidative stress. Chromium-tolerance bacteria (*S. aureus*) increased the significant potential of Fe-NPs by transformation of chromium (Cr^{6+}) ion into less toxic form of chromium (Cr^{3+}). The bacterial inoculation decreased the accumulation of Cr by plant roots via adsorption of Cr ions.	Alharby and Ali (2022)
Chromium	Sunflower (*Helianthus annuus* L.)	*Staphylococcus aureus* with CeO_2-NPs (25-50 ppm)	CeO_2-NPs significantly enhanced plant performance and crop productivity, decreased oxidative stress, and increased antioxidative enzymatic activities during chromium stress condition. *S. aureus* upregulated the potential role of NPs in mitigating metal toxicity. The highest enhancement was observed in applied NPs and *S. aureus*. Increased Chl content and reduced leaf membrane stability index.	Ma et al. (2022)
Cadmium polluted soil	White clover (*Trifolium repens* L.)	*Pseudomonas fluorescens* (10^7 CFU/kg soil) and TiO_2 -NPs (100-1000 mg/kg)	Interactive role of TiO_2-NPs and PGPR upgraded plant development and Chl level as compare to control. Application of TiO_2 NPs to rhizospheric soil potentially enhanced the uptake efficiency of *T. repens*. TiO_2 NPs and PGPR can decrease the TiO_2-NPs for phytoremediation of toxic ions. Combined application maintained *T. repens* growth in polluted soil and increased accumulation of Cd in plants.	Zand et al. (2020)
No stress	Okra (*Abelmoschusesculentus* L.)	*Pseudomonas libanesis* with Se-NPs	Enhanced phytochemicals (25–35%), height of shoot and root (25–35%), and fruit quality with the application of Se-NPs (75 ppm) to avoid the Se-NPs bioaccumulation in the agro-ecosystems.	Sonali et al. (2023)
No stress	Maize (*Zea mays* L.)	*Bacillus megaterium, Bacillus brevis, Pseudomonas fluorescens* and *Azotobacter vinelandii* with Si-NPs	Enhanced the efficiency of seed germination, plant development and productivity	Karunakaran et al. (2013)
No stress	Wheat (*Triticum aestivum* cv. Stava)	*Bacillus thuringiensis* AZP2, *Paenibacillus polymyxa* A26 with TiO_2-NPs	Upreguleted PGPR activities and their colonization	Timmusk et al. (2018)
No stress	Oilseed rape (*Brassica napus* L.)	*Bacillus amyloliquefaciens* subsp. plantarum UCMB5113 with TiO_2-NPs	Protected plants from the fungal infection, i.e., *Alternaria brassicae*	Palmqvist et al. (2015)
No stress	Cowpea (*Vigna unguiculata* L.)	P*seudomonas monteilii* with Au-NPs	Increased the production of IAA by *P. monteilii* with Au-NPs (50 µg/mL). Au-NPs upgraded plant agronomic responses	Panichikkal et al. (2019)
No stress	Maize (*Zea mays* L.)	*Bacillus* sp. with nanozeolite (50 ppm)	Agronomic response, such as length of plants, leaf area expansion, leaf numbers, photosynthetic pigments and leaf protein were significantly upregulated. Biochemical activities of soil were significantly enhanced, i.e., dehydrogenase, fluorescein diacetate hydrolysis and alkaline phosphatase activities.	Khati et al. (2018)
No stress	Soybean (*Glycine max* L.)	*Bradyrhizobium japonicum*, *Pseudomonas putida*, *Azospirillum lipoferum* with Zn-NPs (0.3-0.9g/mL^1	Plant length, number of nodules, grain yield-weight enhanced	Seysdsharifi and Khoramdel (2016)
No stress	Maize (*Zea mays* L.)	*Bacillus* sp. with nanochitosan	Increased the frequency of seed germination (60 - 97%), length of plants (1.5-fold), and leaf area expansion (2-folds). Soil biochemical activities, i.e., dehydrogenase, fluorescein diacetate hydrolysis and alkaline phosphatase were upregulated. Plant metabolites increased, such as alcohols, acid ester and aldehyde compounds.	Khati et al. (2017)

Nutrient Enhancement in Soil: The Role of Nanoparticles and PGPR

Numerous demonstrations assessed the impact of co-applying PGPR and NPs on soil quality, characterizing the physical, chemical, and biological characteristics of soil (Table 1). When NPs and PGPRs are applied together, generally found to enhance the level of mineral nutrient content in rhizospheric soils when compared to a single application of NPs or PGPR (Akhtar et al., 2021; Singh et al., 2021; Alharbi et al., 2023; Tang et al., 2023). The combined use of NPs and PGPRs resulted in increased soil nitrate levels compared to a single dose of nitrogen. However, PGPR and NPs enhanced organic carbon, phosphorus, and nitrogen availability compared to normal plants. Nanoparticles sprayed on plant leaves at low concentrations since they are economical and environmentally beneficial (Ameen et al., 2021; Liu et al., 2022; Verma et al., 2023a). NPs acquired more interest from plant physiologists because of their usage as nano-growth regulators to improve the growth and development of plants, mainly owing to the effective supply and accumulation and translocation of required minerals (Awan et al., 2020; Kupe et al., 2023). When mixed in soil, they dissolve nutrients due to dissolution and decomposition under the influence of soil properties and the activity of microbes. PGPR, especially those that solubilize organic phosphate, accelerates the accumulation of mineral nutrients from NPs. By utilizing PGPR and NPs together, soil microorganisms are spared from the need to seek out certain nutrients, enabling them to focus on the uptake of other essential elements. This shift enhances enzyme activity and facilitates the release of additional nutrients, thus promoting a more efficient nutrient acquisition process and overall soil fertility enhancement (Ameen et al., 2021; Liu et al., 2022; Rajput et al., 2023a).

Improving soil water retention with nanoparticles and PGPR

With their higher surface area-to-volume ratio, NPs can potentially increase soil water-holding capacity (WHC), especially those with coarse textures. The ameliorative impact of NPs on water retention capacity has also been found in multiple states of the experiments evaluating the co-application of NPs and PGPR. In comparison to PGPR and NPs application only, combined application of PGPR and NPs enhanced soil water holding capacity (WHC) (Nayana et al., 2020; Tang et al., 2023). While single use of PGPR has never been shown to improve soil WHC and water content, it can increase water-deficit resistance capacity to crop plants. However, enhanced WHC by the application of NPs, synergize with PGPR given that the nutrient cycling, breakdown of soil organic matter, and microbial signaling considering higher soil moisture levels [Figure 2] (Verma et al., 2020b; Ahmad et al., 2022; Chieb and Gachomo, 2023). It should be highlighted that the indirect significance of NPs with PGPR application has yet to be conducted.

Modulating soil microbial communities

Nanoparticles enhance various physicochemical characteristics of soil, eventually facilitating the function of indigenous soil microbial populations. NPs can boost WHC, soil pH, substrate, and nutrient availability, enhancing microbial biomass, abundance, and diversification (Rajput et al., 2023a). Furthermore, it has been demonstrated that NPs upregulate the nodulation of the natural rhizobia with legume plants. This is due to the enhancement in aeration provided by NPs that access more air to nodule bacteria, which can persist for a long time on the porous surface of NPs before colonization in the roots (Swarnalakshmi et al., 2020; Flores-Duarte et al., 2022).

The mutualistic relationship between existing microorganisms and plants may be further enhanced by adding NPs (Ameen et al., 2021). The phosphate-solubilizing bacteria *Pseudomonas* sp. increased the availability of phosphorus in soil presumably by solubilizing it from the NPs thereby increasing the colonization of roots and overall plant development (De Souza-Torres et al., 2021). The general abundance of some microbial groups in soil may increase with the interactive role of NPs and PGPR, which improves soil health throughout (Table 1). The authors attributed it increase in advantageous bacteria to the improved soil organic matter content and its breakdown due to the interacting effect of NPs and the inoculant (De Souza-Torres et al., 2021; Ahmad et al., 2022; Rajput et al., 2023b).

Catalyzing soil enzyme activity with nanoparticles and PGPR

Research studies on various intra- and extracellular enzymes have also been used to evaluate the potential benefits of applying NPs and PGPR simultaneously. Soil urease and dehydrogenase enzymes activity was enhanced by applying PGPR and NPs compared to single-use of any of them (Alharbi et al., 2023; Upadhayay et al., 2023). Jabborova et al. (2021) observed that co-inoculation of PGPRs with NPs resulted in higher levels of protease, alkaline, and acid phosphomonoesterase than a single application of PGPRs. Interactive application of *Bacillus subtilis* with NPs was significantly observed in the activities of invertase and catalase in soil than in single use of NPs (Medina-Velo et al., 2017; Khanna et al., 2021). Ultimately, it has been revealed that the PGPR with NPs increases the enzymatic activity of soil phosphomonoesterase in acidic and alkaline conditions, as well as that of sucrase, urease, protease, and invertase. By defining the direction and intensity of nutrient transformation processes in soil, these enzymes can improve soil fertility by stimulating biochemical processes within the ecosystem. There is a direct correlation between soil nutrients and the enhanced enzyme activity caused by PGPR and NPs (Mushtaq et al., 2020; Ahmad et al., 2022; Rajput et al., 2023b). Research is still lacking on how co-application of NPs and PGPRs affects leucine aminopeptidase and N-acetyl-glucosaminidase,

significant N-cycling enzymes. These enzymes catalyze the complex proteinaceous compounds in soil. The amount of mineral nitrogen added to the NPs can be determined by measuring the activity of these enzymes in the presence of PGPR since the NPs are organic materials that contain organic proteins (Figures 1, 2; Table 2).

Nanoparticles and PGPR: a synergistic approach to combat environmental stresses

Crops that grow with low pesticide application concentrations, higher nutritional values, and disease resistance are necessary for sustainable agriculture. In recent decades, the widespread application of expensive agrochemicals in agriculture has prompted the development of more environmentally friendly substitutes, like PGPR and NPs (Medina-Velo et al., 2017; Ahmad et al., 2022). PGPR and NPs have been broadly mentioned for their significant impacts on plants. However, utilizing PGPR and NPs together has been more successful in plant production in recent years than using PGPR or NPs individually. Multiple research projects have documented the significant role of PGPR and NPs in enhancing crop productivity (Mushtaq et al., 2020; Khanna et al., 2021; Rajput et al., 2023a). Similarly, the combined use of *Alcaligenes* sp. with NPs promoted fresh and dry mass, plant length, yield, and quality of fruits over a single use of PGPR. PGPRs with NPs have also been evaluated during decreased fertilizer frequency to reduce the greenhouse gas emissions associated with the fabrication of ammoniac fertilizers and their volatilization (Awan et al., 2020; Mushtaq et al., 2020; Khanna et al., 2021; Shah et al., 2021).

Application of *Enterobacter, Pseudomonas, Azospirillum, Agrobacterium* and NPs enhanced sustainable agriculture production. The interactive use of PGPR and NPs may enhance seed germination frequency, height of plants, dry-fresh biomass, and crop productivity than the single use of PGPR or NPs (Medina-Velo et al., 2017; Fadiji et al., 2022; Sharma et al., 2023). This combination can work in different directions. In the direct mechanisms, the usual generation of plant hormones by the PGPR, such as indole acetic acid, siderophores, etc., and enhancing soil minerals' availability through phosphate solubilization and N_2 fixation contributes to better plant performance and productivity. The existence of NPs can assist the withstanding of the PGPR in larger numbers in addition to providing nutrient-rich substrate thereby leading to improved efficiency by the PGPR ultimately boosting the production of plants (Mushtaq et al., 2020; Khanna et al., 2021; Ahmad et al., 2022; Rani et al., 2023).

It has been widely recognized that the PGPR reducing a wide range of environmental stresses that impede the growth and development of plants. Many researchers have demonstrated their effectiveness in combating drought, soil flooding, salinity, low and high light intensities, nutritional imbalance, and heavy metal contamination. Plants can develop stress tolerance efficiency by exploiting the ability of PGPR to release exopolysaccharides in dry

TABLE 2 Some of the stress-responsive genes in plants that can be regulated by NPs and PGPRs.

Gene Category	Functions	Examples of Genes/Proteins	Source
Antioxidant Genes	Neutralize reactive oxygen species (ROS)	Superoxide dismutase (SOD), Catalase (CAT), Peroxidases (POD), Ascorbate peroxidase (APX)	Akhtar et al., 2021; Ali et al., 2021; Azmat et al., 2022; Sun et al., 2022
Heat Shock Proteins (HSPs)	Protect plants against high temperature and other stressors	*HSP70, HSP90*	Zhao et al., 2012
Water Stress Genes	Regulate water flow in cells	Aquaporins, Dehydrins, *P5CS, CAT1, DREB2*, dehydration-responsive element-binding proteins, *"HsfA1a," "SlAREB1," "LeNCED1," and "LePIP1"*	Mahakham et al., 2017; Raeisi Sadati et al., 2022; Subotic et al., 2022; Mohamed and Abdel-Hakeem, 2023
Salinity Stress Genes	Maintain ion homeostasis and salt tolerance	Pathway of Salt Overly Sensitive (SOS) genes, auxin responsive proteins (ARP), cAPX, DREB, MnSOD, and GST genes	Hezaveh et al., 2019; Moradi et al., 2022
Ethylene Responsive Factors (ERFs) and biosynthesis genes	Regulate plant responses to adverse agroclimatic conditions	*ERF1, ERF5, ACC deaminase*	Fadiji et al., 2022
Pathogenesis-Related (PR) Proteins	Involved in defense against pathogens and stress responses	*PPO, PR1, PR5*, β-1,3-glucanase	Elmer et al., 2018
ABA-Related Genes	Regulate responses to abiotic stress via the ABA hormone	ABA insensitive (*ABI*) genes, *PYR/PYL* receptors	Azmat et al., 2022
Salicylic Acid (SA) responsive and Pathway Genes	Defense responses modulation	SA responsive PR (pathogenicity related proteins) genes (PR1 and PR2)	Cai et al., 2020
Nitrogen Assimilation Genes	Uptake and metabolism of nitrogen	Nitrate reductase (NR), Glutamine synthetase (GS), nitrification related *amoA1 and amoC2* genes	Yang et al., 2013
Phytochelatin Synthase	Chelation of heavy metals for tolerance	PCS (Phytochelatin Synthase) genes, siderophores	Zand et al., 2020
Glutathione S-Transferases (GSTs)	Detoxification processes within plant cells	GST family genes	Moradi et al., 2022

It provides an overview of various gene categories that may be impacted by the presence of NPs and PGPRs in plants, along with their general functions in stress response.

environments (Vurukonda et al., 2016; Ahmad et al., 2022; Chieb and Gachomo, 2023). In saline environments, it increases water absorption, decreases stomatal conductance, boosts potassium accumulation at the cost of sodium, reduces the direct negative impacts of soil salinity, and increases antioxidant enzyme activities. All these alterations assist in the improved growth of plants in saline environments. Similarly, it has been observed that the PGPR improves overall nutrient uptake while also immobilizing and reducing the uptake of heavy metals by plants, thereby reducing the toxicity caused by heavy metals (Bishnoi, 2014; Islam et al., 2014; Kaushal and Wani, 2016; Kumar et al., 2017; Etesami and Maheshwari, 2018). *Bacillus pumilus* in linked with Ag-NPs performed better for the stimulation of onion plant growth. Combined application exhibited a reduction in the salt-induced retention in the moisture of rhizospheric soil and enhanced protein content of bulb, reduced leaf flavonoids. Ag-NPs enhanced sugar and proline levels. *Bacillus pumilus* proved to be more significant during control conditions to all growth agronomic responses but *Pseudomonas moraviensis* potentially coped in response to saline conditions (Jahangir et al., 2020).

Numerous articles have reviewed these findings discussed in the Table 1. Additionally, it has been demonstrated that NPs improve salt tolerance, reduce drought stress, and minimize the toxicity that organic and inorganic soil contaminants cause in plants (Rasheed et al., 2022). Drought stress benefit in NPs-amended soils occurs through higher water holding capacity to large surface area-to-volume ratio of NPs (El-Saadony et al., 2022; Verma et al., 2022b, c; Verma et al., 2023a). Similarly, plants in soil amended with NPs mitigate soil saline conditions due to reduced osmotic stress to increased soil water content and decreased Na^+ absorption caused by Na^+ transient binding on sorption sites on NPs. The primary approach by which NPs reduce the toxicity stress of organic and inorganic heavy metals sorption. Multiple studies have reviewed all these applications of NPs against diverse environmental stressors (Azameti and Imoro, 2023; Faizan et al., 2023; Rajput et al., 2023b). Recently, some studies have explored the possibility of combining PGPR and NPs to mitigate the environmental circumstances for plant development with the assumption that both additives would act synergistically (Table 1). Synergies between PGPR and NPs have been actively explored in these studies.

Soil quality enhancement: the combined power of PGPR and nanoparticles

Improved soil quality occurs from the combined application of PGPR and NPs, which perform multiple functions to mitigate the effects of drought stress (Table 1). When the soil water content was half the field capacity (50% of soil moisture level), applying NPs and PGPR jointly substantially improved the pH, EC, nitrate, phosphorus, extractable K, and organic matter when compared by applying NPs and PGPR separately [Figures 1, 2] (Akhtar et al., 2021; Rajput et al., 2023a). Sun et al. (2022) reported that when the FeO-NPs were treated with arsenic (As)-contaminated soil with the PGPR *P. vermicola*, the outcomes were notably enhanced, yielding more effective results. Consequently, results demonstrated that the *P. vermicola* and FeO-NPs applied combined may mitigate As (arsenic) toxicity in seedlings of *Trachyspermum ammi*, improving plant growth and composition under metal stress observed by balanced exudation of organic acids (Sun et al., 2022). The developing direction from this research suggests that the higher water holding capacity and concurrent reduction in drought stress strengthen the survival and abundance of the PGPR, which perform their activities better (Ahmad et al., 2022).

In terms of soil quality, salt influences the composition of the microbial community in the soil and reduces biomass and microbial activity. Furthermore, under saline conditions, Na^+ exceeds K^+ transport channels, resulting in decreased and inhibited development of plants (Al-Turki et al., 2023). Despite this, it has been demonstrated that co-application of PGPR and NPs in salty conditions induces salt tolerance and plant development, mostly through lowering Na^+ absorption and elevating the K^+/Na^+ ratio. Using two endophytic bacteria, such as *Burkholderia phytofirmans* or *Enterobacter* sp., and NPs drastically reduced saline stress in crop plants by minimizing the uptake of xylem Na^+ content. On the contrary, combined application greatly enhanced the K^+ and K^+/Na^+ ratio, reducing plant saline stress (Kumawat et al., 2022; Al-Turki et al., 2023; Rajput et al., 2023b). In the same demonstration, the sodium adsorption ratio and Na^+ in soil solution were reduced by the latter application to adsorption sites and desorption of K^+ by interactive combination. When inoculated with NPs and *Paraburkholderia phytofirmans*, which can produce exopolysaccharides—significantly reduced the level of Na^+ in the soil solution, alleviating plant salinity stress (Fu and Yan, 2023).

Combined PGPRs and NPs have synergistic benefits on soil quality because they reduce the Na^+ level and increase colonization efficiency. PGPR strains co-applied with NPs in salty soil showed higher colonizing efficacy than PGPRs without NPs in the soil (Kumawat et al., 2022; Rajput et al., 2023b). *Enterobacter* sp. with 5% NPs demonstrated increased colonizing efficiency in saline soil than *Burkholderia phytofirmans* with and without NPs. Compared to PGPR inoculation individually; co-applicating an endophytic PGPR with NPs produced in salty soil caused an increase in PGPR colonization in the rhizosphere, root, and shoot interior bacterial population of about 150–250%. In rhizospheric soil, NPs demonstrated downregulation in the Na^+/K^+ ratio and improved PGPR root colonization efficiency, reducing soil salinity stress. By releasing mineral elements like K^+, Ca^{2+}, and Mg^{2+} from the soil solution, NPs, and PGPRs maintain the nutritional balance by lowering the concentration of Na^+ in the soil. As a result, the soil K^+/Na^+ ratio gradually improved. Na^+ in soil binds by exopolysaccharides formed by PGPRs under stress (deMoraes et al., 2021; Kumawat et al., 2022; Fu and Yan, 2023). The specific genes responsive to stress in plants under the influence of NPs and PGPRs can widely depend on the type of stress, plant varieties/species, type of NPs, type of application, and applied PGPRs strains. Some of the stress-responsive genes in plants can be regulated by NPs and PGPRs which are shown in Table 2. It provides an overview of various gene categories that may be impacted by the presence of NPs and PGPRs in plants, along with their general functions in stress response.

In polluted soils, the application of PGPR together with NPs has also been explored (Table 1). Based on the findings; it is an effective approach for lowering soil contamination from heavy metals. *Enterobacter* sp. microbe with NPs could efficiently expedite the restoration of soil contaminated with cadmium (Cd) toxicity. The combined application of *Bacillus* sp. and NPs enhanced soil enzyme (dehydrogenase) more than NPs, leading to improved biological remediation. This combination also reduced HOAc-extractable Cd levels than independent applications of NPs and PGPR. By lowering the heavy metal's availability, *Bacillus* sp. applied with NPs significantly reduced the detrimental impact of chromium and improved plant health. PGPRs and NPs immobilize metals via metal-immobilizing bacteria, adsorption, co-precipitation, and complexation, therefore restricting their availability in soil for uptake and translocation (Mitra et al., 2018; deMoraes et al., 2021; Zulfiqar et al., 2023).

Synergistic role of PGPR and nanoparticles enhancing physiological and yield characteristics

Several studies have demonstrated that the role of PGPR and NPs on plant performance in adverse agroclimatic conditions (Table 1). The impact of applying PGPR and NPs together on plant development and productivity has been reviewed, and different physiological and biochemical responses have been triggered (Rajput et al., 2023a). However, a consequence of drought stress is increased plant ethylene levels. It has been demonstrated that the use of ACC-producing deaminase-producing PGPR with NPs may reduce the higher ethylene level in plants caused by drought because the latter increases colonization in the plant rhizosphere and promotes the inoculant survival rate (deMoraes et al., 2021; Al-Turki et al., 2023). In terms of comparison, this resulted in higher plant yields than using PGPR or NPs alone. Comparably, using *P. aeruginosa* and NPs jointly decreased electrolyte leakage substantially compared to applying independently. Also, co-application improved fresh and dry leaf-shoot-root weight compared to a single usage of NPs or PGPR, according to deMoraes et al. (2021). When combined with NPs, several additional PGPRs that produce ACC deaminase, such as *Agrobacterium fabrum* and *Bacillus amyloliquefaciens* have also been shown to increase wheat productivity during severe water stress conditions [Table 1] (Al-Turki et al., 2023; Rajput et al., 2023a).

PGPR applied along with NPs to stressed plants, enhanced relative water content, stomatal conductance, Ca^{2+} and K^{+} levels, and reduced proline level. Additionally, researchers observed the reduced electrolyte leakage assisted plants in adapting to drought stress conditions. Drought causes plants to release more ethylene and electrolytes, which inhibits plant growth. By reducing ethylene concentration and electrolyte leakage in plants, co-application of PGPR with NPs may mitigate drought stress in plants. According to experiments carried out by Ahluwalia et al. (2021); Alharbi et al. (2023) and Chandrashekar et al. (2023), PGPR with NPs increased the relative water content, stomatal conductance, chlorophyll, and carotenoids in plants.

The salty soil influences plant development, growth, photosynthesis, lipid metabolism, and protein synthesis (Figures 1, 2). Hormonal imbalances and osmotic changes harm plant growth in saline soils (Singh et al., 2013). It also results in specific toxicity of ions and malnutrition. A different reason is that both sodium and chloride ions restrict plant growth. In certain plants, only chloride ions accumulate in the shoot, while sodium ions are retained in the roots and stems (Kumawat et al., 2022; Fu and Yan, 2023). When PGPRs and NPs are applied in common, their combined effects normally have a more beneficial impact on plant productivity and the elimination of salt stress than separate applications. Under salinity stress, combining NPs and a siderophore-producing strain of *Burkholderia phytofirmans* enhanced the plant height, grain yield, photosynthetic leaf gas exchange, and root and shoot dry weight, respectively. The synergistic ability to integrate PGPR and NPs to ameliorate soil salinity stress for plants has also been confirmed by evidence from multi-year field research experiments (deMoraes et al., 2021; Kumawat et al., 2022; Alharbi et al., 2023). PGPR and NPs play a major role in restoring toxic ions in plants. They can alter, accumulate or eliminate heavy metals (Gulzar and Mazumder, 2022; Wang et al., 2022; Rai et al., 2023).

When combined with NPs, *Enterobacter* sp. significantly facilitated the development of *Brassica napus* in cadmium-contaminated soil (Saeed et al., 2019). Compared to soil without PGPR and NPs under stress conditions, the co-application greatly enhanced shoot and root length, respectively. In addition, PGPR with NPs application reduced Cd uptake in root and shoot under Cd stress conditions relative to individual use of PGPR and NPs, respectively. An increment was observed in ryegrass biomass than the application of NPs, and minimum Cd level was noticed in the interactive application as relative to NPs, PGPR, and control (Jin et al., 2016; Rizwan et al., 2021).

Recent studies performed by Timmusk et al. (2018) underscore the transformative potential of integrating titania nanoparticles (TNs) with Plant Growth-Promoting Rhizobacteria (PGPR) in agriculture. This novel approach has demonstrated a significant boost in plant biomass, particularly under challenging conditions such as drought, salt, and pathogen stress. The synergy between TNs and PGPR not only fortifies plant resilience but also paves the way for sustainable yield improvements. By harnessing the combined power of these two agents, we can unlock new pathways to bolster plant health and productivity, marking a significant step forward in our quest for sustainable agricultural practices.

Deciphering the interactions: how PGPR and nanoparticles influence plant biology

When NPs are applied with PGPR strains, they provide a habitat for PGPR (i.e., colonization, reproduction, and growth) due to its porous structure, more surface area, and the capacity to absorb microorganisms and organic compounds. Several studies mentioned in Table 1 demonstrated that the adding NPs to soils increases the growth and abundance of PGPR inoculants. NPs also

protected them from other dangerous bacterial infections. It provides energy and the essential nutritional building blocks for inoculants' survival and growth. Furthermore, using NPs influences the physicochemical characteristics of soils and may increase soil microbial biomass and enzymatic activity. NPs are rich in different nutritional compositions, such as nitrogen, phosphorus, potassium, calcium, magnesium, zinc, etc., depending on the type and frequency of application (deMoraes et al., 2021; Singh et al., 2021; Alharbi et al., 2023; Al-Turki et al., 2023; Fu and Yan, 2023; Verma et al., 2023a).

PGPRs enhance plant growth through direct and indirect processes during normal and stressful situations. Like NPs, the PGPR can either bring in a nutrient from outside via their direct functions such as nitrogen fixation (by nitrogen-fixing bacteria) or solubilize the immobilized nutrients (by phosphate-solubilizing bacteria) thereby contributing to plant nutrition [Figures 1, 2, Table 1] (deMoraes et al., 2021). In addition, due to their nitrogen-fixing characteristics, nitrogen-fixing PGPRs like *Paenibacillus polymyxa, Rahnella* sp., and *Serratia* sp. can increase the level of mineral nitrogen in soil solution and restrict it from leaching into the soil (Weselowski et al., 2016; Liu et al., 2019; Ribeiro et al., 2020). A wide range of phosphate-solubilizing PGPRs has been demonstrated to solubilize and provide phosphate in soil for plant uptake (Walpola and Yoon, 2013; Alori et al., 2017; Prabhu et al., 2018; Prakash and Arora, 2019; Gupta et al., 2021; Koczorski et al., 2023; Suleimanova et al., 2023).

The direct phosphorus accumulation from NPs using PGPR has yet to be demonstrated. Similar mechanisms of higher availability of potassium may be predicted because PGPR is known for reducing the soil pH and making the soil potassium available to plants and NPs to be rich in essential minerals (deMoraes et al., 2021; Koczorski et al., 2023). Another direct pathway of the production of ACC deaminase reduces the generation of ethylene-enhanced levels obtained during stress conditions via its breakdown into ammonia and alpha ketobutyrate. *Enterobacter* sp., *Alcaligenes* sp., *Pseudomonas fluorescens, Serratia odorifera, Leclerciaade carboxylata, Agrobacterium fabrum, Bacillus amyloliquefaciens, Pseudomonas aeruginosa*, etc., can be produced by ACC deaminase. These strains have beneficial synergistic effects with NPs during environmental stress mitigation. PGPRs by their indirect processes, i.e., pH regulations, production of exopolysaccharides, and defense against biotic stresses are also associated with plant growth enhancement (Alori et al., 2017; Prakash and Arora, 2019; Singh et al., 2021; Kumawat et al., 2022; Alharbi et al., 2023; Suleimanova et al., 2023).

Potential drawbacks and risks associated with their use of NPs and PGPRs for sustainable crop development

Nanoparticles offer transformative potential for agriculture through their unique properties, such as high reactivity and the ability to be engineered for specific tasks. Still, they also present environmental and health risks. Concerns include the toxicity of nanoparticles to non-target organisms like soil microbes, plants, and animals, with studies indicating that certain nanoparticles can disrupt soil microbial communities and accumulate in the food chain. Their small size facilitates mobility, raising the risk of environmental contamination and unknown long-term effects (Tourinho et al., 2012; (Dimkpa et al., 2013; deMoraes et al., 2021). Human health concerns also arise from the potential for nanoparticles to enter the body through food consumption, with evidence of their ability to cross biological barriers. Regulatory challenges are significant, as existing frameworks may not adequately address the risks associated with nanomaterials. Despite these risks, the benefits of nanoparticle use in agriculture are considerable, necessitating comprehensive risk assessments, safe handling practices, and clear regulatory guidelines to ensure their safe and sustainable application ((Grieger et al., 2012; Shah et al., 2021). To ensure a sustainable approach, it is crucial to conduct comprehensive risk assessments, invest in research for understanding long-term effects, and develop nanomaterials that are biodegradable and safe for the environment and human health. Furthermore, establishing clear regulatory guidelines and promoting safe handling practices are essential for mitigating risks (Grieger et al., 2012). By prioritizing safety and sustainability, the agricultural sector can leverage nanotechnology to address global food security challenges while protecting environmental health and biodiversity.

Conclusions and future perspectives

During adverse agroclimatic conditions, reduced plant growth and crop mortality are prevalent through significant food and commercial cash crops. Given the significant potential of PGPR and NPs to enhance crop productivity and soil health under both normal and adverse conditions, our conclusion highlights the necessity of integrating these technologies into sustainable agricultural practices. The synergy between PGPR and NPs not only offers a path to reducing synthetic fertilizer dependency but also promises resilience against climatic stresses by improving plant performance, production, and fruit or grain quality, as well as soil profile. However, as highlighted in this article, limited field demonstrations have been conducted to assess the significance of the PGPR and NPs' interactive role in sustainable agriculture. Mechanistic research on the interaction between PGPR and NPs requires more research. An extremely efficient approach to evaluate the combined impact of PGPR and NPs could be long-duration field demonstrations. The potential of the PGPR and NPs for sustainable food production has been independently assessed in reasonably long-duration field studies.

Future research is poised to delve deeper into the mechanistic underpinnings of PGPR and NP interactions, with a strong emphasis on long-term field trials. These studies are critical for understanding the dynamic interactions in various soil types, especially those lacking in organic matter in tropical and subtropical regions. The aim is to develop tailored application strategies that leverage the unique benefits of PGPR and NPs,

thereby reducing reliance on synthetic fertilizers and enhancing the environmental sustainability of agricultural systems. This detailed exploration and application of PGPR and NPs hold the promise of revolutionizing farming practices to meet the global food demand sustainably.

Author contributions

KV: Writing – original draft, Resources, Methodology, Formal analysis, Data curation, Conceptualization. AJ: Writing – original draft, Software, Resources, Formal analysis, Data curation. X-PS: Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. SS: Writing – review & editing, Software, Resources, Data curation. AK: Writing – review & editing, Software, Resources, Formal analysis, Data curation. JA: Writing – review & editing, Software, Resources, Formal analysis, Data curation. SKS: Writing – review & editing, Software, Resources, Formal analysis, Data curation. MS: Writing – review & editing, Software, Resources, Formal analysis, Data curation. CS: Writing – review & editing, Software, Resources, Formal analysis, Data curation. Y-RL: Writing – review & editing, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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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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A smooth vetch (*Vicia villosa* var.) strain endogenous to the broad-spectrum antagonist *Bacillus siamensis* JSZ06 alleviates banana wilt disease

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Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4), poses a significant threat to banana production globally, thereby necessitating effective biocontrol methods to manage this devastating disease. This study investigates the potential of *Bacillus siamensis* strain JSZ06, isolated from smooth vetch, as a biocontrol agent against *Foc* TR4. To this end, we conducted a series of *in vitro* and *in vivo* experiments to evaluate the antifungal activity of strain JSZ06 and its crude extracts. Additionally, genomic analyses were performed to identify antibiotic synthesis genes, while metabolomic profiling was conducted to characterize bioactive compounds. The results demonstrated that strain JSZ06 exhibited strong inhibitory activity against *Foc* TR4, significantly reducing mycelial growth and spore germination. Moreover, scanning and transmission electron microscopy revealed substantial ultrastructural damage to *Foc* TR4 mycelia treated with JSZ06 extracts. Genomic analysis identified several antibiotic synthesis genes, and metabolomic profiling revealed numerous antifungal metabolites. Furthermore, in pot trials, the application of JSZ06 fermentation broth significantly enhanced banana plant growth and reduced disease severity, achieving biocontrol efficiencies of 76.71% and 79.25% for leaves and pseudostems, respectively. In conclusion, *Bacillus siamensis* JSZ06 is a promising biocontrol agent against *Fusarium* wilt in bananas, with its dual action of direct antifungal activity and plant growth promotion underscoring its potential for integrated disease management strategies.

KEYWORDS

smooth vetch, *Bacillus siamensis*, *Fusarium* wilt of banana, mechanism of inhibition, pot experiment, LC-MS/MS, biological control

1 Introduction

Banana (*Musa* spp.) stands as a prominent tropical and subtropical fruit crop worldwide. It holds the distinction of being one of the most produced, traded, and consumed fruits globally (FAO, 2023). Following rice, wheat, and maize, bananas have ascended to become the fourth major food crop in developing nations (Aguilar-Hawod et al., 2019; Zhou et al., 2023). China holds the position of the largest global importer and the second-largest manufacturer of bananas. The economic progress of China's tropical regions is significantly dependent on the banana industry (Zheng et al., 2018). *Fusarium* wilt of banana, induced by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), poses substantial threats to production by diminishing yields in terms of quantity and quality. This can result in regional and global fluctuations within the banana industry (Ghag et al., 2015; FAO, 2023). *Foc* pathogens are categorized into four pathogenic microspecies, namely *Foc* race 1, 2, 3, and 4, based on variations in banana infestation (Zhou et al., 2023). In particular, the TR4 – a variant of tropical race 4 – possesses the capability to infect a wide range of banana genotypes combinations susceptible to TR4 infection (Zhou et al., 2023). Currently, it is spreading swiftly across all global banana regions, posing a threat to approximately 80% of banana plants due to *Foc* TR4 (Damodaran et al., 2019; García-Bastidas et al., 2020).

Fusarium oxysporum f. sp. *cubense* Tropical Race 4 (*Foc* TR4) is a soil-borne pathogen that rapidly disseminates through the transportation by susceptible plants, soil, water, agricultural tools, and other human activities (Swarupa et al., 2014; Zhang et al., 2021a). The spores of the pathogen can persist in the soil for decades, and currently, there is no established effective method for controlling *Foc* TR4 (Ghag et al., 2015; Ploetz, 2015). At present, strategies for controlling banana wilt primarily revolve around the development and breeding of new disease-resistant varieties, as well as agricultural, physical, chemical, and biological control methods, etc (Davis and Grant, 1996; Ploetz, 2015; Shen et al., 2019). Chemical control stands out as the most effective approach among these strategies; however, it contributes to environmental pollution and other issues (Latz et al., 2016; He et al., 2021). On the other hand, agricultural and physical management is intricate and challenging to execute (Bubici et al., 2019). Moreover, considering the triploid nature of bananas and the associated difficulty in cultivating disease-resistant varieties (Zhou et al., 2023), this contributes to the constraint in *Foc* TR4 control. Conversely, in light of the growing emphasis on sustainable agricultural development, beneficial microorganisms are currently acknowledged as one of the most promising approaches for *Foc* TR4 control, characterized by safety, environmental friendliness, and efficiency (He et al., 2021; Yun et al., 2022).

During the last decades, *Bacillus* spp. have been extensively used as biocontrol agents (Bubici et al., 2019; He et al., 2021). In particular, the genus *Bacillus* has long been recognized as an ideal biological control agent due to the abundance and stability of its metabolites and its ease of cultivation (Fan et al., 2021; Li et al., 2021a). Studies have shown that soil administration of *Bacillus subtilis* increases the diversity of soil microorganisms, stimulates native antagonistic flora, and enhances tolerance to the propagation of banana wilt (Gadhave et al., 2018; Tao et al., 2020). *Bacillus endophyticus* may cause systemic stress in crops through the production of cyclic lipopeptides or the jasmonone/ethylene signaling cascade (van Loon, 2007; Sun et al., 2022). Some *Bacillus* species produce antibiotics through the Non-Ribosomal Peptide Synthase (NRPS) and Polyketide Synthase (PKS) genes, for example, *Bacillus velezensis* produces surfactin, *Bacillus amyloliquefaciens* contains bacillomycin D, and fungitoxin is effective in antagonizing banana wilt (Xu et al., 2013; Li et al., 2021a). Furthermore, certain *Bacillus* strains promote banana growth by producing IAA and other volatile compounds after colonising the plant (Idris et al., 2007; Tan et al., 2013). However, these *Bacillus* species' antimicrobial activity can still be affected by their complex growth environment and different pathogenic fungi (Saravanan et al., 2003). Thus, it is necessary to identify and isolate *B.* spp. with antagonistic activity against various plant pathogenic fungi.

Many scholars have widely isolated endophytic *Bacillus cenocepaci* from banana plants (Souza et al., 2014) or their inter-root soils (Zhang et al., 2011), and these endophytic bacteria tend to show advantages of bioprophylaxis and colonization (Xue et al., 2015). However, it is possible that efficient antagonistic bacteria still exist in other plant materials. Some studies have reported the isolation of potent antagonists from weeds, medicinal plants, and chili peppers (Adler, 1980; Zeng-Hui et al., 2007). Endophytic *Burkholderia cenocepacia* 869T2 isolated from the roots of *Vanilla planifolia* showed good biocontrol efficacy in a field trial (86%) (Ho et al., 2015). Endophytic strains of *Serratia marcescens* ITBB isolated from rubber trees B5-1 also showed high inhibitory activity in the greenhouse (79%) and in the field (70%) (Tan et al., 2015). Two strains of endophytic nitrogen fixers – namely *Burkholderia* spp. and *Herbaspirillum*-like – isolated from pineapple roots and stems have also been reported promising candidate strains for biocontrol and biofertilizer of banana, and both of them were isolated from plants other than banana (Weber et al., 2007). Therefore, banana endophytic antagonists are widely present in various plant species, and the isolation of efficient and green endophytic antagonists is still a research direction for banana biocontrol. Smooth vetch (*Vicia villosa* var.) is an annual legume that can enhance soil quality, reduce use of mineral fertiliser and improve plant resistance, which is essential for ensuring high plant yields and creating a good agro-ecological environment (Gao et al., 2023; Liang et al., 2023). Earlier research has shown that planting smooth vetch can recruit large quantities of soil plant growth regulating rhizobacteria (PGPR) (Xiao et al., 2017; Shi et al., 2022), Some of the PGPR can colonize into the interior of smooth vetch to generate dominant strains, which can play the function of promote plant growth and resisting diseases, thus improving crop yield and resistance (Abeywickrama et al., 2023; Pu et al., 2023). Other studies have found that intercropping bananas with green manure reduces the *Fusarium spinosum* population and thus substantially lowers the prevalence of banana wilt (Yang et al., 2022a). It is possible that smooth vetch harbors a large number of endophytic bacteria with good disease resistance-promoting properties (Yang et al., 2022b; Liang et al., 2023). Thus, smooth vetch can be used as a good material for the isolation of biocontrol-promoting bacteria.

A new strain of *Bacillus siamensis* JSZ06, isolated from the stem of smooth vetch, was subjected to taxonomic classification based on 16S rRNA gene sequencing, supplemented by morphological, physiological, and whole genome data. Preliminary studies evaluated its *in vitro* inhibitory effect on *Foc* TR4 and its *in vivo* biocontrol and growth-promoting effects on potted plants. Additionally, the fungicidal activity of the fermentation broth extract was assessed, focusing on its impact on *Foc* TR4 mycelium and spores, and elucidating its bactericidal mechanism. Chemical composition analysis was performed using liquid chromatography-mass spectrometry (LC-MS). This study aims to provide scientific evidence supporting the potential of *B. siamensis* JSZ06 as an endophytic antagonist for the biological control of banana wilt disease.

2 Materials and methods

2.1 Smooth vetch plant sample collection

The smooth vetch plant samples (stem, leaf, root) were collected from Songming County, Kunming, Yunnan Province, China (102° 41' E, 25° 28' N). Plant material was kept in insulated containers at -80°C after being transferred to the lab in clean polyethylene bags. The banana variety was 'Baxi' (*Musa* spp. AAA), which was propagated by the Agricultural Biodiversity Research Laboratory of Yunnan Provincial Agriculture Academy.

2.2 Phytopathogenic fungi

The banana research team of the Institute of Agricultural Environment and Resources, Yunnan Academy of Agricultural Sciences, isolated, identified, and stored *Foc* TR4 strain 15-1 (NCBI Accession Number: SRX9739537) as the test banana pathogen, from Brazilian banana varieties grown in Xishuangbanna fields. The samples were preserved strains with high virulence and pathogenicity based on guidelines described in Zheng et al. (2018).

2.3 Isolation of plant endophytes

The isolation of endophytes was initially performed by rinsing the plant surface with tap water. The plant was then split into roots, stems and leaves. To sterilize the surface of the plant material, 10 g of washed plant material was weighed and soaked in 75% (v/v) alcohol for 2 minutes, followed by 1% HgCl for 2 minutes, then rinsed three times with sterile distilled water. Subsequently, the roots, stems or leaves were excised and thoroughly crushed with the addition of quartz sand (0.2 g) and sterilized in a saline solution (pH = 7.4). A grinding solution prepared in 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} concentration gradients was applied to Luria-Bertani solid medium (LB; tryptone 10 g/L, yeast powder 5 g/L, sodium chloride 10 g/L and 15 g/L agar). To verify the effectiveness of the surface sterilization, the water used for the final rinse of the plants was spread on LB agar plates. All procedures were replicated thrice. Petri dishes were then incubated for 72 hr at 30°C. Single colonies were purified on LB medium and stored at -80°C in 50% (v/v) glycerol (Christakis et al., 2021).

2.4 Preliminary screening of endophytic bacteria antagonistic to *Foc* TR4

Using a dual culture assay, we evaluated the antagonistic effect of screened endophytic bacteria against *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4) (Wei et al., 2020). We utilized a perforator to cut *Foc* TR4 mycelial discs of 5 mm diameter from the periphery of actively growing *Foc* TR4 plates. These discs were then centrally placed in the centre of potato dextrose agar (PDA; 200 g/L potato powder, 20 g/L dextrose or 15 g/L agar). Subsequently, the selected strains were inoculated at four symmetrical points around the *Foc* TR4 fungal cake, at a distance of about 2.5 cm. As a control, we used *Foc* TR4 plates without inoculation of the isolated strains. All procedures were replicated thrice. The diameter of pathogenic bacteria was measured by the crossover method after 5-7 days of incubation at 28°C. Use Equation 1 to calculate the rate of inhibition (GI) of TR4 growth in each treatment group:

$$\boldsymbol{GI} = [(\boldsymbol{C} - \boldsymbol{T})] \times \mathbf{100}\,\% \quad (1)$$

The diameters of fungal mycelial growth in the treated and control plates were denoted by T and C, respectively.

2.5 Secondary screening of endophytic bacteria antagonizing *Foc* TR4

The preparation of crude extracts with antimicrobial activity was performed according to the method previously reported by Li et al. (2021c). A small-scale fermentation process of endophytic bacteria initially screened for antifungal activity was carried out. The specific method involved culturing the antagonistic endophytes in LB liquid medium at 30°C and 180 rpm for three days. Subsequently, the culture was centrifuged at 13,000 rpm and filtered through a 0.22 μm sterilizing filter to obtain the fermentation broth. This fermentation broth was then mixed with an equal volume of ethyl acetate, and crude extracts were obtained using organic solvent extraction. The mixture was subjected to ultrasonication for 1 hr and shaking for 8 h. Ten extraction was repeated three times. Post-extraction, the organic solvent layer was concentrated using a rotary evaporator in a 40°C water bath, resulting in a precipitate. This precipitate was dissolved in methanol (AR) and dried in a fume hood for further use (Li et al., 2021b). In order to find the extract's antifungal activity tests, the crude extract was evaluated by agar pore spreading technique for its inhibitory activity on mycelial growth (Li et al., 2021c). Initially, 10 mg of the extract were dissolved in 1 M Dimethyl sulfoxide (DMSO),

achieving a concentration of 10% (v/v), and thereafter, it was sterilized using a 0.22 μm filter membrane. Subsequently, four 5 mm microwells were meticulously punched on PDA agar plates employing a sterile punch. Consequently, 50 μL of the sterilized extract solution was carefully aspirated into each well. A similar volume with DMSO 10% v/v without 10 mg of raw extract served as untreated control. Inoculate a 5 mm diameter TR4 cake into the center of the petri dish. The antimycotic effect was evaluated by measuring the growth of hyphae after 7 days of incubation at 28°C. All products of the replicated experiments were incubated to inoculate three petri dishes.

2.6 Biolog identification and morphological analysis of biocontrol strains

To gain more insight into the morphological characteristics of endophytic bacteria that most effectively antagonise *Foc* TR4, endophytic bacteria were cultured on different agar media commonly available in the laboratory, including LB, PDA, Reasoner's 2A, Nutrient Agar, Czapek-Dox Agar, Tryptic Soy Agar and Gao's No. 1. Transverse sections of bacterial strains on LB dishes were taken from 1 cm^2 and processed for 3 h at 4°C using 2.5% glutaraldehyde in order to obtain scanning electron microscope (SEM) images of the strains.

The purified JSZ06 strain was sent to Guangdong Microbial Strain Collection Center (Guangdong, China) for physiological and biochemical identification. The identification system was GEN III MicroPlatesTM (Biolog, Hayward, CA, USA), which contained 49 carbon sources and 20 biochemical assays.

2.7 Genome sequencing of strain JSZ06

The isolated strains were cultured in Luria-Bertani (LB) broth for 24 h at 30°C and 200 rpm with shaking. Subsequently, bacterial DNA was extracted from 1 mL of the culture using the Qiagen DNA extraction kit, following the manufacturer's instructions. The extracted DNA was then subjected to whole genome sequencing, which was performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. Bioinformatics analysis of the sequencing data was conducted using the Majorbio cloud platform (www.majorbio.com) (Ren et al., 2022).

2.8 Construction of phylogenetic tree

16S rRNA sequences were extracted from the genome of strain JSZ06. To find the homology sequences, the 16S rRNA isolate was checked against NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) database and EzBioCloud server (https://www.ezbiocloud.net/identify) (Qi et al., 2021). Phylogenetic trees were created with the MEGA 7.0 neighbor-joining algorithm (Wei et al., 2020), with the Bootstrap value set to 1000 and the remainder of the parameters at default setting.

2.9 Characterization of PGP traits of endophytes

Using the technique described by Sheng et al. (2008), isolates were evaluated for the production of Indole Acetic Acid (IAA). Endophyte culture inocula were added to LB medium that had been 500 mg/L tryptophan added, the samples were then cultured at 30°C for 72 h. The supernatant from the centrifugation of fermentation broth at 8,000 rpm was used to detect IAA.

The production of iron carriers by isolated bacteria was evaluated using techniques described by previous authors Luo et al. (2011). An equal amount of CAS solution was mixed with bacterial supernatant cultured in CDA liquid medium. After incubation in the dark at room temperature for 3 h, the absorptivity level at 630 nm was measured. Subsequently, the readings were contrasted with the control's OD_{630} (λ/λ0).

In a modified Pikovskayas media enriched with tricalcium phosphate, the endophytes' ability to solubilize minerals was measured (Wang et al., 2018). After incubation of the test strains in phospholysis medium for 72 h at 30°C, dissolved phosphate in the supernatant was determined by the molybdenum blue method. The strains were tested for their potassium capacity (Zhang and Kong, 2014). The test strains were incubated in medium containing potassium feldspar powder for 72 h, and the amount of effective potassium in the supernatant was determined by flame spectrophotometry.

Endophytic bacteria were inoculated in a basic medium containing 10 mL of Dworkin-Foster (DF) with 3 mM ACC instead of ammonium sulphate as the N source and incubated at 30°C for 72 h (Wang et al., 2018). Samples for the assay were prepared according to the method described by the Belimov et al. (2005). Bacterial Acetyl-CoA Carboxylase (ACC) was quantified using the Bacterial ACCdase ELISA kit (Shanghai Huabang Biotechnology Co., Ltd.).

The 12 primer pairs (Supplementary Table S3) were employed to identify the antimicrobial genes of the microorganism in reference to the study of antibacterial synthases and regulatory genes by Li et al. (2021a). Fan et al. (2021) described the method, reaction conditions and procedure for genomic DNA extraction.

2.10 Optimization of culture conditions for fermentation of antagonistic bacteria

The impact of varying starting pH-buffers, temperature, incubation speed and inoculum volume on the growth (OD_{600}) and the yield of inhibitory substances of *Bacillus siamensis* JSZ06 were investigated on the basis of the optimal growth medium using a one-way test. A one-way test was set up with six ndifferent pH values of 4.5, 5.5, 6.5, 7.5 and 8.5, and seven temperature levels of 21, 24, 27, 30, 33, 36 and 39 °C, six rotational speeds of 140, 160, 180, 200, 220 and 240 rpm, and five levels of inoculum concentrations of 1%, 2%, 3%, 4% and 5%. The inoculation was carried out in triplicates in 100 mL of medium for 72 h. Bacterial growth (OD_{600}) and the inhibition of *Foc* TR4 growth by the fermentation broth were determined under different culture

conditions to determine the optimal initial pH, temperature, rotation speed and inoculum concentration (Li et al., 2021c).

2.11 Determination of broad-spectrum antifungal activity of antagonist strain JSZ06 and its extracts

To assess the wide-ranging fungicidal effectiveness of this antagonistic strain and its extracts, we have chosen 10 species of plant pathogen fungus, among them *Botrytis cinerea* (ATCC 11542), *Rosellinia necatrix* (ATCC 28386), *Fusarium equiseti* (ATCC 26104), *Phytophthora nicotianae* (ATCC 66202), *Colletotrichum fragariae* (ATCC 58689), *Fusarium oxysporum*(ATCC MYA-1198), *Setosphaeria turcica* (ATCC 64836), *Fusarium oxysporum* f. sp. *lycopersici* (ATCC 16322), *Cercoseptoria zingiberis* (ATCC 46262) and *Fusarium solani* (ATCC 52628). All phytopathogenic bacteria were isolated and preserved by the Kunming Comprehensive Experimental Station of the National Green Fertilizer Industry Technology System. Four symmetrically positioned PDA plates were inoculated with antagonistic bacteria and strain fermentation extracts at a final concentration of 200 mg/L of extracts, followed by inoculation of 5 mm diameter discs of phytopathogenic fungi in the center of the PDA plates and incubated at 28°C for 7 days. The broad-spectrum resistance of JSZ06 strain and strain extracts was assessed by *in vitro* fungicidal action.

2.12 Influence of raw extract on *Foc* TR4 mycelium development

The growth inhibition of *Foc* TR4 was assessed by measuring the rate of mycelial growth. A variety of quantities of the 100% methanol-isolated strain extracts were then mixed with the purified PDA fluid, at the levels of 0.78, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0, 100.0, and 200.0 mg/L. Each group's negative control was the identical concentration of DMSO. In the middle of the plate, inoculate with 5 mm of *Foc* TR4 diameter patties. After 7 days at 28°C, the *Foc* TR4 growth diameter was assessed. Three copies of every treatment were used. A regression equation for toxicity was formulated using the method of least squares (Vanewijk and Hoekstra, 1993). Using the toxicity regression equation, the minimum concentration for 50% of the maximal effect (EC_{50}) was calculated.

2.13 Effect of JSZ06 extract on the ultrastructure of *Foc* TR4 mycelia

The ultramorphological characterization of *Foc* TR4 mycelia, treated with extracts from the fermentation broth of strain JSZ06, was meticulously observed using Scanning Electron Microscopy (SEM; ZEISS Sigma 300, Germany). For the experimental setup, a 5 mm piece of *Foc* TR4 mycelium was inoculated at the centre of each PDA plate which contained 50 mg/mL of the strain extract. In contrast, DMSO with an equivalent concentration served as control. Following a 7-day incubation period at 28°C, a 1 cm^2 block of agar, inclusive of the mycelium, was carefully excised from the periphery of the *Foc* TR4 growth. The ultramorphology of both the extract-treated *Foc* TR4 mycelium and the control mycelium was subsequently analysed via SEM (Fan et al., 2021).

2.14 Impact of strain exposure on the mycelial cell structure of *Foc* TR4

The effect of extracts on the cell architecture in *Foc* TR4 was observed using a TEM (HT7700, Hitachi Ltd, Japan). Referring to the relevant methodology (Li et al., 2021c), samples were collected, fixed, and dehydrated. After 12 h at 35°C, 24 h at 45°C, and 48 h at 60°C, the samples were aggregated after being implanted in Epon812 resin. The material was then sliced into 80 nm sheets. Uranyl acetate and lead citrate solution were used to double-stain these sections. Mycelial cells' ultrastructure was examined by TEM after the samples had dried naturally at room temperature.

2.15 Impact of strain extracts on *Foc* TR 4 spore germination

We determined the effect of *Bacillus siamensis* JSZ06 extracts on the germination rate of *Foc* TR4 spores. Extracts of strains with concentrations of 1, 2, 4, and 8 × EC_{50}, were thoroughly mixed with 45 μL of *Foc* TR4 spore suspension (1.0×10^6 CFU/mL) on concave slides and then allowed to stand for 12 h at 28°C. Subsequently, the extracts were mixed with 45 μL of *Foc* TR4 spore suspension (1.0×10^6 CFU/mL). Equivalent concentrations of dimethyl sulfoxide-treated spore suspensions were used as control. All experiments were repeated three times. The germination of 100 spores on each concave slide was examined using a light inverted microscope. Inhibition was assessed on the basis of spore germination (Zou et al., 2021).

2.16 Annotation of functional genes

The identification of protein encoding sequences (CDS) within the genomic structure was achieved using Glimmer v3.02 analysis tools (Delcher et al., 2007). Based on the whole genome sequence, estimation of gain was made of the G + C content of the strain genome. The bacterial genomes were annotated using GO (cloud.majorbio.com), COG (http://www.ncbi.nlm.nih.gov/COG) and KEGG (http://www.genome.jp/kegg/) databases (Li et al., 2021c). Employing the web-based tool antiSMASH, the prediction of secondary metabolite gene clusters in genomes was constructed (Huang et al., 2019).

2.17 Metabolite composition analysis of JSZ06

Metabolites of anti-*Foc* RT4 in JSZ06 fermentation broth were determined using a metabolomic approach (Yun et al., 2023). The

analysis of the specimens was performed with an Ultra High Pressure Liquid Chromatography (Agilent 1290 Infinity, USA) tandem Fourier Transform Mass Spectrometer (AB Triple TOF 6600, USA) UHPLC -Q Exactive system. Sample extraction and detection was carried out using LC-MS/MS (Wang et al., 2019; Xie et al., 2019; Yun et al., 2023). Metabolite profiles were generated by aligning mass spectrometry data with data from publicly available metabolic databases, namely HMDB (http://www.hmdb.ca/), Metlin (https://metlin.scripps.edu/), and Lipidmaps (http://www.lipidmaps.org). Metabolic routes, via KEGG database pathway annotation, were also performed to identify the routes implicated in the compounds. The data analysis was constructed using the Majorbio Choud platform, which was available for free online (cloud.majorbio.com) (Ren et al., 2022).

2.18 Banana seedling experiment with pot inoculation

From August to October 2023, the effectiveness of the fermentation solution of the antagonistic strain against banana wilt was assessed at 70% relative humidity and a standard room temperature of 28°C in a controlled environment. He et al. (2021) contributed the overexpression procedure of the fluorescent green protein gene *Foc* TR4 (GFP-*Foc* TR4). One seedling of histocultured 'Baxi' banana (AAA) from the seedling bag was transplanted into a plastic pot (5 cm × 8 cm) containing 2 kg of sterile soil (Fan et al., 2021). Four different types of intervention were designed as follows: distilled water, GFP-*Foc* TR4 (1.0×10^6 CFU/ml), antagonist strain (1.0×10^8 CFU/ml) + GFP-*Foc* TR4 (1.0×10^6 CFU/ml), and antagonist strain (1.0×10^8 CFU/ml). As recommended (Fan et al., 2021), 100 mL of the combination were applied to the roots of banana plants. For a total of 45 days, the development of banana seedlings was measured every 7 days in 30 plants per treatment.

2.19 Evaluation of biological control and growth promotion effects

On days 0, 7, 14, 21 and 45 of inoculation, banana roots and bulbs were collected and cut as finely as possible. A confocal laser scanning microscopy (Leica TCS-SP, Wetzlar, Germany) was used to rapidly detect the level of GFP-*Foc* TR4 infection and settlement (Zhou et al., 2023).

Forty-five days after inoculation, the infection of banana leaves and bulbs was observed and recorded separately. The disease index and control effect of each group were computed following the methods of Fan et al. (2021) and Zhou et al. (2023). The following standards for assessing leaf diseases were applied: grade 0: no indications; grade 1: no more than 50% of the whole area with wilting of true leaves and cotyledons; grade 2: more than 50% of the whole area with wilting of true leaves and cotyledons; grade 3: just the growth tips remained, with the leaves withered or dead; grade 4: the whole plant seemed very withered or dead. Bulb disease grading: grade 0: no lesions on the bulb; grade 1: the area of lesions on the bulb is 1% - 10%; grade 2: the bulb lesion area is 11%~30%; grade 3: the bulb lesion area is 31%~50%; grade 4: the bulb lesion area is more than 50%. Plant height, fresh weight, stem thickness, leaf length and leaf width of the banana plants in all treatments were measured and recorded on days 7, 21 and 45 after inoculation.

2.20 Data analysis

Data were subjected to one-way ANOVA, with the significance of differences between treatments being assessed using Duncan's multiple range test ($P< 0.05$). Therefore, both Excel and SPSS Statistics 20.0 software were utilized for statistical analysis and orthogonal experimental design. Consequently, graphical representations were created using Origin 2021 and GraphPad Prism 8 software.

3 Results

3.1 Isolation of endophytes

Smooth vetch antagonistic endophytes were isolated using a correlation procedure at the green manure long-term localization test site in Songming County, Kunming, Yunnan Province (102° 41'E, 25° 28'N) (Figure 1A). Sixty strains of endophytic bacteria were isolated using taproots, branches and leaves of smooth vetch (Supplementary Figure S1). All strains were conserved at the Kunming Comprehensive Experimental Station, National Green Fertilizer Industry Research and Development Center, Kunming, China (Figure 1B).

3.2 Determination of antifungal activity of isolated endophytes

Firstly, all isolated endophytic strains were verified against *Foc* TR4. The inhibitory capacity of mycelial growth was determined for TR4. Eleven of the 60 endophytic strains exhibited a significant antifungal efficacy in the plate antagonism test (Supplementary Figure S2A), representing 18.30% of screened endophytes. Furthermore, the suppressive effects of the 11 antagonistic endophytic strains against *Foc* TR4 were assessed. The strain numbered JSZ06 showed the strongest inhibitory activity with 70.20% inhibition.

Simple fermentation of 11 endophytic strains from a preliminary screening with significant antagonistic effects was carried out. Their crude extracts were further investigated for their antagonistic ability against Foc TR4. The results showed that the crude extracts of all strains inhibited *Foc* TR4 in the range of 35.36-70.60% (Supplementary Figure S2B). Strong inhibitory circle size was observed in the extracts of five isolates (JSZ06, JSZ01, JSZ02, YSZ10 and YSZ19). The inhibition rates were 70.56, 58.69, 57.14, 60.00 and 56.43%, respectively. Combining the results of initial and rescreening, strain JSZ06 was selected as the most suitable for subsequent studies.

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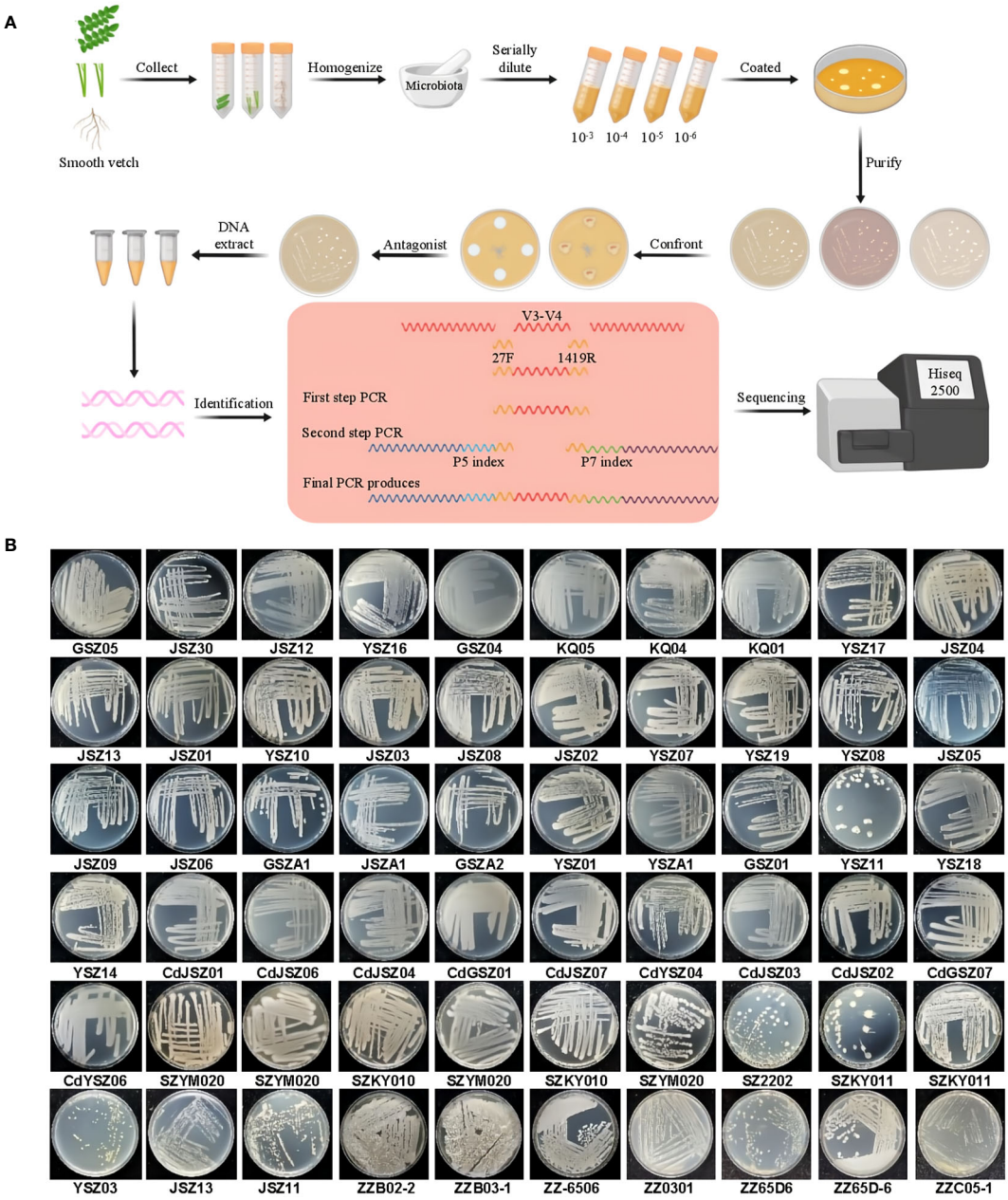

FIGURE 1
Growth characteristics of 60 endophytic bacteria isolated from smooth vetch. **(A)** Smooth vetch can be cultivated for the isolation and identification of endophytic bacteria for detailed analysis of experimental procedures. **(B)** Growth characteristics of 60 strains of endophytic bacteria.

3.3 Characteristics and identification of strain JSZ06

3.3.1 Characteristics of culture

The growth characteristics of strain JSZ06 have been measured on 7 selected media (Supplementary Table S1). It grew well on LB and TSA media and moderately on the rest of the media except R2A, CDA and Gause's no.1 media. The colonies showed an overall colour ranging from yellow to grey on all the media. Colonies were light yellow on PDA and TSA media, light grey on LB, yellowish white on NA, beige on let R2A and lemon yellow on CDA and Gause's no.1. Both irritating odour and biofilm were produced on the LB medium, and the colony bulge was the largest in diameter. Multiple notched edges, irregular growth, rough appearance, opacity, and wrinkled surface of the colonies were observed on all media, this provides an additional means of distinguishing the genus *Bacillus* (He et al., 2021).

3.3.2 Features of physiological and chemical systems

In order to further investigate the physiological and biochemical activities of JSZ06, we assayed JSZ06 using the Biolog method. The results showed that in terms of physicochemical aspects, the strain was able to be positive at pH 5 and pH 6, and at 1% NaCl, 4% NaCl,

and 8% NaCl, i.e., JSZ06 could grow normally within these limits. In terms of sugar utilization, JSZ06 was able to catabolize and utilize D-Maltose, D-Trehalose, D-Cellobiose, α-D-Glucose, D-Mannose, D-Fructose and other sugars, on the other hand it could not fully utilize D-Galactose, D-Turanose, Stachyose and others. In addition, JSZ06 can also utilized D-sorbitol, D-mannitol and glycerol, but not D-arabinitol. In terms of amino acid utilization, a variety of amino acids including L-alanine, L-arginine, L-aspartic acid, and L-glutamic acid can be utilized, but not L-histidine, L-pyroglutamic acid, and D-serine (Supplementary Table S2). In summary, JSZ06 can adapt to a variety of micro-environments and can decompose and utilize different kinds of organic matter, which is consistent with the physiological and biochemical characteristics of *Bacillus* spp.

3.3.3 Genomic characterization and phylogenetic analysis of isolates

Electron microscope scanning (SEM) showed that strain JSZ06 organisms were short rod-shaped or kidney-shaped, with an unsmooth bacterial surface. Their lengths ranged from 0.75 to 1.20 μm and widths from 0.52 to 0.75 μm (Figure 2A). We then analysed the 16S rRNA region of the whole genome of JSZ06 with the aim of precisely identifying the species (Figure 1A). The strain JSZ06's 16 S RNA sequences compared to the GenBank library revealed the greatest degree of similarity to *Bacillus siamensis*. Predicted on the sequences' evolutionary tree, JSZ06 was found to be similar to *Bacillus siamensis* (GenBank accession number: MN 176482). By examining the morphological and genetic features mentioned above, bacterium JSZ06 was recognized as *Bacillus siamensis* (GenBank accession number: PP226927) (Figure 2B). The whole genome of the antagonist bacterium JSZ06 was sequenced with a total distance of 3,935,105 bp and a 46.5% G + C ratio (Login number: CP143783). The 3,749 genes that code for proteins, 86 tRNA genes, and 27 rRNA genes made up the whole genome (Figure 2C).

3.4 Detection of antibiotic synthesis gene of strain JSZ06 and its functional test results

The PGP traits of strain JSZ06 were assessed and represented in Figure 3A. Strain JSZ06 was able to produce IAA at 6 h of incubation and up to 64.23 mg/L at 48 h with a total IAA production ranging from 27.27 to 64.23 mg/L (Figure 3B). A CAS assay is shown in Figure 3C and the λ/λ0 value of JSZ06 after 48 h was 0.33, which is in the category of a high iron carrier producing bacteria. Also, ACC utilisation attained a peak of 2.07 U/L at 48 h

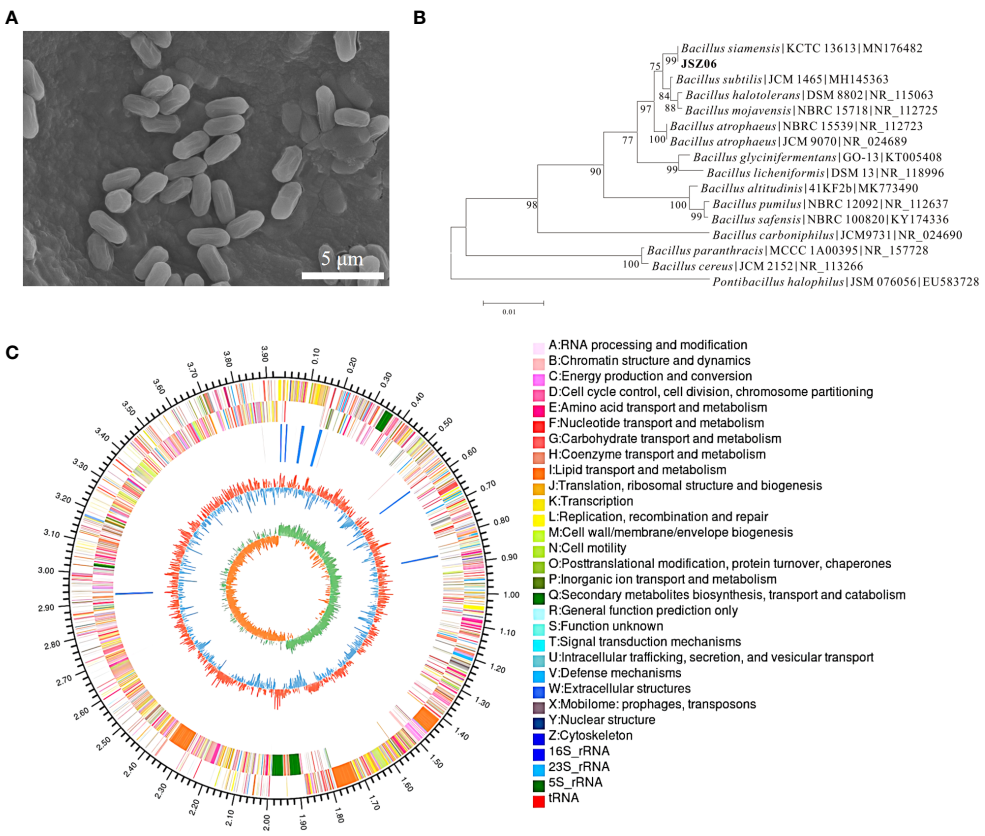

FIGURE 2
Characterization of strain JSZ06. **(A)** Scanning electron micrograph of JSZ06. **(B)** Phylogenetic tree of 16S rRNA gene of JSZ06. **(C)** JSZ06 strain genome ring diagram. Genome size identification is represented by the outermost circle of the ring diagram; CDS on the positive and negative strands are represented by the second and third circles; rRNA and tRNA are represented by the fourth circle; GC content is represented by the fifth circle; and the GC-Skew value is represented by the innermost circle.

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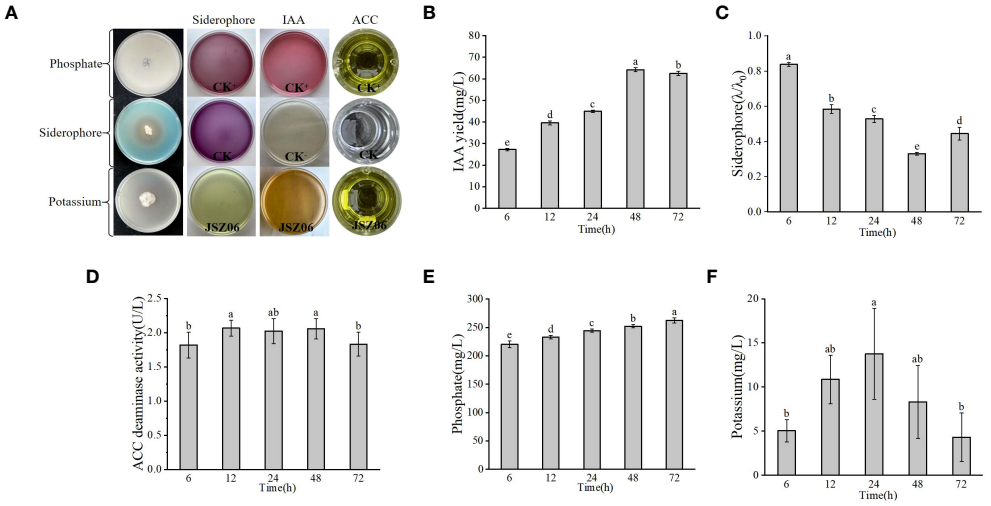

FIGURE 3
Evaluation of PGP traits and genetic detection of antimicrobial active substances in plant endophytes. **(A)** PGP traits of JSZ06. **(B–F)** Determination of IAA, iron carrier, ACC, phosphate solubilisation and mineral potassium solubilisation yields during culture of endophyte JSZ06. The error bars represent the standard deviations of the means from three independent experiments. Different lowercase letters indicate a significant difference at the level of $P< 0.05$. Production of siderophores (λ/λ_0) ranges from small (0.8-1.0), low (0.6-0.8), moderate (0.4-0.6), high (0.2-0.4), to extremely high (0-0.2).

(Figure 3D). In addition, strain JSZ06 was able to solubilise mineral phosphate and mineral potassium up to a maximum of 262.47 mg/L and 13.77 mg/L (Figures 3E, F), which indicated that the endophytic strain JSZ06 possesses good growth-promoting properties and has the capacity to enhance stress tolerance in plants and encourage plant development.

PCR of the strain showed that strain JSZ06 had seven Non-Ribosomal Peptide Synthetases (NRPS) producing genes (Supplementary Figure S3; Supplementary Table S4), four Polyketide Synthase genes (PKS) for four Polyketide metabolites, and growth-promoting related genes for Ribosomal Peptide Synthetases (RPS), including *srfAA*, *yndJ*, *fenD*, *ituC*, *yngG*, *bamD*, *dhb* and *dfn*, *bae*, *mln*, *bac* and *sboA*. This is instructive for subsequent genome-wide prediction and isolation and identification of antimicrobial substances.

3.5 Inhibitory growth optimisation of the antagonist bacterium JSZ06

The process of creating bactericidal materials by the antagonist JSZ06 was favoured when the culture conditions were at a suitable pH level, fermentation temperature, culture speed and inoculum amount. At a pH below 4.5 and above 8.5, the concentration in the broth was considerably less than in the other concentration gradients, and the OD_{600} values were only 2.06 and 2.10. The result suggested that both too low and too high pH would inhibit the growth of the strain. At the pH of 7.5, the OD_{600} value of the fermentation broth was the highest at 2.58 and was the highest inhibition rate of fermentation broth against pathogens at 61.46% (Supplementary Figure S4A). When the temperature was at 33°C, The strain's highest OD_{600} value for broth production was 2.64, and the inhibition rate was 64.48%, which was the best effect (Supplementary Figure S4B). Different rotational speeds had different effects on the OD_{600} value and the inhibition rate of the incubation fluid of the microorganisms. The OD_{600} values of the fermentation broths were similar when the rotational speeds were 180 and 200 rpm, but the inhibition rate was larger at 200 rpm, which was 62.66% (Supplementary Figure S4C). Whereas, when the inoculum was 2%, the strain fermentation broth OD600 value and inhibition rate were higher at 2.73 and 56.58%, respectively (Supplementary Figure S4D). Therefore, the optimal pH, fermentation temperature, culture speed and inoculum amount of the strain were comprehensively defined as 7.5, 33°C, 200 rpm and 2.0%, respectively.

3.6 Assay for wide-spectrum fungicide action

Strain JSZ06 and its extracts showed good broad-spectrum resistance and significantly inhibited the growth of 10 phytopathogenic bacteria (Figure 4). Strain JSZ06 showed the highest inhibition of 82.29% against *Rosellinia necatrix* and the lowest inhibition of 52.12 against *Fusarium oxysporum* (Figure 4A). The extract of strain JSZ06 showed the highest inhibition of 62.44% against *Rosellinia necatrix* and the lowest inhibition of 28.85% against *Setosphaeria turcica* (Figure 4B).

3.7 Inhibition of mycelial growth of *Foc* TR4 by extracts of strain JSZ06

Seven days later, extracts from strain JSZ06 greatly slowed the development of *Foc* TR4's mycelium. The inhibitory ability became more pronounced with a gradual increase in the concentration of the extract (Figure 5A). In contrast to the *Foc* TR4 development width in the control plate (7.00 cm ± 0.20), the mycelial growth diameter in

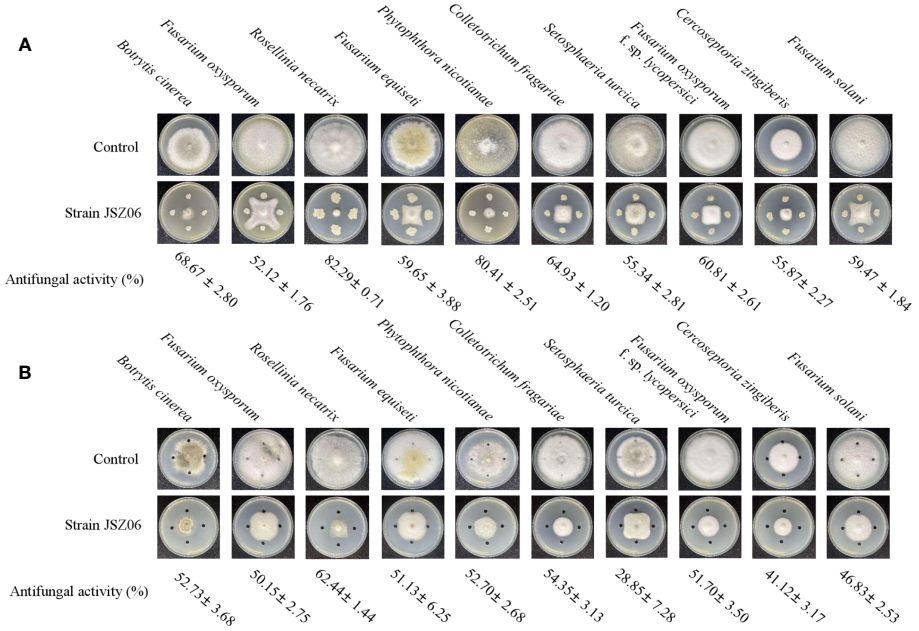

FIGURE 4
Wide-ranging antifungal action of JSZ06, an antagonistic fungus. **(A)** The ability of strain JSZ06 to inhibit a certain plant-pathogenic fungus. **(B)** Crude extract's antifungal efficacy against a specific plant-pathogenic fungus.

the 12.5 mg/L extract treatment group was significantly reduced to 4.94 cm ± 0.07. Upon achieving 200 mg/L of strain JSZ06 extract level, considerable suppression of *Foc* TR4 development was observed, with a suppression rate of 88.3% ± 0.02 (Figure 5B). The toxicity regression equation was further established ($y = 1.17861x + 3.27375$, $R^2 = 0.94537$), and the JSZ06 extract's EC_{50} value against *Foc* TR4 was 29.15 mg/L, was defined as 1 × EC_{50} in the follow-up study.

3.8 Effects of JSZ06 extracts on *Foc* TR4's mycelial morphological features

By electron microscopy scanning, the *Foc* TR4 mycelium sampled from the inhibitory zone's edge were different to the control. Normal mycelia in the control group had a smooth surface and normal, smooth, and plump tops. The mycelia

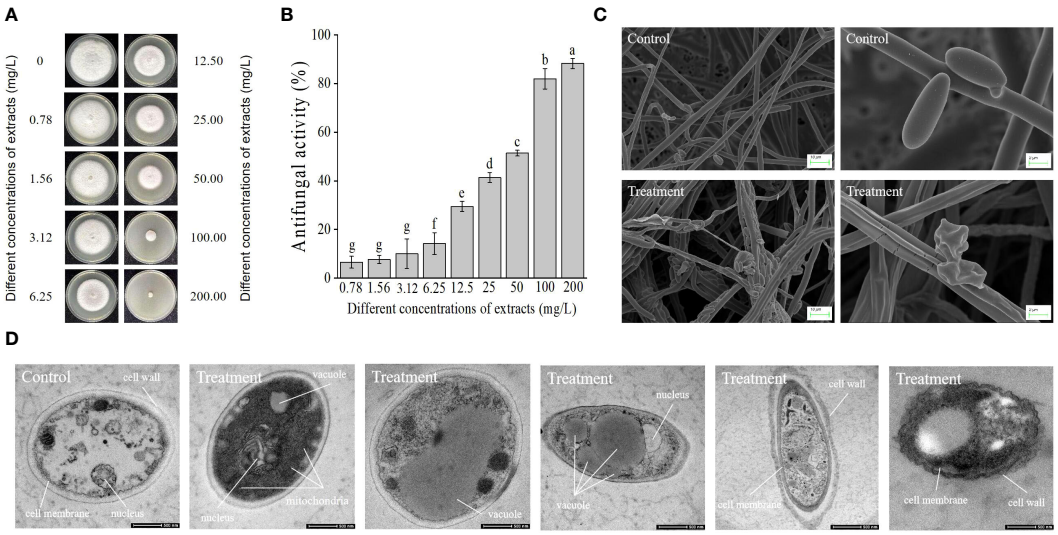

FIGURE 5
Impact of strain JSZ06 extracts on *Foc* TR4 mycelial development and ultramorphology. **(A)** *Foc* TR4 growth inhibition on PDA medium after different extraction dosages were applied. **(B)** Growth inhibition rate of *Foc* TR4 after treatment with different extraction doses. **(C)** SEM observation of the mycelial morphology of *Foc* TR4. **(D)** TEM micrographs showing 50 mg/L of strain JSZ06 treated *Foc* TR4 cells. The *Foc* TR4 mycelium treated with 10% DMSO is characterized by blanks. *Foc* TR4 treated with 50 mg/L of strain JSZ06 extract and its mycelial morphology. At the significance threshold of $P < 0.05$, different lowercase letters indicate a significant difference.

subjected to JSZ06 extract treatment, were hard, swollen irregularly, and the cell walls had crumbled. The branches on the mycelial tips were in the form of webbed feet and had spherical, fusiform swelling (Figure 5C). Furthermore, the exposed surface of the *Foc* TR4 spores to the strain extracts exhibited depression and deformation. The spore surface in the control group, however, was uniform, regular, and normal, indicating that the extract significantly impacted *Foc* TR4 growth (Figure 5C).

3.9 Influence of JSZ06 strain isolates on the ultrastructure of *Foc* TR4

The texture of the *Foc* TR4 mycelium after 50 mg/L of treatment of the extract was visualized by transmission electron microscopy. The untreated treatment's cell walls and membranes were still visible. There were also several undamaged organelles on display, including the nucleus and vacuole (Figure 5D). After treatment with extracts of strain JSZ06, the number of mitochondria with aberrant cell walls increased significantly and the nucleus disintegrated (Figure 5D). The vacuole grew until it eventually ruptured (Figure 5D). Organizational disarray, matrix blurring, and organelle lysis were observed in *Foc* TR4 cells, with plasma-wall segregation, and disruption and progressive deterioration of the cell membrane and membrane integrity (Figure 5D).

3.10 Impact of strain extracts on *Foc* TR4 spore morphological features and germination

In this study, the impact of the crude extract from the antagonistic strain JSZ06 on both the growth and spore formation of the pathogenic fungus *Foc* TR4 was thoroughly investigated (Supplementary Figure S5A). A considerable variation in the impact of various crude extract levels on the development of spores ($P< 0.05$) was found. As the concentration of the bacterial strain extract increases, the growth rate of spores reduces as a consequence. The highest concentration that proved most effective for the growth of spores was 8 × EC_{50}. This fungus' maximal spore development inhibition towards *Foc* TR4 was 10%. As a result, the 10% DMSO (v/v) treatment served as a means of control and had no effect on the harmful fungi's ability to germinate.

The shape of *Foc* TR4 before and after 1 ×, 2 ×, 4 × and 8 × EC_{50} treatment was further studied by SEM (Supplementary Figure S5B). The untreated spores that received a 10% DMSO treatment had a smooth surface and were undamaged. Greater morphological changes, such as stretching, loss of volume, disintegration, folding, bending, and cell rupture, were observed in spores treated with 2 ×, 4 ×, and 8 × EC_{50} crude extracts, significantly disrupting the normal morphology and integrity of the spores.

3.11 Genome analysis using bioinformatics

By annotation, GO, COG, and KEGG groups were assigned to 2,286; 3,077, and 2,377 encoding genetics, correspondingly. In the GO category, the following types of anticipated genes were identified: molecular roles (1,861), parts of cells (1,145), and processes in biology (1,235) (Supplementary Figure S6A). These genes were divided into four groups of 23 genes under the COG category. The biggest subcategory was physiology (1,479), which was followed by communication and processes in cells (957) and information storage and processing (641). Ultimately, 392 genes were classified as inadequately described (Supplementary Figure S6B). Based on KEGG evaluation, 41 pathways including 2,377 genes that encode proteins were identified (Supplementary Figure S6C). The major components of resistance to disease include the processing of signals, secondary metabolite production, and terpenoids and polyketide degradation. The genome of bacterium JSZ06 was projected to include twelve metabolic gene groups by antiSMASH software compared with GenBank, including NRPS gene cluster, PKS-like gene cluster, gene cluster for terpenes, gene cluster lanthipeptide-class-ii, TransAT-PKS gene cluster, the T3PKS gene cluster, Betalactone gene cluster, NRP-metallophore gene cluster and RIPP-like gene cluster (Supplementary Table S5). Eight of these gene clusters could be compared to similar gene clusters capable of producing Bacillaene, Fengycin, Difficidin, Bacillibactin, Surfactin, Butirosin A, B, Macrolactin H, bacilysin and other anti-substances (Supplementary Figure S8). The corresponding chemical structural formulae are shown in Supplementary Figure S7.

3.12 Detection of bioactive compounds by LC-MS/MS from strain JSZ06 extract

JSZ06 metabolites' anti-*Foc* TR4 active components were examined using LC-MS. The positive and negative ion flow chromatograms are shown in Supplementary Figure S9. The final statistical results showed that, via missing value filtering of the initial data, lacking appreciate converting, data normalization, Quality Control (QC) confirmation, and information change, the POS (+) and NEG (-) models produced 1,259 and 1,050 metabolites, accordingly, of which 1,086 and 1,021 compounds have been added to publicly accessible databases like HMDB and Metlin (Table 1).

Assigning the above metabolites to the KEGG and HMDB databases, 542 and 449 metabolites were categorized into 18 KEGG secondary pathways, respectively, of which 509 metabolites were categorized as "metabolites". Among the metabolites, the most abundant was "Amino acid metabolis", followed by "Biosynthesis of other secondary metabolites", "Metabolism of cofactors and vitamins", "Xenobiotics biodegradation and metabolism", "Lipid metabolism", "Metabolism of other amino acids" and "Carbohydrate metabolism" processes (Supplementary Figure S10A). Within the KEGG secondary pathway, 63 metabolites are designated as "biosynthesis of other secondary metabolites". Of these, there are a variety of antimicrobial metabolites in the KEGG Significant Pathway Statistics modeling synthesis pathway, including "biosynthesis of various other secondary metabolites" (13), "biosynthesis of various alkaloids" (12), "biosynthesis of various antibiotics" (10), "biosynthesis of neomycin, kanamycin, and gentamicin" (6), "biosynthesis of carbapenems" (4), "Biosynthesis

TABLE 1 Data about detected metabolites.

Ion Mode	Every Peak	Determined Metabolites	Libraries' Metabolites	KEGG's metabolites
POS	6751	1259	1080	542
NEG	10919	1050	1016	449
Total	17670	2309	2096	991

Number of POS and NEG; number of metabolites annotated in the corresponding database.

of Penicillins and Cephalosporins" (4), "Vancomycin Resistance" (3), "Staurosporine Biosynthesis" (3), "Novobiocin Biosynthesis" (3), "Taurine and Low Taurine Metabolism" (3), "Prodigiosin Biosynthesis" (3), and others (Supplementary Figure S11). These pathways involved in the synthesis of antimicrobial metabolites play crucial roles in exerting antagonism against pathogenic bacteria.

The 1080 and 1016 metabolites identified in the secondary analysis were cross-referenced with the 16 HMDB superclasses available in the HMDB database. Among these, 657 (31.35%), 443 (21.14%), and 375 (17.89%) metabolites were categorized under "Organic acids and derivatives," "Lipids and lipid-like molecules," and "Organoheterocyclic compounds," respectively. These classifications constituted the predominant portion within all categories, as illustrated in (Supplementary Figure S10B).

3.13 Identification of antimicrobial metabolites of strains

To further identify antimicrobial substances in the fermentation broth, a total of 2,309 identified substances were analyzed. The findings revealed a wide range of main intermediates in the strain JSZ06 extract. Several antibacterial compounds were found among the secondary metabolites, all of which have demonstrated antimicrobial activity. These substances include Validamycin A, Neamine, Apramycin, Hygromycin B, and Surfactin in the POS, and Cephamycin C, Neomycin in the NEG, as well as Novobiocin, Oxytetracycline, Ribostamycin, Aerobactin, and others (Supplementary Table S6). These results suggest that strain JSZ06 is an advantageous strain with significant potential for biocontrol, as it produces a variety of antagonistic substances capable of preventing the spread of harmful fungi.

3.14 Effect of fermentation broth of strain JSZ06 on the biological prevention of banana wilt disease

After a total of 45 days of inoculation, severe wilting and necrosis symptoms were observed on leaves of the banana plants treated solely with *Foc* TR4 (Figure 6B). In contrast, banana leaves treated simultaneously with the fermentation broth of strain JSZ06 and *Foc* TR4 remained green and healthy (Figure 6E). Banana leaves treated with sterile water and the fermentation broth of strain JSZ06 separately showed no signs of disease (Figures 6A, F). Upon longitudinal sectioning of the banana pseudostems subjected to different treatments, those treated with *Foc* TR4 exhibited wilting, with the longitudinal sections turning brown-black (Figure 6D), while no significant infection was evident in the banana pseudostems treated with the fermentation broth of strain JSZ06 along with *Foc* TR4 (Figure 6G). Additionally, there were no signs of infection on pseudostems treated solely with sterile water or strain JSZ06 (Figures 6C, H). Furthermore, following treatment with the fermentation broth of strain JSZ06, both the disease severity indices of banana leaves and pseudostems showed a significant decrease (Figure 6I), with biocontrol efficiencies reaching 76.71% and 79.25%, respectively (Figure 6J).

3.15 Effect of the antagonistic bacterium JSZ06 on TR4 infestation of banana roots and bulbs

Forty-five days after inoculation with GFP-*Foc* TR4, JSZ06 drastically reduced the incidence of *Foc* TR4 infection on bananas (Figure 7). TR4 presence was detected in both banana roots and bulbs 7 days post-inoculation with TR4 alone. Conversely, in the JSZ06 treatment group, no TR4 hyphae were observed. Following a 14-day inoculation period, a significant amount of mycelia was observed in the banana roots and bulbs that had received TR4 injections only. However, mycelial growth was limited in banana roots treated with JSZ06 inoculation. Subsequent observations at 21 and 45 days post-inoculation revealed progressively larger mycelial infestations in the roots and bulbs of bananas treated with TR4 alone. In contrast, minimal mycelium was observed in both roots and bulbs of bananas inoculated with JSZ06, with the highest infestation observed only at 45 days, but still considerably lower compared to the TR4-alone treatment. Based on the tracking of TR4 infestation over time, we hypothesized that treatment with strain JSZ06 could gradually enhance banana plant resistance to TR4 mycelial invasion.

3.16 Effect of fermentation broth of strain JSZ06 on the development of banana plants

Pot trials were conducted to assess the impact of JSZ06 broth fermentation on banana development. Application of JSZ06 fermentation broth treatment resulted in a significant increase in plant height, leaf size, stem thickness, leaf width, above-ground biomass, and below-ground biomass during the banana growth

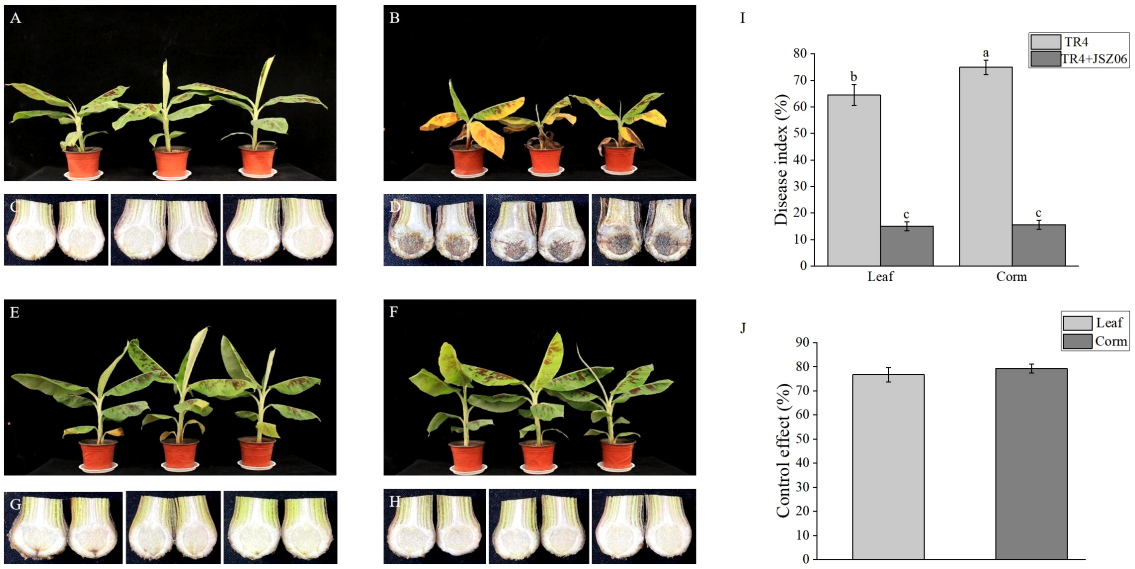

FIGURE 6
The growth and resistance to *Foc* TR4 in banana seedlings were affected by the antagonist strain JSZ06 fermentation broth. **(A, C)** Blank control without the JSZ06 or TR4 vaccination. The injection of TR4 (1 × 10^6 cfu/mL) in **(B, D)**. Inoculation with JSZ06 (1 × 10^8 cfu/mL) and TR4 (1 × 10^6 cfu/mL) **(E, G)**. JSZ06 (1 × 10^8 cfu/mL) inoculation **(F, H)**. **(I)** Indexes of banana leaf and bulb disease 45 days after *Foc* TR4 inoculation. **(J)** JSZ06 inoculation's controlling impact on banana bulbs and leaves. At the significance threshold of $P< 0.05$, different lowercase letters indicate a significant difference.

period compared to both the control and TR4-alone treatments (Figures 8A–F). It is noteworthy that the application of strain JSZ06 in the JSZ06 + TR4 treatment still significantly enhanced banana growth compared to the TR4-alone treatment, albeit to a lesser extent than the JSZ06-alone treatment. Thus, the antagonist bacterium JSZ06 was able to mitigate the inhibitory effect of TR4 on banana growth and promote growth to a certain extent.

3.17 Conceptual modeling

Based on the aforementioned results, a conceptual model illustrating the mechanism of action of the biocontrol bacterium JSZ06 against banana wilt was developed (Figure 9). The conceptual model indicates that JSZ06 reduces the incidence of banana wilt by producing antibiotic-type bacteriostatic agents that disrupt the structure of *Foc* TR4 mycelia and inhibit spore germination, thereby reducing the infestation of TR4 mycelia on bananas. Additionally, JSZ06 promotes banana growth by producing beneficial substances that enhance the plant's resistance to pathogenic bacteria. This symbiotic relationship contributes to the overall health and vigor of the banana plant, aiding in its defense against wilt disease.

4 Discussion

Fusarium oxysporum f. sp. *cubense* Tropical Race 4 (*Foc* TR4), a soil-borne fungus, poses significant threats to banana crops and currently lacks practical control methods (Dita et al., 2010). Sources of infection for this pathogen include bacterial sucking buds, diseased plant residues, and contaminated soil (Zhou et al., 2023). While chemical management of TR4 banana wilt is often quick and effective, it can lead to soil and aquatic environmental damage, and excessive usage may result in the development of resistance (Zhang et al., 2021a; Li et al., 2021b). In pursuit of sustainable agricultural practices, attention has shifted towards green and pollution-free biological agents. In recent years, endophytic bacteria have gained prominence due to their ability to enhance plant growth, manage soil-borne infections, and their environmentally friendly, safe, and non-toxic characteristics (Glick, 2012; Papik et al., 2020). Therefore, the selection of highly potent and broad-spectrum resistant bacteria is crucial for the development of effective biocontrol formulations.

Bacillus endophyticus is a potential biocontrol resource characterized by high resistance, broad-spectrum antimicrobial resistance and low pathogenicity to plants (Mon Myo et al., 2019; Fan et al., 2021). Furthermore, they have the capacity to generate a wide range of secondary metabolites that are capable of successfully impeding plant diseases and causing plant colonization, it encourages plant development (Ma et al., 2017). They are thought to be the best biocontrol agents for preventing diseases caused by soil-born pathogens like *Foc* (Fan et al., 2021). Banana wilt is now effectively controlled by a wide variety of biocontrol *bacilli*, such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus velezensis* and many others (Fan et al., 2021; He et al., 2021), all of which have better control effect on banana wilt. Therefore, the use of *Bacillus* to control banana wilt has a promising prospect. In this study, 11 strains of antagonistic bacteria were screened from 60 strains of smooth vetch (Figure 1; Supplementary Figure S2). Among which strain JSZ06 and its secondary metabolites exhibited strong inhibitory activity against *Foc* TR4 and 10 phytopathogenic fungi (Figure 4). After morphological and molecular biological,

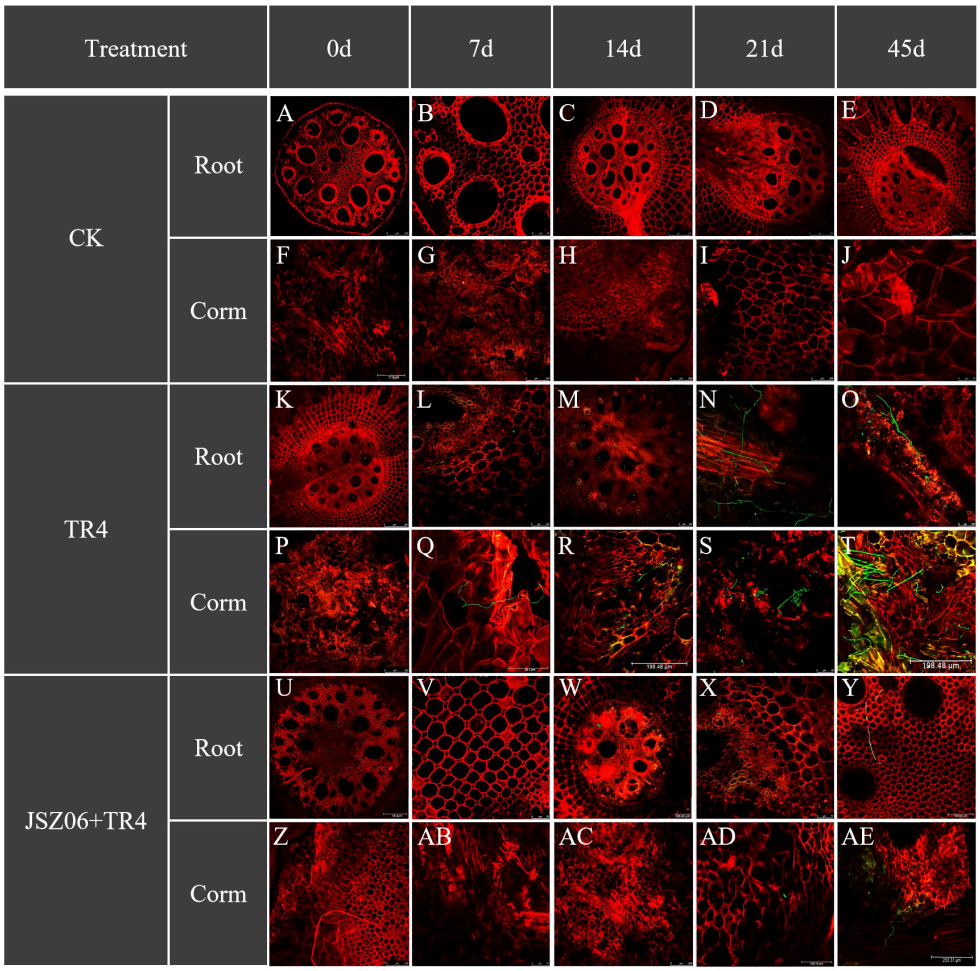

FIGURE 7
Infestation process of banana roots and bulbs at different time points (0 dpi, 7 dpi, 14 dpi, 21 dpi and 45 dpi) after inoculation with GFP-*Foc* TR4 and strain JSZ06. Bar = 100 μm in **(N, O)**; Bar = 126.14 μm in **(F, AD)**; Bar = 198.48 μm in **(R, T)**; Bar = 250 μm in **(A–E, G–M, P, S, U–Z, AB, AC)**; Bar = 253.31 μm in **(Q, AE)**.

physiological, and biochemical characterization, strain JSZ06 was identified as *Bacillus siamensis* (Figure 2; Supplementary Tables S1, S2). Antibiotic drugs and their precursor substances produced by *Bacillus* spp. are widely used in agriculture, industry and medical industry (Sharon et al., 1954; Tiffin, 1958). The extracts isolated in this study by ethyl acetate and methanol showed effective antifungal activity, which was consistent with the *in vivo* inhibitory activity, and the effectiveness of the blockage was positively correlated to each extracts' quantity. 200 mg/L of extract completely stopped the development of *Foc* TR4 hyphae (Figures 5A, B) and completely inhibited spore growth of *Foc* TR4 at concentrations equal to or greater than 8 × EC_{50} (Supplementary Figure S5). Inhibition of pathogen spore germination is important to protect crop and soil health (Chang et al., 2021; Fan et al., 2023). It reduces crop infestation caused by spore germination and the number of dormant spores in the soil and is an effective way to control banana wilt (Kammadavil Sahodaran et al., 2019; Chang et al., 2021). Similar findings showed *Bacillus* extracts could significantly inhibit *Foc* TR4 mycelial and sporulated growth (Ponnusamy et al., 2018; Shen et al., 2022).

Biofilm systems and organelles are crucial for preserving the cell shape and function of pathogenic fungi (Li et al., 2021b). In this research, a *Foc* TR4 mycelium treated with extracts of JSZ06 showed deformities, cross-linking, swelling, shortened internodes and increased branching, and degradation of cell walls, cell membranes and organelles (Figures 5C, D). By PCR amplification, it was detected that strain JSZ06 possessed 12 antimicrobial substance-related synthesized genes (Supplementary Figure S3, Supplementary Table S4), which may use gene expression to block the proliferation of *Foc* TR4 and produce antimicrobial compounds (Fan et al., 2021; Li et al., 2021a). Many investigations have shown that metabolites produced by antagonistic bacteria all lead to the loss of cellular integrity of pathogenic fungi (Chen et al., 2018; Wei et al., 2020; Li et al., 2021c; Zou et al., 2021), and the possible mechanism is to induce the activation of chitinase to hydrolyse the *Foc* TR4 cell wall (Zhang et al., 2021a), inhibit *Foc* TR4 biosynthesis and induce oxidative stress and apoptosis (Chen et al., 2018; Chen et al., 2020), which ultimately leads to the damage of the fungal outer wall membrane structure and the function of the internal organelles, the leakage of the contents, and the inhibition of the growth of phytopathogenic fungi. The

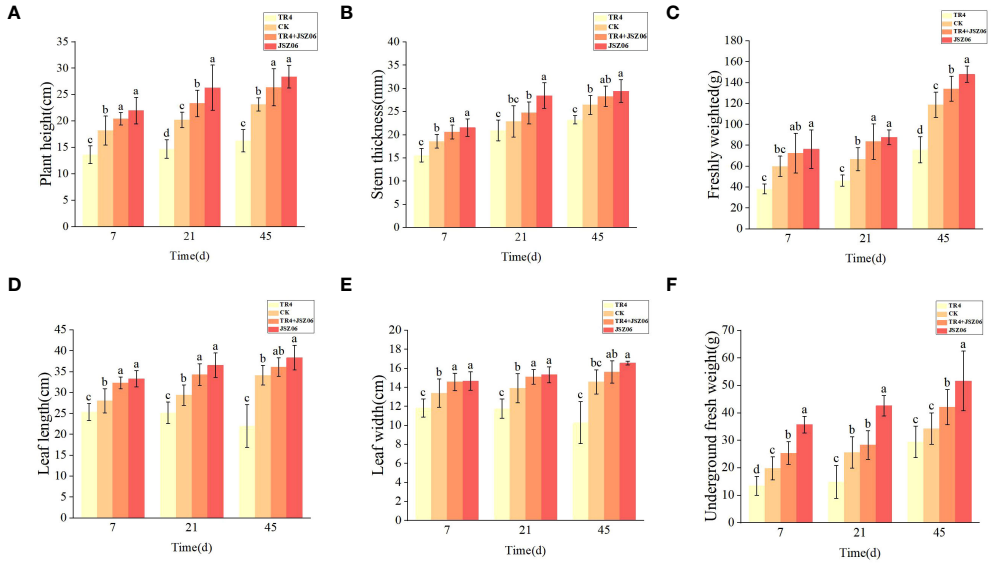

FIGURE 8
The development and growth of banana seedlings was greatly enhanced by the antagonist bacteria JSZ06 fermentation broth. Physiological indicators in the control and treatments of banana seedlings: **(A)** plant height, **(B)** stem thickness, **(C)** above-ground biomass, **(D)** leaf length, **(E)** leaf width, and **(F)** root biomass, measured after 7, 21, and 45 days, respectively. At the significance threshold of $P< 0.05$, different lowercase letters indicate a significant difference.

identification of antimicrobial substance-related synthesis genes in the genome of the antagonist fungus JSZ06 supports this assumption. In conclusion, these findings further reveal the potential antagonistic mechanism of strain JSZ06 against *Foc* TR4.

In order to further reveal the mechanism, the antifungal properties of JSZ06 were preliminarily characterized using LC-MS coupling technique (Supplementary Figures S10, 11, Supplementary Table S6). LC-MS plays an important role in the

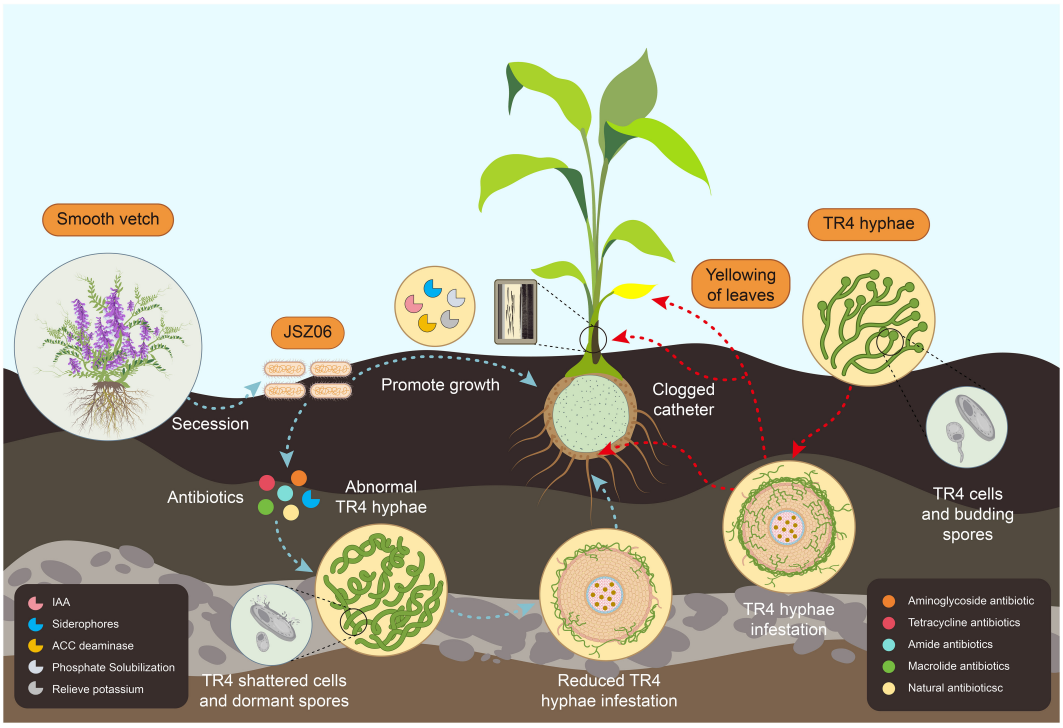

FIGURE 9
A conceptual model of the mechanism of action of strain JSZ06 in reducing banana wilt disease.

discovery of microbial metabolites (Caldeira et al., 2011). The extracts of strain JSZ06 contained a variety of antibacterial chemicals, the largest group being aminoglycoside antibiotics, which exhibits potent antibacterial activity as has been extensively shown (Khan et al., 2020; Wang et al., 2022). Zimmermann et al. (2016) reported that the synthesis of Neamine antibacterial amphiphilic AG enables the synthesis of new antifungal derivatives. It has also been found that Gentamicin and Hygromycin B can be used as antifungal drugs (Farzaneh, 2021), and that Hygromycin B binds to steroids on the fungal cell membranes, altering the membrane permeability and causing disruption of the fungus, and thereby actimg as a fungicide (Brodersen et al., 2000; Armillas Canseco et al., 2015). Additional tetracycline antibiotics include Sarecycline, Rolitetracycline, Minocycline, and Oxytetracycline, of which Oxytetracycline has been shown to inhibit the growth of fungal populations in the soil (Wang et al., 2017). Two amide antibiotics, Macrolactin A (7-O-Succinyl) with Cycloheximide, were also found, and Olishevska et al. (2019) discovered that 7-O-Succinyl macrolactin A, which *Bacillus amyloliquefaciens* was capable to generate, has an antifungal efficacy against both plant infections and damaged fungal cells (Kawai et al., 2023). Validamycin A and Actinonin were also found to be two types of natural antimicrobial agents. Actinonin as an antimicrobial agent has not yet been fully elucidated in terms of its mechanism of resistance to phytopathogenic fungi, but numerous investigations have shown possible mechanisms that can lead to inhibition of fungal growth through the inhibition of fungal protease activity and interference with fungal cell wall synthesis and metabolic pathways (Chen et al., 2000). Validamycin A, as a broad-spectrum antifungal pesticide, has been used in large quantities for the control of plant diseases, which can prevent fungus development and reproduction by inhibiting the synthesis of fungal wall polysaccharides, and interfering with fungal sugar metabolism and biosynthesis pathways (Wu et al., 2017; Bian et al., 2021). Some ester antibiotics, including Erythromycin C, Epothilone C and Surfactin, were also identified (Nayeem et al., 2009). Surfactin in particular is thought to have significant activity antagonistic to *Foc* (Vitullo et al., 2012), which can rupture fungal cell membranes, resulting in damage to the cell membrane leading to cell death and lysis (Fatin et al., 2017; Chen et al., 2022), thereby inhibiting spore germination and impeding the reproduction and infestation of the fungus (Romero et al., 2007). Surfactin additionally induces the upregulation of genes associated with plant defence and stimulates the synthesis of antimicrobial compounds in plants (Ongena and Jacques, 2008). Notably, the presence of surfactin in both the extracts of the strain identified by LC-MS and the gene cluster products predicted by the above mentioned antiSMASH further validates the above described inference that surfactin plays a substantial role in the antimicrobial effect exerted by strain JSZ06 (Supplementary Figure S8, Supplementary Table S5). However, there were also some substances that failed to match each other, likely as a result of the detection technique's inability to detect all of strain JSZ06's compounds (Li et al., 2021c). It has also been shown that the yield and number of antagonistic substances can be further increased by optimization of fermentation conditions (Duan et al., 2020; Zhang et al., 2021b) (Supplementary Figure S4). It is therefore assumed that all of these substances are important in supporting JSZ06's wide-ranging antifungal action.

The pot inoculation test showed that the antagonist bacterium JSZ06 possesses a powerful biocontrol impact against banana wilt (Figure 6). In this study, GFP-*Foc* TR4 was employed to track the TR4 infection process in banana cultivars (He et al., 2021) (Figure 7). These findings demonstrated that TR4 may pierce the root epidermis, invade the xylem conduit to form mycelium, and expand and grow a mycelium in banana tissues. At the later stage of disease development, TR4 would form new spores in banana tissues, thus starting a new round of the infestation process. These occurrences generally concur with the findings of earlier research (Ploetz and Pegg, 1997; Ploetz, 2006; Li et al., 2011; Zhou et al., 2023). Prior studies on the TR4 infection pathway have focused on the invasion of plant bulbs (Dita et al., 2018; Zou et al., 2021; Li et al., 2021b). In our investigation, we noted that the TR4 mycelium entered the bulb through the vasculature from the root, and the amount of TR4 mycelium in the root system was significantly greater compared to the bulb. Meanwhile, the number of mycelia in banana roots and bulbs (JSZ06 + TR4) when JSZ06 was applied was noticeably less than that in the TR4 treatment (TR4), proving that strain JSZ06 can further prevent the pathogen from infecting banana.

In addition, the pot inoculation test greatly aided in the development of banana seedlings in terms of thick stems, plant height, leaf length, leaf width, as well as above-ground and below-ground fresh biomass (Figure 8). In addition to greatly reducing banana wilt, the antagonist bacteria promoted the development of banana plants (Fan et al., 2021). Further investigations into the mechanism of action suggest that it may be related to the creation of IAA, iron carrier, ACC deaminase, solubilized inorganic phosphorus as well as activated potassium by the antagonist bacterium JSZ06 (Figure 3). Biocontrol bacteria with production of iron carriers and IAA can promote plant growth and increase stress resistance (Hassan, 2017; Lü et al., 2023). Iron carriers not only compete for pathogenic iron and interact with pathogenic iron-associated enzymes, and thereby inhibiting growth-limiting pathogens, but also enhance plant health and productivity by improving the plant's iron nutritional status (Compant, 2005; Shen et al., 2022). Bacteria with the ability to detoxify phosphorus and potassium have great potential in regulating plant growth (Rodríguez and Fraga, 1999; Vessey, 2003; Hassan, 2017), while ACC deaminase reduces ethylene in plants and improves rooting and tolerance for plant growth (Glick, 2014). Therefore, the antagonist bacteria JSZ06's showed strong bacteriostatic and life-promoting properties, which may have many uses in agricultural production. It is worth noting that the promotion of endophytic strain JSZ06 may also be related to the finalization mechanism of the strain in plant roots (Sahu et al., 2023), to be followed up with further studies. Several studies have also found that plants recruit specific microorganisms from surrounding soil species to colonize the plant, become core microorganisms and perform specific functions (Lundberg et al., 2012; Bulgarelli et al., 2013), which further cinfirms the study of endophytes in smooth vetch.

5 Conclusion

The antagonist bacterium *Bacillus siamensis* JSZ06, along with its extracts, exhibited potent bacteriostatic effects against *Fusarium oxysporum* f. sp. cubense Tropical Race 4 (*Foc* TR4), effectively inhibiting mycelial development and spore germination of this pathogen. Treatments with extracts from the JSZ06 strain led to notable deformations of mycelial cells and the dissipation of internal structures. Consequently, all ten tested phytopathogenic fungi demonstrated susceptibility to the broad-spectrum suppressive activity of both the JSZ06 strain and its crude extracts. Strain JSZ06 was found to possess 12 pairs of bioprophylaxis-promoting related genes, which play a key role in the synthesis of antibiotics, as well as key growth hormone synthase genes. Further investigations into the growth-promoting abilities of the JSZ06 strain uncovered a significant potential, marked by the capacity for indole-3-acetic acid (IAA) production, iron chelation, ACC deaminase activity, and the solubilization of inorganic phosphorus and potassium. Moreover, the application of the fermentation broth derived from *Bacillus siamensis* JSZ06 notably enhanced the resistance of banana plants to *Foc* TR4 infection and stimulated the growth of banana seedlings. Twenty-seven primary inhibitory active substances were identified within the extracts of the JSZ06 strain. This study demonstrates that *Bacillus siamensis* JSZ06 can serve as an effective biocontrol agent against *Foc* TR4 infestation in bananas, while concurrently promoting banana growth.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

YR: Formal analysis, Writing – original draft, Writing – review & editing. CN: Conceptualization, Data curation, Investigation, Software, Writing – original draft. AJ: Visualization, Writing – review & editing. HF: Methodology, Writing – original draft. LF: Writing – original draft, Project administration, Supervision. SZ: Supervision, Methodology, Resources, Writing – review & editing. SL: Methodology, Supervision, Writing – review & editing, Data curation. ZW: Data curation, Methodology, Supervision, Writing – review & editing, Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Software, Validation, Visualization, Writing – original draft.

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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.2024.1410197/full#supplementary-material

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A novel exopolysaccharide-producing bacterium, *Pseudescherichia liriopis* sp. nov. isolated from *Liriope platyphylla*, enhances the growth of *Daucus carota* subsp. *sativus* under drought and salinity stress

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Biological and abiotic stresses in plant growth are associated with reduced crop yields. Therefore, improving plant stress resistance can be a crucial strategy to improve crop production. To overcome these problems, plant growth-promoting bacteria are emphasized as one of the alternative tools for sustainable agriculture. This study found a novel strain ($L3^T$) of a plant growth-promoting bacterium in fermented *Liriope platyphylla* fruit. Strain $L3^T$ showed the ability to promote plant growth. The $L3^T$ strain promoted plant growth of *D. carota* subsp. *sativus*, increasing the length (increase rate compared to the control group, 36.98%), diameter (47.06%), and weight of carrots (81.5%), ultimately increasing the edible area. In addition, we confirmed that plant growth was improved even in situations that inhibited plant growth, such as salinity and drought stress. Strain $L3^T$ performed indole production, siderophore production, phosphate solubilization, and nitrogen fixation, all characteristics of a strain that promotes plant growth. Genome analysis revealed genes involved in the growth promotion effects of strain $L3^T$. Additionally, the properties of exopolysaccharides were identified and characterized using FTIR, TGA, and UHPLC. Our results demonstrated that L3 isolated from fermented *L. platyphylla* fruit can be used to simultaneously alleviate drought and NaCl stress.

KEYWORDS

PGPB, plant-microbe interaction, stress tolerance in plants, exopolysaccharides, phylogenetic analysis

Introduction

In recent years, many research teams have shown increasing interest in exploring the potential of microorganisms to continuously improve agricultural productivity (Mustapha et al., 2020; Yan et al., 2020; Thakur et al., 2023). Microorganisms play an important role in various ecological processes, such as promoting plant growth and nutrient cycling. Microorganisms like plant growth-promoting bacteria (PGPB) can positively affect plant growth and development. Among PGPB, the L3^T strain we discovered was isolated from fermented *Liriope platyphylla* fruit. This is claimed to be a new bacterial strain with remarkable properties that have the potential to benefit both plant and human health. The plant growth-promoting (PGP) strain ability was demonstrated by fermenting *L. platyphylla* fruits near Dongguk University in the Republic of Korea and then inoculating the isolated strain L3^T into carrot plants. In addition, this paper is a continuation of research that isolated a novel species from *L. platyphylla* (Kim et al., 2020b, 2023a).

L. platyphylla is used as a traditional medicine for cough and lung inflammation diseases in some Asian countries, including the Republic of Korea (Hur et al., 2004). As a result of recent research, anti-obesity, anti-inflammatory, and the potential for estrogenic, antiplatelet, and antiviral effects were discovered in *L. platyphylla* (Tsai et al., 2013; Huang et al., 2014; Kim et al., 2016; Le et al., 2021). The *L. platyphylla* fruit, which is relatively less studied than the root, contains anthocyanins, has excellent antioxidant potential, and has been shown to inhibit collagenase (Lee and Choung, 2011; Truong et al., 2023). Collagen, which primarily constitutes the dermal layer of the skin, is broken down by an enzyme called collagenase. Therefore, inhibiting collagenase activity is a promising strategy to prevent skin aging, as it plays a crucial role in maintaining skin elasticity and strength (Ohtsuki et al., 2008; Morikawa et al., 2020). Some bacteria are known to produce collagenase. These microorganisms provide nutrients by breaking down collagen-like proteins around plant roots or can influence the regulation of the plant's immune response (Duarte et al., 2016; Bhagwat et al., 2018; Pequeno et al., 2019). Strain L3^T, isolated from the fruit of *L. platyphylla*, has the ability to promote plant growth and is a producer of novel exopolysaccharides (EPS).

EPS are high-molecular-weight natural polymers produced by microorganisms, including bacteria, fungi, and blue-green algae (Angelin and Kavitha, 2020). Some microbial-derived EPS have several physiological functions attributed to their antiviral, anti-inflammatory, and antioxidant activities (Wu et al., 2021; Kim et al., 2022). In addition, EPS production is less affected by seasons than polysaccharides made from animals or plants is easy to handle and manage, and has industrial advantages and potential for various applications in medicine, food, and agriculture (Daba et al., 2021). In particular, EPS produced by PGPB greatly help promote plant growth and play a role in protecting plants from abiotic stress (Liu et al., 2017). EPS produced by plant-derived bacteria protects plants from abiotic stresses caused by adverse conditions. EPS from PGP bacteria alleviate NaCl stress by inhibiting Na^+ uptake in plant roots and preventing translocation to leaves (Bhat et al., 2020). Moreover, bacterial EPS production is a crucial mechanism that aids survival under microenvironmental changes (Roberson and Firestone, 1992). EPS provide high moisturizing power, preventing both plants and bacterial cells from drying out, thereby enhancing their viability (Zhang et al., 2022). To withstand soil strategies to maintain high water content. This process helps sustain plant growth and prevents root desiccation (Naseem et al., 2018). Additionally, EPS not only protect plants from drought stress but also facilitate bacterial attachment to plant root (Skorupska et al., 2006; Bhagat et al., 2021).

In this study, *Pseudescherichia liriopis* sp. nov., isolated from fermented *L. platyphylla* fruit, demonstrates PGP properties. Additionally, its PGP ability was tested under conditions of salinity and drought overlapping stress, showing efficacy even under simultaneous drought and NaCl stress. This study provides insight into the abiotic stress tolerance of strain L3, suggesting that it can improve crop productivity and be considered a solution for eco-friendly and sustainable agriculture in response to increasing soil drought and salinization (Abdelaal et al., 2021; Hassani et al., 2021).

Materials and methods

Isolation of strain from fermented *L. platyphylla* fruit

A bunch of fresh *L. platyphylla* fruit was collected from a flowerbed located in Goyang, Gyeonggi, Republic of Korea (37° 40′40.9′′ N, 126 °48′24.9′′ E). The collected 10 g of fruit and 10 g of glucose were added to a 50-mL round tube, sealed, and fermented at 25°C for 1 month. Subsequently, a standard dilution method was performed, in which 0.1 mL of the previously fermented fermentation broth was added to 0.9 mL of 0.85% sterile saline and repeated. Aliquots of 0.1 mL of the sample suspension were spread on *Lactobacilli* MRS agar (BD Difco, Franklin Lakes, NJ, USA) plates and incubated at 25°C for 3 days. Colonies were then picked and purified by streaking 3 times under the conditions mentioned above. Selected strains were stored in 25% glycerol (w/v) at −80°C.

Indole-3-acetic acid

Auxin production was determined using a colorimetric method (Sachdev et al., 2009). After inoculating the strain into R2A medium supplemented with 0%, 0.05%, 0.1%, 0.2%, and 0.3% $_L$-tryptophan, it was cultured for 48h at 25°C and 160 rpm with shaking and then centrifuged to collect the supernatant. Equal volumes of Salkowski's reagent (9.8 mL of 35% perchloric acid and 200 µL of 0.5 M $FeCl_3$) were added to the supernatant, mixed, and left in the dark for 30 min. Absorbance at 530 nm was measured using a spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Waltham, MA, USA). The indole-3-acetic acid (IAA) concentration values of the strains were determined by

substituting them into the IAA standard curve (5 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, and 100 μg/mL).

Selection of PGPB candidates

IAA-producing strains were evaluated for PGP properties, such as phosphate solubilization, siderophore production, and nitrogen fixation. To check phosphate solubilization, PVK (Pikovskaya, 1948) was used, and the composition of the medium is as follows: $FeSO_4{\cdot}7H_2O$ (0.001%), yeast extract (0.05%), dextrose (1%), $(NH_4)_2SO_4$ (0.05%), $Ca_3(PO_4)_2$ (0.5%), KCl (0.02%), $MgSO_4{\cdot}7H_2O$ (0.0%), agar (1.5%), and $MnSO_4{\cdot}7H_2O$ (0.001%). Cultures were incubated at 25°C for up to 7 days to develop a clear zone around colonies grown in PVK medium. Siderophore production was confirmed using CAS agar (Schwyn and Neilands, 1987). Briefly, qualitative tests for siderophore production were performed: strain cultures were plated on plates supplemented with 10% CAS and incubated at 25°C for 1 week. An orange halo around a strain colony indicates a positive result. The nitrogen fixation capacity of the strains was studied on Jensen's medium, and the composition is as follows: agar (1.5%), $Na_2MoO_4{\cdot}2H_2O$ (0.2%), $FeSO_4{\cdot}7H_2O$ (0.01%), NaCl (0.05%), $MgSO_4{\cdot}7H_2O$ (0.05%), K_2HPO_4 (0.1%), sucrose (2.0%), and $CaCO_3$ (0.2%). Briefly, the strain culture was plated on Jensen's medium and cultured at 25°C for 7 days. Visual confirmation of colony growth on Jensen's medium indicates positive nitrogen fixation.

Strains for NaCl and drought tolerance

The method for selecting NaCl tolerance strains followed the protocol described by Kim et al (Kim et al., 2020a). NaCl (from 0% to 10% at 1% intervals; w/v) and was measured at various concentrations. In summary, various concentrations of NaCl were added to 10 mL of R2A liquid medium, then sterilized, and 200 μL was transferred to a 96-well plate. Next, 2 μL of the strain culture cultured for 24h was inoculated and incubated at 25°C for 48h and measured at 600 nm using a UV-vis spectrophotometer. R2A broth was used as a negative control at each concentration supplemented with NaCl without strain injection. To assess drought resistance, R2A medium was supplemented with PEG 6000 (Steinheim, Germany) at different concentrations (from 0% to 25% at 5% intervals; w/v), and 2 μL of each of strain $L3^T$ that had been incubated for 24h was added, followed by incubation at 25°C for 48h. The growth of the strains was compared to the negative control by measurement at 600 nm using a UV-vis spectrophotometer.

Identification and growth condition of the isolate

Genomic DNA was extracted from strains using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver. 3.0 (Takara Bio, Kusatsu, Japan). The 16S rRNA gene sequence of *Pseudescherichia* sp. $L3^T$ was amplified using the universal bacterial primer sets 27F, 518F, 805R, and 1492R (Weisburg et al., 1991). The sequence was further verified by screening the 16S rRNA gene from the genome using ContEst16S, and the 16S rRNA gene sequence obtained from the genome yielded the same results as before (Lee et al., 2017). It was also used to assess the contamination of the genome sequence. Multiple sequences were aligned using MEGA 11 software and analyzed using Cluster X (Tamura et al., 2021). Phylogenetic trees were reconstructed based on neighbor-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) algorithms. The NJ and ML algorithms were implemented using the Kimura two-parameter model (Saitou and Nei, 1987). A min-mini heuristic was applied to the MP to compare with the NJ and ML phylogenetic trees (Fitch, 1971) Phylogenetic tree topologies were evaluated via bootstrap analysis based on 1000 replications (Felsenstein, 1985). The reference strain *P. vulneris* JCM 1688^T was obtained from the Japan Collection of Microorganisms (JCM, Tsukuba, Japan).

Taxonomic analysis

The growth of the novel strain in different media, temperatures, NaCl concentrations, and pH levels followed the protocol (Kim et al., 2023b). The hydrolysis of Tween 80, DNA, casein, chitin, and carboxymethylcellulose, as well as Gram reaction, motility, oxidase, and catalase tests, were performed (Chhetri et al., 2021). Cells cultured on R2A agar at 25°C for 2 days were negatively stained using 3% uranyl acetate, and the morphology of the $L3^T$ strain was observed using a transmission electron microscope (Libra 120; Zeiss, Oberkochen, Germany). Biochemical and enzymatic tests were performed using the API 20NE kit according to the manufacturer's instructions (bioMérieux, NC, USA). To investigate the oxygen requirement of the strains, the oxygen in the anaerobic chamber was removed using oxygen absorption strips (Mitsubishi Gas Chemical, Tokyo, Japan) and monitored continuously.

Whole genome sequencing and annotation

Genomic DNA of *Pseudescherichia* sp. $L3^T$ was extracted using the TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit Ver. 3.0 (Takara Bio, CA, USA) following the manufacturer's protocol. The concentration and quality of the genomic DNA were checked using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). Genome sequencing of strain $L3^T$ libraries was performed using the (Illumina, CA, USA) HiSeq X platform and assembled using the SPAdes ver. 3.15 *de-novo* assembler (Bankevich et al., 2012). The genome contamination and completeness of the novel strain $L3^T$ were analyzed using the bioinformatics tool CheckM (https://ecogenomics.github.io/CheckM) (Parks et al., 2015). The genome of *Pseudescherichia* sp. $L3^T$ was annotated using the NCBI Prokaryote Genome Automatic Annotation Pipeline (ncbi.nlm.nih.gov/genome/annotation_pork). The draft genome was analyzed using Prokaryotic Genome Annotation (Prokka) v1.14.6 (Seemann, 2014). The ANI of the novel strain and its phylogenetically close relatives was calculated using EzBioCloud's

e-service and KBase wrapper (Goris et al., 2007). The genomes of strain L3^T and the reference strain, genes involved in secondary metabolism were predicted using antiSMASH 6.0 (Blin et al., 2019).

Plant inoculation experiment of strain L3^T

The plant growth promotion study was conducted for 2 months, from August to October 2022. A flower pot with a diameter of 18 cm and a height of 15 cm was used in the experiment. Strain inoculation was divided into two groups: non-inoculated (control) and inoculated with strain L3^T. Seeds were used after sterilization with minor modifications, as described by Chhetri et al (Chhetri et al., 2022). Briefly, seeds were first defatted with distilled water and then disinfected with 70% ethanol. Then, 0.5 mL of Tween 20 was added to 50 mL of 20% Clorox (v/v) and sterilized by shaking for 20 min. Cells of the strain L3^T were grown by shaking and culturing at 30°C and 160 rpm for 24h. The cells were collected by centrifugation at 8,000 × *g* for 5 min and washed with sterile distilled water to maintain an $_{OD}600$ close to 0.7. Strains were inoculated by spraying 25 mL of cell suspension (or 25 mL of sterilized R2A medium as a control) into the soil around the plants once every 2 weeks.

Inoculation under drought and salt stress

Carrot seeds were surface-disinfected with 70% ethanol for 3 min, dried, shaken with 20% Clorox (0.5 mL of Tween 20 added) for 20 min, and then washed five times with sterile distilled water. The seeds were soaked for 30 min under conditions i to viii, respectively, and then sown. Starting one week later, each group of plants was inoculated by spraying 25 mL of each of conditions i–viii into the soil around the plant roots once a week as follows: (i) 0.514 M NaCl; (ii) 0.514 M NaCl + L3^T; (iii) 5% PEG; (iv) 5% PEG + L3^T; (v) 10% PEG; (vi) 10% PEG + L3^T; (vii) 0.514 M NaCl, and 5% PEG; (viii) 0.514 M NaCl, 5% PEG, and L3^T. Strain L3^T used for plant inoculation was cultured for 48h, and then cells were collected by centrifugation at 8,000 x g for 5 min and washed with sterile distilled water to maintain the OD600 close to 0.7.

Exopolysaccharide extraction and purification

To extract the EPS of strain L3^T, the strain was first cultured in R2A medium for 24h. Then, 10 mL (1% of 1 L) of the culture was inoculated into 1 L of R2A supplemented with 1% glucose and cultured for 76h. The supernatant was obtained by centrifugation (8,000 × *g*, 15 min) and then centrifuged again to obtain a cell-free supernatant. Next, 14% trichloroacetic acid was added to the supernatant and shaken at 90 rpm for 30 min at 25°C. Afterward, centrifugation was performed again under the same conditions to remove denatured proteins, and this was repeated twice. Cold absolute ethanol stored at −20°C was added in an amount three times the volume of the upper layer, and the mixture was allowed to precipitate overnight in a 4°C refrigerator. The process was repeated twice. The precipitated EPS was separated, placed in a 50 mL round tube, and centrifuged (10,000 × *g*, 10 min) to completely remove ethanol. The separated EPS was dialyzed (3.5 kDa MWCO; SnakeSkin Dialysis Tubing, NJ, USA) for 48h against ultrapure water, which was changed every 12h. After confirming the presence of protein by spectrophotometry (NanoDrop spectrophotometer, Thermo Fisher Scientific, MA, USA), the completely purified EPS was lyophilized, weighed on a scale to determine the yield, and stored in a freezer at −80°C for further experiments.

Antioxidant activity

ABTS radical scavenging activity

The ABTS*+ scavenging capacity of EPS produced by strain L3^T was determined according to a previously reported method with some modifications (Yang et al., 2021). Briefly, 7 mM ABTS (2,2′-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) and 2.45 mM potassium persulfate were mixed at a ratio of 1:1 (v/v), then incubated in the dark for 16h at 25°C to generate ABTS radicals. 0.1 mL each of 1 mg/mL EPS solution and ABTS*+ solution was added, incubated for 5 min in a dark room at 37°C, and then measured at 734 nm using a spectrophotometer. Distilled water was used as a blank, and ascorbic acid (PC) was used as a positive control.

Hydroxyl radical scavenging activity

The OH* scavenging activity of strain L3^T was investigated (Tarannum et al., 2023). Briefly, 40 µL of 9 mM $FeSO_4$, 40 µL of H_2O_2 (0.03%, v/v), and 20 µL of 9 mM salicylic acid–ethanol solution were mixed. Then, 160 µL of the supernatant of strain L3^T was added to the mixture, mixed vigorously, and incubated at 37°C for 30 min. Afterward, absorbance was measured at 510 nm using a spectrophotometer. Distilled water and PC were used as negative and positive controls, respectively, and all experiments were repeated three times. The scavenging of OH* by the EPS from strain L3^T was calculated according to the following equation:

Hydroxyl radical scavenging capacity of EPS (%) = [1 - (A_1/A_2)] × 100

where A_1 is the absorbance of the sample, and A_2 is the absorbance of the control.

DPPH radical scavenging activity

The DPPH radical scavenging activity of EPS was determined by slightly modifying the method proposed by Kim et al (Kim et al., 2023b). Briefly, samples were dissolved in distilled water at concentration of 1 mg/mL. After adding the same volume of 0.2 mM DPPH solution as each sample, it was mixed and incubated for 30 min at 25°C in the dark. Then, absorbance was measured at 517 nm using a spectrophotometer. PC served as a positive control, and distilled water was used as a negative control. The scavenging of DPPH radicals by the EPS from strain L3^T was calculated according to the following equation:

DPPH radical scavenging capacity of EPS (%) = [1 − (A_1 − A_2)/A_3] × 100

where A_1 is the absorbance of the DPPH solution mixed with the EPS solution, A_2 is the absorbance of the DPPH solution, and A_3 is the absorbance of the control.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 11.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA). According to Duncan's test, significant differences between treatment groups were: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Isolation and identification of strains

About 11 microbial colonies were obtained from fermented fruit samples. 16S rRNA gene sequencing and morphological analysis were performed. Additionally, we were able to select six types of bacteria presumed to secrete EPS around cells. These six strains were tested on media with different concentrations of NaCl and polyethylene glycol (PEG) to investigate whether they would grow under salinity and drought stress conditions (Supplementary Table S1) (Kim et al., 2023c, 2023a). Through repeated experiments, strain L3^{T} was shown to maintain sustainable growth in salinity conditions up to 1.198 M NaCl. It was also selected for PGP testing because it maintained consistent growth even under 22% PEG conditions. Molecular identification of strain L3^{T} was performed by analyzing the 16S rRNA gene base sequence and whole genome sequence of strain L3^{T}. Strain L3^{T} showed the highest 16S rRNA gene similarity to *Buttiauxella izardii* CCUG 35510^{T} strain at 98.63%, followed by 98.56% and 98.56% similarity with the *Enterobacter ludwigii* EN-119^{T} and *Pseudescherichia vulneris* NBRC 102420^{T} strains, respectively. The phylogenetic tree was reconstructed using the 16S rRNA gene sequence. Strain L3^{T} showed clustering with *P. vulneris* NBRC 102420^{T}. In addition, the phylogenetic tree also showed that it was clustered with *P. vulneris* NBRC 102420^{T}. These results indicated that strain L3^{T} was a novel species of the genus *Pseudescherichia* (Figure 1A). The bootstrap values shown in the phylogenetic tree and phylogenomic tree were 86 and 92, respectively, which support the above results (Figures 1A, B). Therefore, to place strain L3^{T} in the phylogenetically correct position, we performed a taxonomic comparison with the closest species, *P. vulneris* NBRC 102420^{T} (Supplementary Table S2). Although these two *Pseudescherichia*

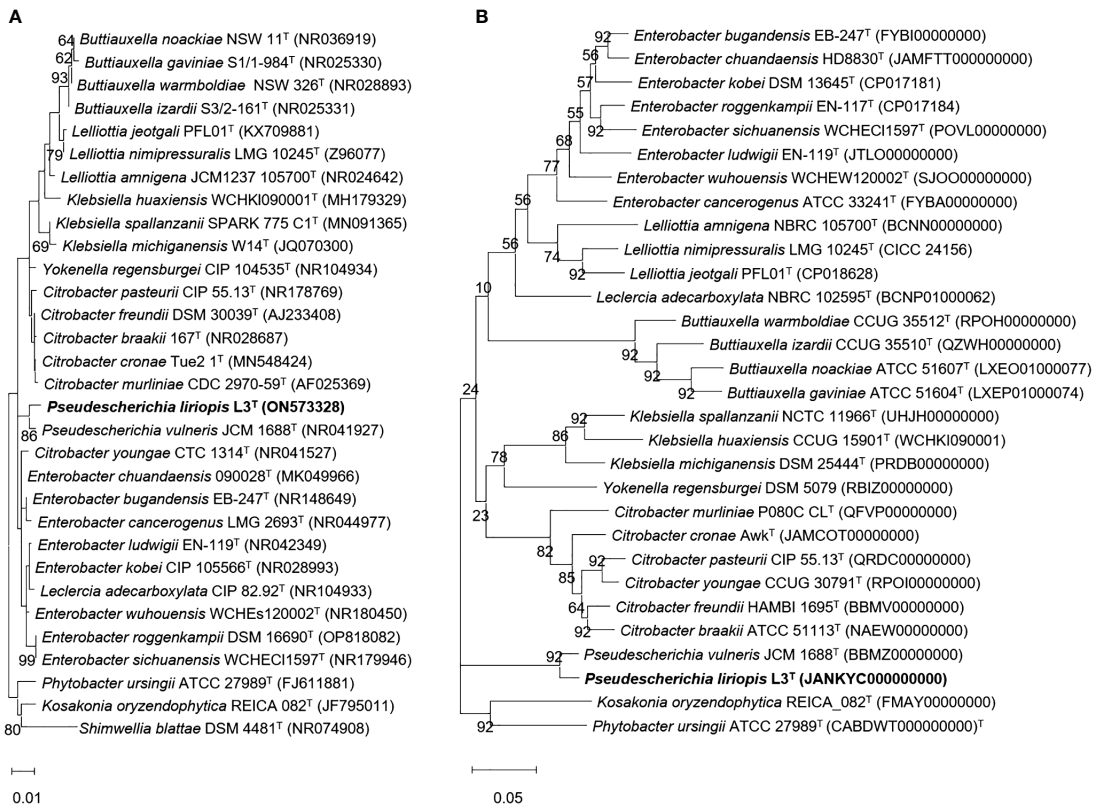

FIGURE 1
(A) Neighbor-joining (NJ) phylogenetic tree of strain L3^{T} within the genus *Pseudescherichia*. Bootstrap values are shown as percentages of 1,000 replicates (only values >50% are shown). Filled circles indicate that the corresponding nodes were recovered in trees generated with the maximum-likelihood (ML), and maximum-parsimony (MP) algorithms. Empty circles indicate that the corresponding nodes were recovered using the ML algorithm. Bar, 0.02 substitutions per nucleotide position. **(B)** Phylogenomic tree was reconstructed using the coding sequences of 92 protein clusters showing the position of strain L3^{T} among closely related species. Parentheses indicate the NCBI accession number for the genome of each strain.

species showed mostly similar experimental results, only strain L3^T was found to grow at pH 4.0, 7% NaCl, and 15°C, revealing the differences between the two strains. In addition, according to the results of the API 20NE test, the only other difference was whether L-arginine was hydrolyzed. The almost full-length 16S rRNA gene sequence (1470 bp) of strain L3^T was deposited in the GenBank database under the accession number ON573328 and the whole genome sequence JANKYC00000000, respectively.

Genomic features of strain L3^T

The draft genome sequence of strain L3^T contained 10 contigs, with a total size of 4,304,575 bp, and encoded 4,131 genes in total, 3,989 of which were protein-coding genes. The genome information of strain L3^T and its related species is detailed in Supplementary Table S1. CheckM version 1.2.2 revealed that the completeness of the strain L3^T genome was 100.0%, with a contamination level of 0%. After calculating average nucleotide identity (ANI) values with FastANI using orthogonal mapping, we visualized the genome conservation of two strains that are phylogenetically close to strain L3^T (Supplementary Figure S1). The results showed the most orthologous mapping between strain L3^T and *P. vulneris*, suggesting that they are the closest species, and there were relatively few orthologous mappings between strain L3^T and *B. izardii* and *E. ludwigii*, respectively. As shown in Supplementary Table S3, ANI and digital DNA–DNA hybridization (dDDH) comparisons between strain L3^T and 29 genomes were performed. The ANI and dDDH values between strain L3^T and the *P. vulneris* genome were 55.1% and 93.9%, those between strain L3^T and the *B. izardii* genome were 19.9% and 75.9%, and those between strain L3^T and the *E. ludwigii* genome were 22.4% and 79.5%, respectively. The recommended cutoff values for delineating novel species are 70% (dDDH) and 95% (ANI). The Antibiotic and Secondary Metabolite Analysis Shell (antiSMASH) server revealed a secondary metabolite biosynthetic gene cluster for non-ribosomal peptide metallophores, non-ribosomal peptide synthetase, two other unspecified ribosomally synthesized and post-translationally modified peptides, redox-cofactors, such as pyrroloquinoline quinone (PQQ), and terpene. A comparison of secondary metabolite predictions between strain L3^T and phylogenetically close species can be seen in Supplementary Figure S1B.

Plant growth promotion characterizations

Strain L3^T produces IAA and has been shown to perform siderophore production, phosphate solubilization, and nitrogen fixation. Confirmation of IAA production was measured using a slight modification of the method described by Gordon and Weber (Gordon and Weber, 1951). The IAA value was highest at 20.37 ± 0.96 µg/mL when 0.05% L-tryptophan was included. When the L-tryptophan concentration was 0.1%, 0.2%, and 0.3%, the IAA concentration values were 19.16± 0.14, 14.22 ± 0.38, and 14.97 ± 0.91 µg/mL, respectively, confirming that the IAA concentration did not increase as the L-tryptophan concentration was increased (Figure 2A). Strain L3^T had the ability to secrete IAA, but only synthesized IAA in the presence of L-tryptophan and could not produce it alone. Strain L3^T showed clear areas around colonies grown on Pikovskaya (PVK) agar plates, indicating its ability to utilize inorganic phosphate in the medium (Figure 2B). Additionally, the L3^T strain grows quickly on PVK agar plates and produces halozone quickly within 24h. The development of a halo around the bacterial colony of strain L3^T grown on chrome azurol S (CAS) plate medium indicated its ability to produce siderophores (Figure 2C). Strain L3^T was able to grow in 1 day on a nitrogen medium at 30°C, indicating its nitrogen fixation ability (Figure 2D).

Effect of strain L3^T on the growth of plant

Based on IAA production, siderophore production, phosphate solubilization, and a nitrogen fixation study, it was investigated whether strain L3^T could promote carrot (*D. carota* subsp. *sativus*) plant growth. The results confirmed that the stem length, carrot diameter, carrot weight, and carrot length were significantly increased (Figures 3A–D). Inoculation with strain L3^T increased the weight of carrots by 81.5% compared to the control group, and the shoot length, diameter, and length of carrots increased by 30.13%, 47.06%, and 36.98%, respectively. These results suggest that strain L3^T promoted the growth of carrots. In addition, among the results of the pot experiment, the PGP ability of strain L3^T was confirmed in photos of the inoculated carrots with leaves and soil removed (Figure 3E). There were also clear differences in leaf length and abundance between the L3^T-inoculated group and the control group (Figures 3F, G).

Effect of strain L3^T inoculation on alleviating salinity and drought stress

The plants inoculated with strain L3^T cultures showed an improvement in plant growth under salinity and drought stress. When inoculated with the L3^T strain under NaCl stress (0.514 M), carrots' total shoot length increased by 14.84% compared to the control group (Figure 4A). Moreover, unlike the plants inoculated with strain L3^T, the leaves of the control plants turned yellow due to stress caused by salt (Figure 4E). Drought stress was assessed by treatment with PEG at 5% and 10% concentrations (referred to as the 5PEG and 10PEG groups, respectively). Plants inoculated with strain L3^T under drought stress showed a 12.84% (5% PEG) and 16.37% (10% PEG) increase in carrots' total shoot length growth compared to the control group (Figure 4A). In addition, carrots' weight increased by 80.01%, 46.52%, and 50.98% under salinity, 5% PEG, and 10% PEG conditions, respectively (Figure 4B). The 5PEG group also had yellow and dry leaves compared to the L3^T-inoculated group (Figure 4F). Moreover, in the 10PEG group, the total length of shoots was significantly reduced, leaves dried, and stems also dried and tilted to the side (Figure 4G). Carrot length also increased by 51.96% (0.514 M NaCl), 38.1% (5% PEG), and 35.28% (10% PEG), under the indicated stress compared to the control group (Figure 4C). Furthermore, there was an increase in the

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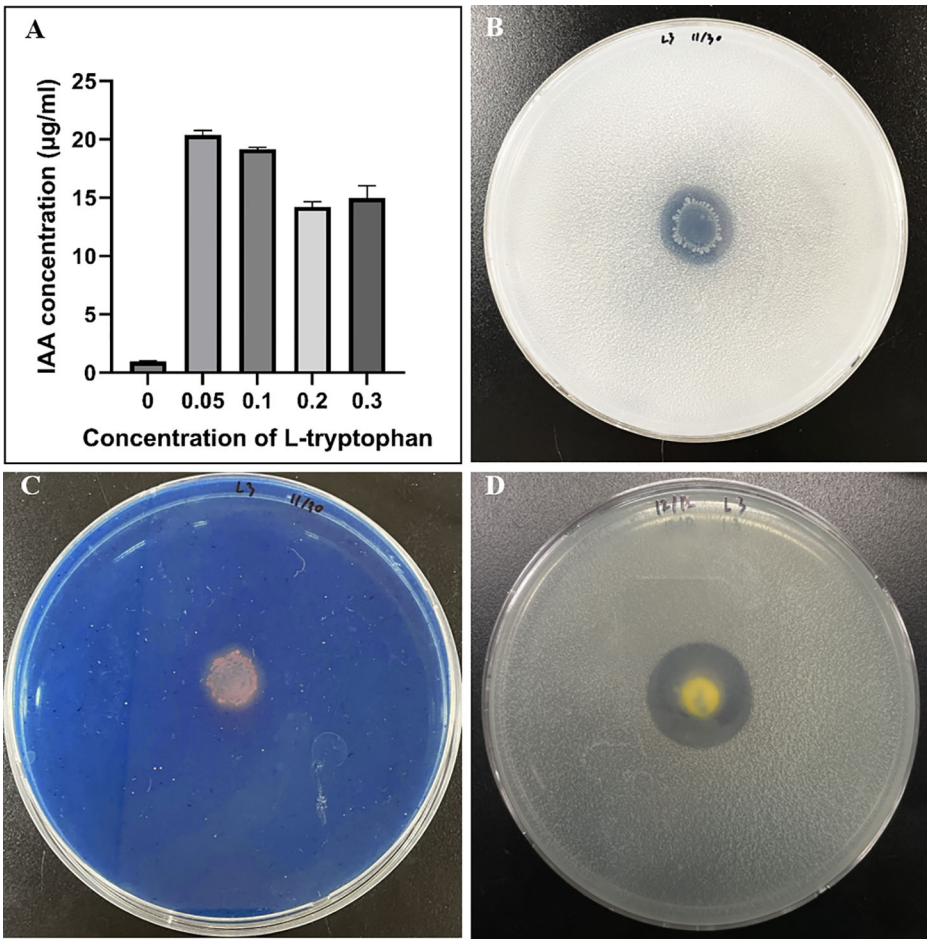

FIGURE 2
Traits associated with plant growth promotion ability of strain L3^T. **(A)** IAA production is dependent on the concentration of tryptophan. **(B)** Phosphate solubilization assay (PVK agar plate). **(C)** Siderophore qualitative assay. **(D)** Nitrogen fixation.

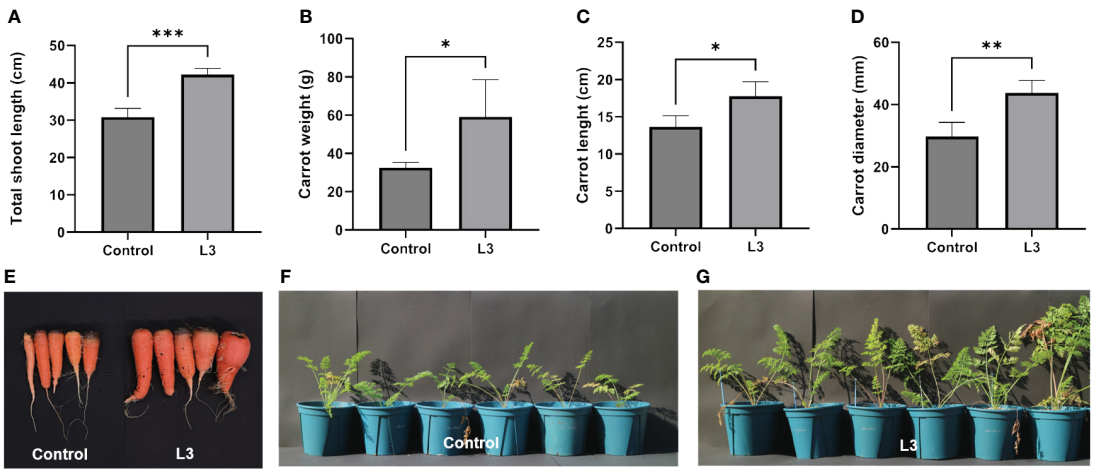

FIGURE 3
Effect of inoculation with strain L3^T on growth promotion of carrot plants. **(A)** Total shoot length of carrot plants in L3^T-inoculated and control groups. **(B)** Carrot weight of L3^T-inoculated group and control group. **(C)** Effect of carrot length in L3^T-inoculated group and control group. **(D)** Effect of carrot diameter according to strain L3^T inoculation, **(E)** carrots with leaves and soil removed, **(F)** control group not inoculated with L3^T, **(G)** group inoculated with L3^T. Data are presented as mean + SD of five replicates. *P < 0.05, **P < 0.01, ***P < 0.001.

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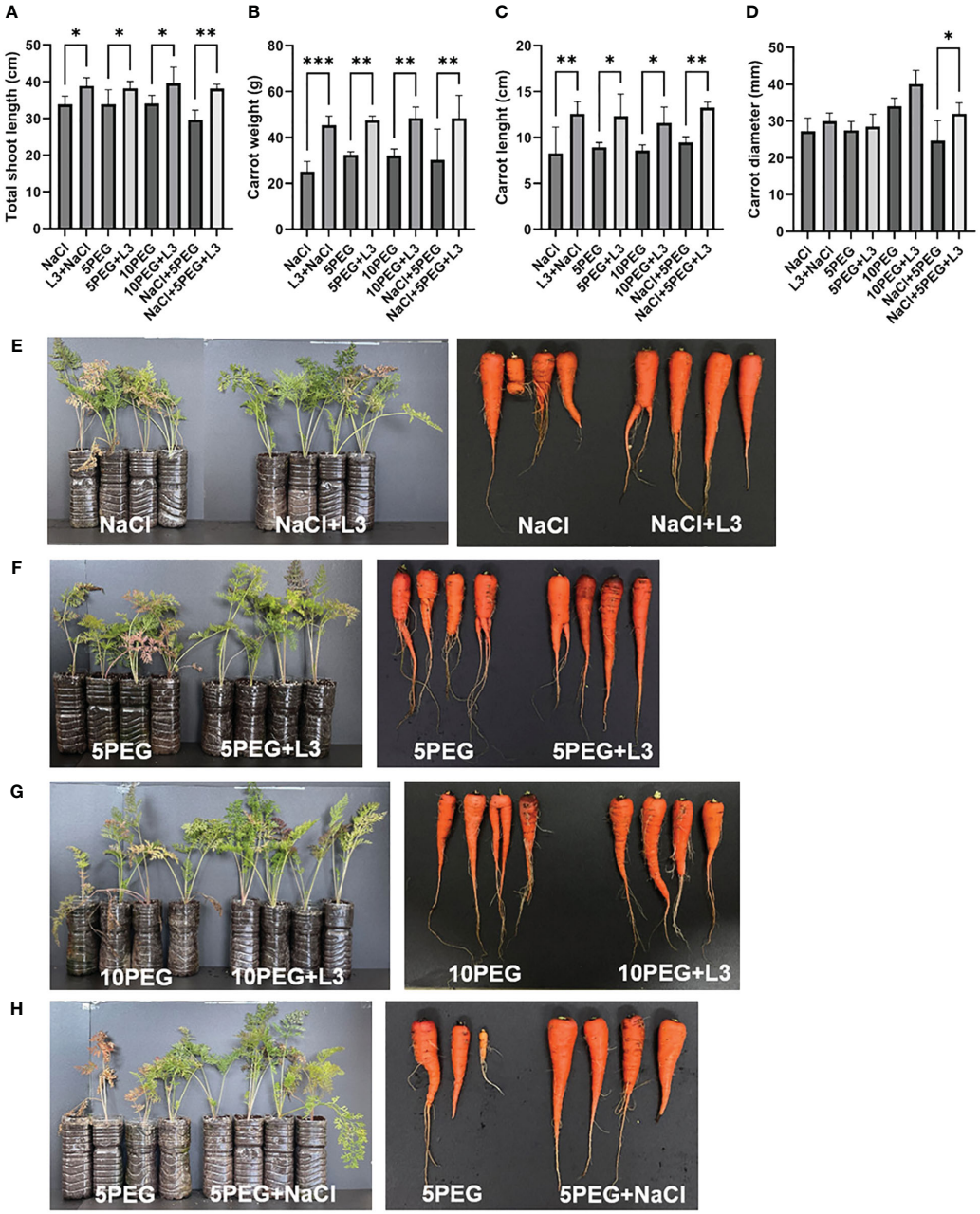

FIGURE 4
Inoculation effect of strain L3^T under drought and NaCl stress: **(A)** effect of total shoot length under drought and salt stress, **(B)** carrot weight, **(C)** carrot length, and **(D)** carrot diameter. Data are presented as mean + SD of four replicates. Effect of inoculation of strain L3^T on growth promotion of carrot plants under salinity and drought stress. **(E)** NaCl (0.513 M), **(F)** 5% PEG, **(G)** 10% PEG, **(H)** 5% PEG + NaCl (0.513 M). *P < 0.05, **P < 0.01, ***P < 0.001.

diameter of the thickest part of carrots compared to the control group, albeit the value was not significant (except for the NaCl + 5PEG group; Figure 4D). Inoculation with strain L3^T under conditions of overlapping salt and drought stress significantly increased total plant sprout length, carrot length, carrot weight, and carrot diameter by 28.54%, 40.21%, 60.49%, and 29.73%, respectively, compared to the control group (Figures 4A–D). In contrast, in the control group, one of the plants did not grow, and the overall length of the leaves and the size of the edible parts of the carrots also showed differences from the L3^T-inoculated group (Figure 4H). These results suggest that inoculation with strain L3^T may be a countermeasure against crop damage caused by salinity and drought.

Genome analysis and plant growth promotion insight

As a result of this study, the new strain L3^T promoted the growth of carrots. The genome of strain L3^T was examined to identify genes involved in plant growth promotion. A variety of PGPB exhibit IAA production, phosphate solubilization, nitrogen fixation, and

siderophore production. In the strain L3^T genome, a set of genes for IAA production was discovered, and IAA production was confirmed experimentally, showing consistency between these two results (Table 1). A set of genes responsible for phosphate solubilization and transport were also included in the genome (Table 1). Additionally, it was experimentally confirmed that the L3^T strain dissolves insoluble phosphate on PVK agar plates. Bacteria that solubilize phosphate release gluconic acid, which converts the insoluble phosphate into a soluble form (Ahemad and Kibret, 2014). Additionally, PQQ is known to be a PGP factor that is associated with antioxidant properties (Choi et al., 2008). The set of PQQ-related genes was contained in the strain L3^T genome (Table 2). In the draft genome of strain L3^T, genes related to nitrogen metabolism, nitrogen fixation, and siderophore production were found, of which only one gene was related to nitrogen fixation (Table 2).

TABLE 1 Genes involved in IAA production and phosphate solubilization predicted from the strain L3^T genome.

Properties	Name	Locus tag
Indole-3-acetic acid	Bifunctional anthranilate synthase glutamate amidotransferase component *TrpG*, anthranilate phosphoribosyltransferase *TrpD*	NR795_07870
	Anthranilate synthase component 1	NR795_07865
	Bifunctional indole-3-glycerol-phosphate synthase *TrpC*, phosphoribosyl anthranilate isomerase *TrpF*	NR795_07875
	N-methyl-L-tryptophan oxidase *solA*	NR795_03815
	Tryptophan synthase subunit beta *trpB*	NR795_07880
	Tryptophan synthase subunit alpha *trpA*	NR795_07885
	Tryptophan–tRNA ligase *trpS*	NR795_15650
	Tryptophan permease *mtr*	NR795_18040
Phosphate solubilization	Phosphate response regulator transcription factor *PhoB*	NR795_00415
	Phosphate regulon sensor histidine kinase *PhoR*	NR795_00420
	Phosphate starvation-inducible protein *PhoH*	NR795_03675
	Phosphate signaling complex protein *PhoU*	NR795_17295
	Phosphate ABC transporter ATP-binding protein *PstB*	NR795_17300
	Phosphate ABC transporter permease *PstA*	NR795_17305
	Phosphate ABC transporter permease *PstC*	NR795_17310
	Phosphate ABC transporter substrate-binding protein *PstS*	NR795_17315
	Octa prenyl diphosphate synthase *ispB*	NR795_17835
	Phosphonate C-P lyase system protein *PhnH*	NR795_14870
	Exopolyphosphatase *ppx*	NR795_11090
	Polyphosphate kinase 1 *ppk1*	NR795_11085
	Phosphate acetyltransferase *pta*	NR795_10460
	Glucose-6-phosphate dehydrogenase *zwf*	NR795_08600

TABLE 2 Genes involved in pyrroloquinoline quinone biosynthesis, nitrogen fixation, nitrogen metabolism, and siderophore production predicted from strain L3^T.

Properties	Name	Locus tag
Pyrroloquinoline quinone	Pyrroloquinoline quinone precursor peptide *PqqA*	NR795_11860
	Pyrroloquinoline quinone biosynthesis protein *PqqB*	NR795_11865
	Pyrroloquinoline-quinone synthase *PqqC*	NR795_11870
	Pyrroloquinoline quinone biosynthesis peptide chaperone *PqqD*	NR795_11875
	Pyrroloquinoline quinone biosynthesis protein *PqqE*	NR795_11880
	Pyrroloquinoline quinone biosynthesis protein *PqqF*	NR795_11885
Nitrogen fixation	Pyruvate:ferredoxin (flavodoxin) oxidoreductase *NifJ*	NR795_06880
Nitrogen metabolism	P-II family nitrogen regulator *glnK*	NR795_00695
	Nitrogen assimilation transcriptional regulator *NAC*	NR795_09305
	Nitrogen regulatory protein P-II *glnB*	NR795_11375
	PTS IIA-like nitrogen regulatory protein PtsN	NR795_17750
	Nitrogen regulation protein NR(I) *glnG*	NR795_19485
	Nitrogen regulation protein NR(II) *glnL*	NR795_19490
Siderophore production	TonB-dependent siderophore receptor	NR795_00925
	TonB-dependent siderophore receptor	NR795_01210
	TonB-dependent siderophore receptor	NR795_01490
	Fe(3+)-siderophore ABC transporter permease *fepD*	NR795_01525
	TonB-dependent siderophore receptor	NR795_08280
	Catecholate siderophore receptor *cirA*	NR795_10045
	Siderophore-iron reductase *fhuF*	NR795_13900
	Siderophore-interacting protein	NR795_18295

ABTS, hydroxyl, and DPPH radical scavenging by EPS

The antioxidant potential of partially purified EPS was tested. The ABTS*+, OH*, and DPPH*, scavenging activities were investigated *in vitro* and compared with the PC. At a concentration of 1 mg/mL, EPS, and PC had almost similar ABTS*+ scavenging activities (91.2% and 94.6%; Figure 5), and the results indicated that EPS produced by strain L3^T has excellent scavenging activity toward ABTS*+. The OH* scavenging activity of EPS (72.8%) was lower than that shown by PC (92.6%) but was still excellent (Figure 5). As shown in Figure 5 at concentrations of 1 mg/mL, PC showed high DPPH* scavenging ability (87.5%) and EPS showed less (28.5%).

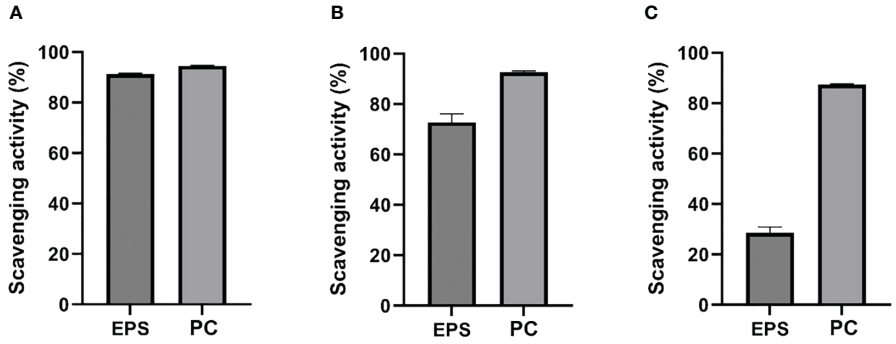

FIGURE 5
Comparison of the scavenging effects of EPS from *Pseudescherichia liriopis* L3^T: **(A)** ABTS*+, **(B)** OH*, and **(C)** DPPH* with that of ascorbic acid (PC).

Composition and monosaccharide analysis of EPS

ATR-FTIR spectra of EPS produced from strain L3^T were collected using a Smiths 70v spectrometer in the spectral range of 650–4000 cm^{-1}. This allowed us to characterize the covalent bond information and confirm the presence of functional groups (Figure 6A). A strong absorption band was observed near 3391 cm^{-1} in the FTIR spectrum, which is due to the —OH group expansion vibration and indicates that the polymer is EPS (Wang et al., 2018). The absorption band appearing at 2937.59 cm^{-1} typically represents the stretching vibration of a hexose, such as glucose, or a methylene group (C—H) (Rajoka et al., 2022). The absorption bands around 1653 and 1367 cm^{-1} are attributed to the stretching vibration of the C═O bond and symmetric CH_3 bending, respectively (Freitas et al., 2009). A peak presumed to be COO^- vibration, evidence of sulfuric acid ester, appeared around 1367 cm^{-1} (Gupta et al., 2021). The absorption band representing the stretching vibration of C—O and the angle change vibration of O—H, indicating pyranose-type glucose and carbohydrates, was observed at approximately 1149 cm^{-1} (Bremer and Geesey, 2009). The absorption bands in the region 900–1150 cm^{-1} are characteristic of carbohydrates and are attributed to C—O—C and C—O stretching bending vibrations (Na et al., 2010). The absorption peak around 860 cm^{-1} is largely formed by α-glycosidic bonds (Cao et al., 2020). As shown in the chromatogram in Figure 6B, the peaks obtained by UHPLC represent the monosaccharide profile of EPS hydrolyzed with 2 M trifluoroacetic acid. Two peaks corresponding to glucose (21.58 min) and mannose (26.66 min) were observed in acid-hydrolyzed EPS, indicating a relative molar ratio of 33.6:1.0.

Thermogravimetric analysis

The thermal properties of EPS are important for its commercial use. In this study, Thermogravimetric (TGA) of EPS produced by the novel strain L3^T was performed from 25°C to 800°C (Figure 7). The first decrease occurred at 27.2°C, which was a mass loss due to gelatinization and swelling, mainly associated with water loss (Fagerson, 1969). Afterward, a loss of 12.75% of the total weight of EPS was confirmed up to 256.8°C, and the greatest energy release occurred between 300 and 321.3°C. The subsequent thermal weight

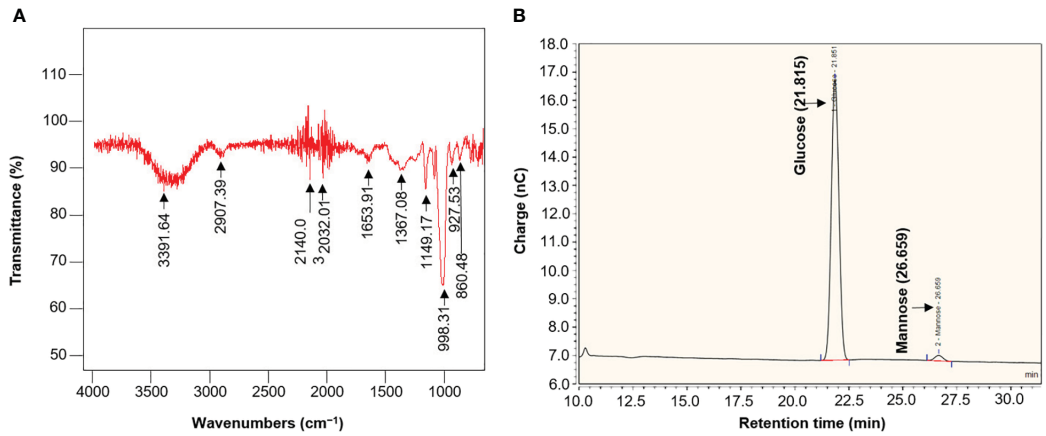

FIGURE 6
(A) ATR-FTIR spectra of EPS from *Pseudescherichia liriopis* L3^T. **(B)** Monosaccharide composition of EPS from *P. liriopis* L3^T by UHPLC analysis.

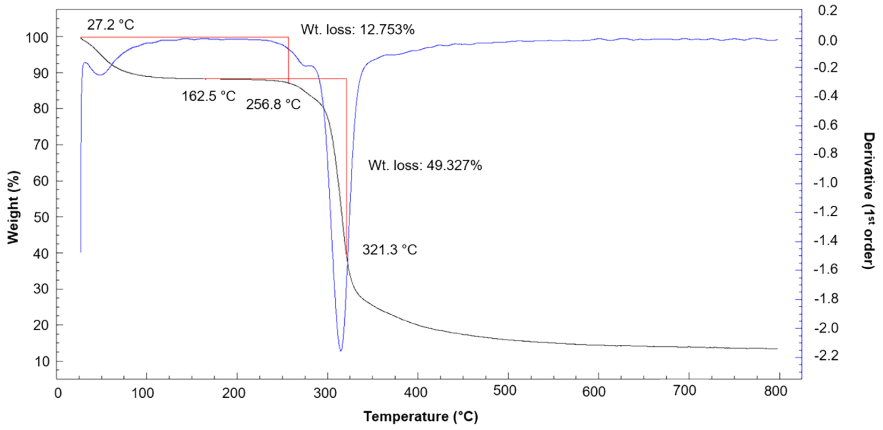

FIGURE 7
Thermogravimetric analysis (TGA) of the EPS produced by *Pseudescherichia liriopis* L3^T. The black line represents TGA, and the blue line represents differential thermal analysis.

loss of EPS represents a mass loss of approximately 49.33% at 321.3°C.

Discussion

Our results of 16S rRNA gene sequence-based phylogenetic analysis, genome comparison of strain L3^T, and the physiological characteristics of strain L3^T showed that it represents a novel species in the genus *Pseudescherichia*. Strain L3^T showed 98.63%, 98.56%, and 98.56% 16S rRNA gene sequence similarity to *B. izardii* CCUG 35510^T, *E. ludwigii* EN-119^T, and *P. vulneris* NBRC 102420^T, respectively. The ANI values between novel strain L3^T and *B. izardii* CCUG 35510^T, *E. ludwigii* EN-119^T, and *P. vulneris* NBRC 102420^T were 75.9%, 79.5%, and 93.9%, respectively, with respective dDDH values of 19.9%, 22.4%, and 55.1%. The ANI values were lower than the ANI threshold of 95%, strongly supporting strain L3^T as a novel species (Chun et al., 2018).

The main goal of this research work is to find ba that promote plant growth, as well as those that have both NaCl and drought stress tolerance. A total of six strains were identified, and strain L3^T, which produced EPS and grew at a concentration of 20% PEG and 6% NaCl, was designated. This novel strain was able to perform phosphate solubilization, IAA production, siderophore synthesis, and nitrogen fixation. It also had the ability to secrete IAA but only synthesized IAA in the presence of $_L$-tryptophan and could not produce it alone. In addition, IAA synthesis does not increase as $_L$-tryptophan concentration increases but rather is synthesized in the largest amount when 0.5% $_L$-tryptophan is present, indicating that it is not concentration dependent. *Enterobacter cloacae* MG00145, another genus in the same family, shows an IAA production of approximately 17.7 µg/mL in the presence of tryptophan. In comparison, strain L3^T shows a higher value (20.37 ± 0.96 µg/mL) (Panigrahi et al., 2020). Auxin is a major plant hormone that plays an important role in regulating plant growth, including leaves, fruits, flowers, and germination (Bottini et al., 2004; Hayat et al., 2010). Auxin production by our strain L3^T was consistent with PGP ability in several species (Chhetri et al., 2022; Tsavkelova et al., 2024). The ability of phosphate solubilization is crucial as it enhances the availability and absorption of essential mineral nutrients by plants (Arif et al., 2017). Genera known as siderophore producing bacteria, such as *Pseudomonas*, *Bacillus*, and *Rhizobium*, improve iron absorption, making plants stronger and more effective in suppressing pathogens (Abo-Zaid et al., 2020; Sun et al., 2022; Xie et al., 2024). We experimentally demonstrated the ability of the L3^T strain to synthesize siderophores, fix nitrogen, solubilize phosphate, and synthesize IAA and identified IAA production, phosphate solubilization, nitrogen fixation, and siderophore gene clusters in the NCBI annotation and genes associated with plant growth promotion. It is suggested that the collective effects of siderophore production, nitrogen fixation, and phosphate solubility may contribute to the growth enhancement shown by inoculation of the novel species L3^T in carrot plants. Therefore, the two results appear to be consistent.

Additionally, various clusters related to PQQ biosynthesis were discovered. PQQ is known to have antioxidant properties and scavenge reactive oxygen species, promoting plant growth and phosphate solubilization (Choi et al., 2008). However, although PQQ-related clusters were predicted in the genome, additional experiments are needed to determine the mechanism(s) by which strain L3^T affects PQQ production and plant growth. Based on these results, the ability of the strain to promote plant growth was verified by inoculating strain L3^T into carrot plants. When carrot plants were inoculated with strain L3^T, the carrot plants' weight, length, diameter, and leaf length increased significantly. The key among these was that the edible part of the carrots increased as the length, weight, and diameter increased. In addition, strain L3^T affected leaf length, weight, and length of carrots even under salt and drought stress conditions, respectively. The size, length, diameter, and leaf length of carrot plants increased compared to control plants subjected to salt and drought stress. Plants inoculated with strain L3^T showed a clear increase in leaf length, leaf weight, carrot length,

and carrot thickness compared to the control group, even under conditions where salt and drought stress occurred simultaneously.

Crop yield and growth are being hindered due to soil salinization, and climate change conducive to salt accumulation might increase the proportion of salinized land (Ilangumaran and Smith, 2017). Agricultural lands with high salt content have a low phosphorus content. Therefore, it is essential to use fertilizers that contain a large amount of phosphorus, but phosphorus can cause water pollution and accumulation of toxic elements during long-term use (Alori et al., 2017). PGPB live in the rhizosphere and internal tissues of plants and promote crop growth and yield through various PGP mechanisms (Pii et al., 2015). This is an environmentally friendly strategy as it leads to a reduction in soil and water pollution resulting from the use of chemical and nitrogen fertilizers (Aminun Naher et al., 2015). Instead of fertilizer, using PGPB, such as strain L3^T, dissolves soil phosphate, providing a sustainable alternative to promote crop growth and increase yields. Drought is a plant stress that inhibits crop growth and has a negative impact on yield (Zhu, 2016). Therefore, the study of PGPB to improve drought stress is important as plant stress directly affects agricultural production.

Strain L3^T also grew well in R2A broth containing 20% PEG. This showed potential for improving plant resistance to drought stress. We demonstrated that inoculation of carrot plants with the L3^T strain under drought stress promoted plant growth compared to the control group that was not inoculated with the strain. This indicates that the L3^T strain has the potential to positively affect water retention in rhizosphere soils, which may be related to the EPS produced by strain L3^T. EPS produced by bacteria may be involved in slowing the rate of dehydration of plants by retaining moisture, which should be studied further. Strain L3^T, an EPS-producing bacterium, not only suggests that it may be useful in the development of biological inoculants to improve abiotic stress in plants but also shows strong antioxidant ability, making it worthy of application in various industries. Microbial EPS production is a physiological adaptation that allows survival under stressful conditions. Therefore, the ability of bacterial cells to produce EPS is associated with the drought resistance of bacteria (Sandhya et al., 2009). The production of EPS on PGP strains plays a crucial role in managing salt stress and can be an effective solution. EPS from chelates with ionic metals such as Na^+ and Cl^-, binding directly to positive ions like Na^+. This action stabilizes the ions and mitigates NaCl stress in plants (Kumar et al., 2021). To the best of our knowledge, this is the first study of PGP activity among *Pseudescherichia* species. So far, many PGP studies have been conducted on improving NaCl stress and drought stress (Kumar et al., 2021; Ashry et al., 2022). However, even when both stresses were applied simultaneously, our strain L3^T showed the ability to promote plant growth. When inoculated with the strain L3^T under conditions where salinity and drought stress overlapped, the total sprout length, carrot length, carrot weight, and carrot diameter of the plants were confirmed to increase by 28.54%, 40.21%, 60.49%, and 29.73%, respectively, compared to the control group.

We also investigated the characteristics of EPS produced by the PGPB strain L3^T. AIR-FTIR analysis was performed to determine the presence or absence of functional groups in EPS. EPS exhibited peaks typical of polysaccharides, suggesting that this polymer is EPS (Wang et al., 2010). It was confirmed that the EPS from strain L3^T consists mainly of glucose and some mannose, which may be heteropolysaccharides. It is similar to EPSe5 produced by lactic acid bacteria but has a slightly different monosaccharide composition (Liu et al., 2023). Additionally, the monosaccharide composition of EPS produced by strain L3^T suggests that it is different from EPS produced by other strains (Kim et al., 2022; Liu et al., 2023). EPS produced by microorganisms is regulated by several genetic factors, carbon sources, and environmental variables and may differ in composition from EPS produced by other strains, even of the same species (Pachekrepapol et al., 2017). Recent plant-related studies have focused on the interactions between plant and microbial secondary metabolites.

Conclusion

PGPB studies are important from environmental and agricultural perspectives. In this study, the new strain L3^T was used to demonstrate its ability to promote plant growth in carrot plants. As a result, the length, weight, and leaf length of the carrots increased, increasing the edible portion. In addition, inoculation with strain L3^T was confirmed to improve these problems associated with drought and salt stress in plants. In addition, biotechnologically important EPS was extracted and purified from *P. liriopis* sp. nov. L3^T. EPS produced by bacteria obtained from fermented *L. platyphylla* fruit was also studied using FTIR, TGA, and UHPLC analysis. The EPS we studied showed excellent antioxidant properties, and its thermal stability was emphasized, supporting its use in multiple industrial applications. The practical application of this research is important for both agriculture and industry. Integrating the L3T strain into agricultural practices can improve crop yields, especially in areas vulnerable to drought and salt stress. This can lead to more sustainable and resilient agricultural systems. Sustainable agriculture: using natural plant growth promoters like L3^T can promote more environmentally friendly agricultural practices by reducing reliance on chemical fertilizers and pesticides. Although the results of this study are promising, several potential limitations need to be addressed. Further research is needed to understand the structure of EPS, its biological activity, and its correlation with plant growth. Additional studies are needed to understand the detailed correlation with plant growth promotion, structure, and biological activity of EPS. In future studies, we should investigate the mechanisms by which EPS promotes plant growth and enhances abiotic stress at the cellular and molecular levels. Because agricultural environments have different conditions depending on the region, plant growth tests must be conducted in a wide range of fields, and microbial community dynamics and nutrient cycling must also be studied to determine how they affect the soil. Economic aspects are also important and the financial efficiency of using L3^T strains in agriculture must be evaluated.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

IK: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Writing – original draft. HW: Data curation, Investigation, Methodology, Writing – review & editing. GC: Data curation, Methodology, Writing – review & editing. SP: Data curation, Writing – review & editing. TS: Writing – review & editing, Funding acquisition, Resources, Supervision.

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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.2024.1417639/full#supplementary-material

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Enhancing drought resistance in *Pinus tabuliformis* seedlings through root symbiotic fungi inoculation

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Background: Drought constitutes a major abiotic stress factor adversely affecting plant growth and productivity. Plant-microbe symbiotic associations have evolved regulatory mechanisms to adapt to environmental stress conditions. However, the interactive effects of different fungi on host growth and stress tolerance under drought conditions remain unclear.

Objective: This study explored the effects of varying polyethylene glycol (PEG-6000) concentrations (0%, 15%, 25%, and 35%) on the growth and physiological responses of two ectomycorrhizal fungi (*Suillus granulatus* (Sg) and *Pisolithus tinctorius* (Pt)) and two dark septate endophytes (*Pleotrichocladium opacum* (Po) and *Pseudopyrenochaeta* sp. (Ps)) isolated from the root system of *Pinus tabuliformis*. Specifically, the study aimed to evaluate six inoculation treatments, including no inoculation (CK), single inoculations with Sg, Pt, Po, Ps, and a mixed inoculation (Sg: Pt : Po: Ps = 1:1:1:1), on the growth and physiological characteristics of *P. tabuliformis* seedlings under different water regimes: well-watered at 70% ± 5%, light drought at 50% ± 5%, and severe drought at 30% ± 5% of the maximum field water holding capacity.

Results: All four fungi exhibited the capacity to cope with drought stress by enhancing antioxidant activities and regulating osmotic balance. Upon successful root colonization, they increased plant height, shoot biomass, root biomass, total biomass, and mycorrhizal growth response in *P. tabuliformis* seedlings. Under drought stress conditions, fungal inoculation improved seedling drought resistance by increasing superoxide dismutase and catalase activities, free proline and soluble protein contents, and promoting nitrogen and phosphorus uptake. Notably, mixed inoculation treatments significantly enhanced antioxidant capacity, osmotic adjustment, and nutrient acquisition abilities, leading to superior growth promotion effects under drought stress compared to single inoculation treatments.

Conclusion: All four fungi tolerated PEG-induced drought stress, with increased antioxidant enzyme activities and osmotic adjustment substances and they promoted the growth and enhanced drought resistance of *P. tabuliformis* seedlings.

KEYWORDS

antioxidant activities, ectomycorrhizal fungi, mixed inoculation, osmotic adjustment, PEG-6000, root symbiotic fungi

1 Introduction

Currently, the trend of global climate change is characterized by rising temperatures and increasing aridity, with precipitation patterns undergoing noticeable changes. The frequency, duration, intensity, and area of droughts have increased significantly. Drought has emerged as a principal factor impacting vegetation growth and recovery (Cook et al., 2014; Huang et al., 2016; Kumar et al., 2024). In arid regions, high summer temperatures adversely affect the survival of tree seedlings and hinder forest regeneration. Simultaneously, soil temperature indirectly influences nutrient synthesis, translocation, and absorption, thereby disrupting the normal physiological processes of young seedlings and eventually impeding vegetation growth and regeneration within the entire forest ecosystem (Chidumayo, 2008). Although drought diminishes soil productivity, constrains water uptake, induces plant osmotic and redox imbalances, and ultimately leads to plant mortality (Alori et al., 2020; Farrell et al., 2017), microbial interventions can effectively alleviate drought stress (Saritha et al., 2021; Li and Liu, 2016; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Studies have demonstrated that when plants establish mycorrhizal associations in their root systems, their capacity to absorb water and nutrients significantly improves, thereby enhancing their drought resistance (Gleason et al., 2006). Consequently, the inoculation of beneficial fungi represents an effective strategy for maintaining plant health and quality, necessitating the selection of fungal species capable of adapting to drought conditions and the screening of strains and strain combinations exhibiting robust drought tolerance.

Ectomycorrhizal fungi (ECMF) establish ectomycorrhizal associations when their mycelia infect the nutrient roots of host plants. The primary host plants of ECMF belong to the Betulaceae, Pinaceae, Salicaceae, and Fagaceae families (Tedersoo and Brundrett, 2017). Existing studies indicate that Pinaceae are among the earliest ectomycorrhizal plant lineages (Tedersoo et al., 2012). Dark septate endophytes (DSE) are a type of minute endophytes that colonize the epidermal cells, root epidermis, cortex, and even the vascular tissue cells or intercellular spaces of plant roots, forming dark-colored, conspicuous structures with laterally septate hyphae and microsclerotia (Addy et al., 2005; Mandyam et al., 2010; Gaber et al., 2023). These fungi exhibit a high colonization rate in plant roots under drought or extreme environmental conditions (Gucwa-Przepióra et al., 2016), with a broad host range encompassing nearly 600 plant species from 320 genera across 114 families (Jumpponen et al., 1998), garnering increasing research attention in recent years (Santos et al., 2021; Huertas et al., 2024). Both ECMF and DSE promote plant growth and improve stress tolerance, playing crucial roles in ecosystem stability and restoration (Yin et al., 2018; Li et al., 2022a, b). Recent studies by Zhang et al. (2024) revealed that inoculating ECMF strains enhanced the drought resistance of *Pinus massoniana* seedlings during the early stages of drought stress by influencing water content, photosynthesis, osmolyte accumulation, and antioxidant enzyme activities in the shoots and roots. Similarly, Wang et al. (2021) reported that ECMF inoculation increased water content, photosynthetic rate, and osmolyte accumulation in *P. tabuliformis* under drought stress. Li et al. (2021) demonstrated that DSE inoculation could increase the biomass of *Isatis indigotica* and mitigate oxidative damage caused by drought stress. However, the interactive effects of these two fungi on host growth and stress tolerance under drought conditions remain unclear, with limited research exploring the co-existence of both fungi and their impact on host plant drought tolerance.

In recent years, the study of co-inoculation with ectomycorrhizal fungi has emerged as a prominent topic in rhizosphere microecology research (Corrales et al., 2022; Tedersoo et al., 2024). The combined application of ectomycorrhizal fungi and Trichoderma has been shown to effectively enhance the antioxidant capacity of Scots pine seedlings (Yin et al., 2016). Deng et al. (2017) reported that dual inoculation with *Suillus luteus* and dark septate endophytes improved the resistance of *Pinus sylvestris* var. *mongolica* seedlings to damping-off disease, reduced seedling blight incidence, and increased seedling survival rates. Our previous studies have demonstrated that inoculating two ECMF and two DSE strains could improve the growth, root development, nutrient uptake, and soil microbial community composition of *P. tabuliformis* seedlings (Xu et al., 2022), and effectively regulate the antioxidant defense response and photosynthesis of these seedlings under cadmium stress. The co-inoculation of the two fungi exhibited a more pronounced synergistic effect on plant tolerance to heavy metals (Zhou et al., 2024). Although the direct effects of beneficial microbial inoculants on plant growth have been widely reported (Santos et al., 2017; He et al., 2019), information regarding the contribution of the ECMF and DSE combination to plant growth under drought stress remains limited.

Pinus tabuliformis, the predominant tree species in North China, holds immense importance for soil and water conservation, landscape

aesthetics, and ecological balance within its distribution range (Gao et al., 2023). *P. tabuliformis* exhibits sensitivity to climate change-induced warming and drying. North China represents the most suitable growth area and concentrated distribution region for *P. tabuliformis*. The recruitment and renewal of *P. tabuliformis* seedlings profoundly influence the structure and species composition of forest communities, bearing great significance for forest resource reserves in this region. The ecological stability of China's northern regions is facing a more serious threat in the face of increasingly severe drought. As the main afforestation species of plantation forests in the northern region, the plantation forests of *P. tabuliformis*, under the influence of prolonged and frequent droughts, have suffered from degradation of forest stands, poor natural regeneration, drying up of tree tops and sparse understory vegetation, which have led to a vicious cycle of ecological environment in the region and further accelerated the depletion of forest resources (Chen et al., 2015). Previous studies have revealed the widespread presence of both ECMF and DSE in the roots of *P. tabuliformis* (Chu et al., 2016, 2019, 2021; Xu et al., 2022).

Therefore, in this study, drought tolerance investigations were conducted on isolated, purified, and identified root symbiotic fungi of *P. tabuliformis* to screen for strains exhibiting robust drought resistance. Subsequently, the root symbiotic fungi were reinoculated into *P. tabuliformis* seedlings, and water control experiments were carried out in a greenhouse. This research aims to provide a reference for further investigation of the drought resistance mechanisms of root symbiotic fungi and the screening of drought-resistant fungi. It also offers theoretical and technical support for *P. tabuliformis* afforestation in arid areas, promoting understory regeneration, preventing soil erosion, and improving forest resource quality. Previous studies on the resistance of mycorrhizal fungi in plants have predominantly focused on single inoculation methods. In contrast, this study introduces a mixed inoculation approach to evaluate the effects of different inoculation methods on plant-microbe symbionts. This experiment primarily examines the physiological and biochemical responses of these symbionts under drought stress conditions. While current research has provided valuable insights, the molecular mechanisms by which fungi enhance drought tolerance in *P. tabuliformis* remain underexplored. Future research should leverage advanced molecular biology techniques, such as transcriptomics, proteomics, and metabolomics, to elucidate these mechanisms.

2 Materials and methods

2.1 Biological material

The four-plant root symbiotic fungi used in this experiment were two ECMF, *Suillus granulatus* (*Sg*) and *Pisolithus tinctorius* (*Pt*), and two DSE, *Pleotrichocladium opacum* (*Po*) and *Pseudopyrenochaeta* sp. (*Ps*), which were isolated from the root of *P. tabuliformis* (Xu et al., 2022). These fungi were identified by morphological characteristics and phylogenetic analysis of nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) sequences (Supplementary Figures S1, S2). The fungi were cultured on potato dextrose agar media and stored in a 27°C mould incubator.

Uniformly sized and plump fresh seeds of *P. tabuliformis* were used in the experiments. The seeds were surface-sterilized and placed in Petri dishes in a 25°C light incubator to accelerate germination. Germinated seeds were sown in transparent plastic pots (15 cm height, 8 cm bottom diameter, 12 cm top diameter) filled with sterilized river sand (2 mm sieve, 121°C, 0.1 MPa, 2 h autoclaving). Each pot contained 1,000 g of the sterile sand medium, with four seeds sown per pot. During the seedling cultivation period, sterile water and Hoagland nutrient solution were supplied to ensure normal seedling growth.

2.2 Design of the experiment

2.2.1 *In vitro* drought stress tolerance assay

The drought stress tolerance of four root symbiotic fungi (*Sg*, *Pt*, *Po* and *Ps*) isolated from *P. tabuliformis* was evaluated through solid and liquid culture experiments using polyethylene glycol (PEG-6000) to simulate drought stress. Media containing 0%, 15%, 25%, and 35% PEG-6000 were prepared using potato, glucose, plant gel, and PEG-6000. The experiment comprised 16 treatments with five replicates per treatment. Well-growing fungal colonies on potato dextrose agar were selected, and 6 mm fungal disks were inoculated into 20 mL solid media and 100 mL liquid media, respectively. Solid cultures were incubated at 27°C for 14 days, during which fungal colony morphology was observed and recorded. Liquid cultures were incubated in a thermostatic incubator at 27°C, with continuous shaking at 150 rpm, for 14 days in the dark. After this period, the mycelia were harvested to determine biomass and physiological indices.

2.2.2 Pot experiment

A greenhouse pot experiment was conducted with different fungal inoculation treatments and moisture levels as variables. The experiment was performed under natural light conditions at Hebei Agricultural University, with temperatures maintained at 30°C during the day and 21°C at night, a 14-h light/10-h dark photoperiod, and relative humidity of 60% during the day and 70% at night. Six inoculation treatments were employed: no inoculation (CK), single inoculations with *Sg*, *Pt*, *Po*, or *Ps*, and a mixed inoculation (*Sg: Pt : Po: Ps* = 1:1:1:1). Liquid inocula was prepared by inoculating three fungal disks of each fungus into 150 mL potato dextrose broth and incubating on a shaking table at 27°C for 14 days. The mycelia were then homogenized and mixed with sterile water (v:v = 3:1) to obtain a mycelial suspension. Seedlings were inoculated by perforated root irrigation with 20 mL of the respective liquid inoculum, while the control group received 20 mL of potato dextrose broth.

One month post-inoculation, root samples were examined to confirm fungal colonization. Drought stress treatments were then imposed, with three soil moisture levels: 70% ± 5% (well-watered, WW), 50% ± 5% (light drought, LD), and 30% ± 5% (severe drought, SD) of the maximum field moisture capacity. Unified watering management was implemented, maintaining soil moisture content within the experimental range by weighing the pots at 17:00 every day. Plastic pots were repositioned every two weeks to

minimize positional effects. The experiment consisted of 18 treatments with five replications each. After 40 days of drought stress, seedlings were harvested for relevant index measurements.

2.3 Antioxidant enzyme activity determination

The superoxide dismutase (SOD) activity in fungi and plants was determined by the nitroblue tetrazolium photoreduction method (Elavarthi and Martin, 2010), and the catalase (CAT) activity was assessed by the ultraviolet absorption method (Zhang, 1990).

2.4 Malondialdehyde and osmolyte determination

The malondialdehyde (MDA) content was quantified using the thiobarbituric acid method (Zhang, 1990), and proline content was determined following the method of Bates et al. (1973). Soluble protein content was measured by the Coomassie brilliant blue colorimetric method (Campion et al., 2017).

2.5 Fungal colonization rate determination

The ectomycorrhizal root colonization rates were calculated by microscopic examination of mycorrhizal and non-mycorrhizal root tips, and the percentages of root tips with evident ectomycorrhizal structures were determined (Brundrett et al., 1996). Dark septate endophyte colonization rates were assessed by trypan blue staining and microscopic quantification of septate hyphae and microsclerotia, with the percentage of colonized root segments calculated (Phillips and Hayman, 1970).

2.6 Mycorrhizal growth response calculation

The mycorrhizal growth response (MGR) was calculated according to the method of van der Heijden (2002). If the total dry weight of inoculated plants (M) exceeded the mean total dry weight of non-inoculated plants (NMmean), then MGR (%) = $100 \times (1 - \text{NMmean}/\text{M})$. If M < NMmean, then MGR (%) = $100 \times (-1 + \text{M}/\text{NMmean})$. A positive MGR value indicated that the inoculation treatment promoted plant growth, while a negative value signified growth inhibition.

2.7 Plant nutrient element analysis

The dried above-ground and below-ground portions of *P. tabuliformis* seedlings were ground into a fine powder and digested using the H_2SO_4-H_2O_2 method until the digestion solution became colorless and transparent (Bao, 2000). The digested solution was filtered and used to determine nitrogen and phosphorus contents in the aboveground and belowground parts by the Kjeldahl method and molybdenum antimony colorimetric method, respectively.

2.8 Statistical analysis

Statistical analysis were performed with SPSS software (Version 24; SPSS Inc., Chicago, Ill., USA). For the *in vitro* experiment, a two-way analysis of variance (ANOVA) was conducted to analyze the effects of PEG-6000 treatment and fungal species on the growth and physiological responses of the four fungi. For the pot experiment, two-way ANOVA examined the effects of drought treatment and fungal inoculation on the growth and physiological responses of *P. tabuliformis* seedlings. Mean values were compared using Duncan's multiple range test at a significance level of $p < 0.05$. Correlation analyses were conducted to assess the influence of different fungal inoculants on plant parameters. Principal component analysis (PCA) was employed to analyze characteristics including fungal colonization rates, plant biomass, enzyme activities, and nutrient element contents. All graphs were generated using GraphPad Prism software (version 10.1.0).

3 Results

3.1 Effects of different concentrations of PEG-6000 on the biomass of root symbiotic fungi

The two-way ANOVA results (Supplementary Table S1) revealed significant effects of PEG stress, fungal species, and their interaction on the biomass of the four root symbiotic fungi. The biomass of the *Sg* and *Ps* strains initially increased and then decreased with increasing PEG-6000 concentration, reaching a maximum under the 15% PEG-6000 treatment in *Ps* strain, and 15% and 25% PEG-6000 treatments in *Sg* strain (Figure 1A). In contrast, the biomass of the *Pt* strain first decreased and then increased, with a 27.0% increase under the 35% PEG-6000 treatment compared to the 0% treatment. The biomass of the *Po* strain showed a gradual increase with rising PEG-6000 concentrations and reached the highest under 35% PEG-6000 treatment. Overall, the biomass accumulation of the *Sg* strain was higher than that of the *Pt* strain, and the *Ps* strain had greater biomass accumulation than the *Po* strain across all PEG-6000 concentrations.

3.2 Effects of different concentrations of PEG-6000 on the antioxidant enzyme activities of root symbiotic fungi

With increasing PEG-6000 concentrations, both SOD and CAT activities in the *Sg* and *Po* strains increased, reaching their

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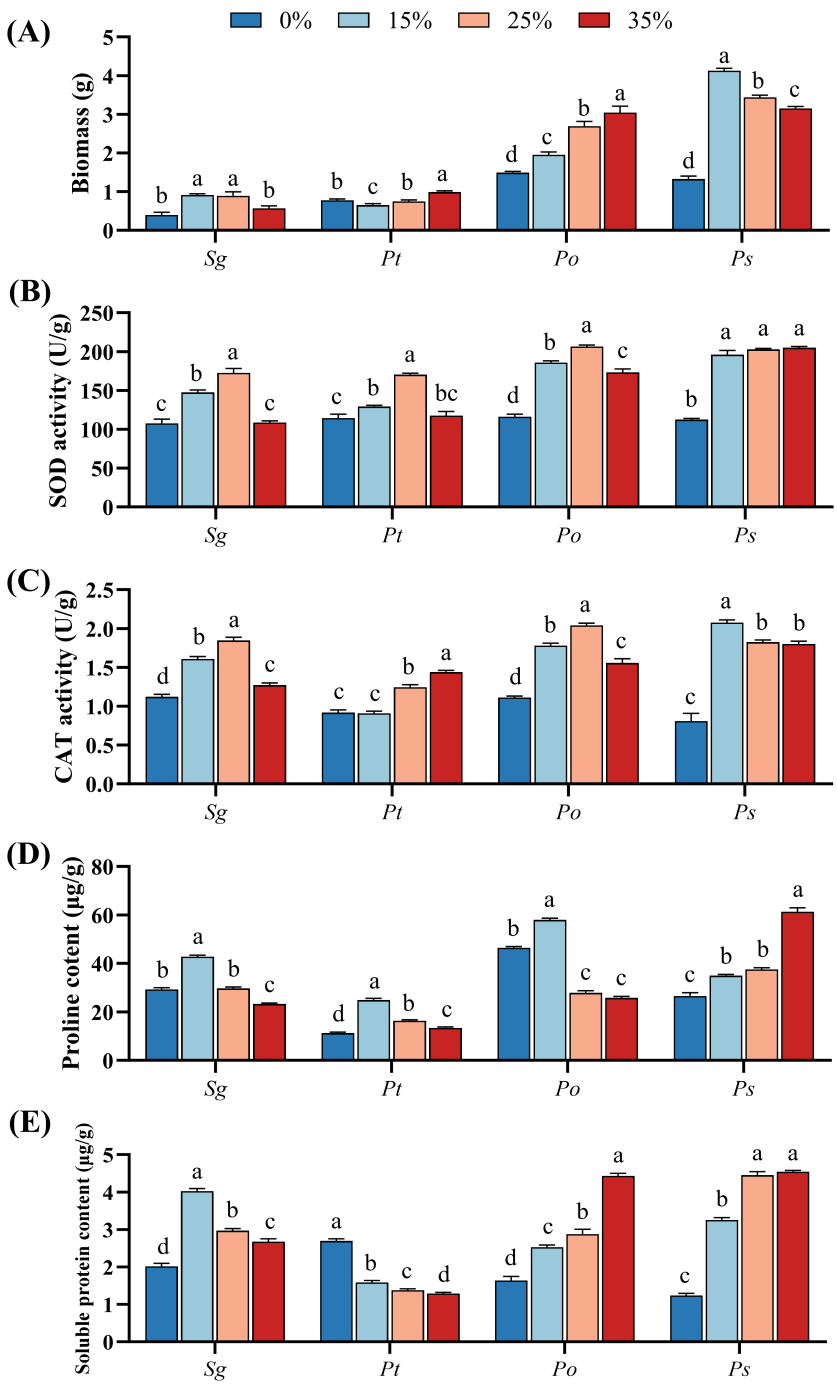

FIGURE 1
Effects of different concentrations of PEG-6000 (0%, 15%, 25%, and 35%) on the biomass **(A)**, superoxide dismutase activity **(B)**, catalase activity **(C)**, proline **(D)** and soluble protein **(E)** of *Suillus granulatus* (*Sg*), *Pisolithus tinctorius* (*Pt*), *Pleotrichocladium opacum* (*Po*), and *Pseudopyrenochaeta* sp. (*Ps*). Data (means ± SD, n = 3) are significantly different ($p < 0.05$) if followed by different letters above the bars.

maximum under the 25% PEG-6000 treatment (Figures 1B, C). Compared to the control treatment, the SOD activity in the *Pt* strain significantly increased under the 15% and 25% PEG-6000 treatments, reaching its peak at 25% PEG-6000. The CAT activity in the *Pt* strain showed no significant difference from the control at 15% PEG-6000 but significantly increased under the 25% and 35% PEG-6000 treatments, with the highest activity observed at 35% PEG-6000. In contrast, the SOD and CAT activities in the *Ps* strain remained significantly higher under all PEG-6000 treatments compared to the 0% PEG-6000 treatment. Overall, the two dark septate endophytes (*Po* and *Ps*) exhibited greater improvements in antioxidant enzyme activities in response to PEG-6000 stress than the two ectomycorrhizal fungi (*Sg* and *Pt*).

3.3 Effects of different PEG-6000 concentrations on osmotic adjustment substances in symbiotic root fungi

All four fungi influenced the accumulation of proline and soluble proteins in response to drought stress induced by PEG-6000 (Figures 1D, E). For the *Sg*, *Pt*, and *Po* strains, proline content peaked at the 15% PEG-6000 treatment, significantly higher than the control, and subsequently decreased with increasing PEG-6000 concentrations. In contrast, PEG-6000 stress consistently and significantly increased proline levels in the *Ps* strain, with no significant difference between the 15% and 25% treatments. However, at 35% PEG-6000, the *Ps* strain exhibited significantly higher proline content compared to the other treatments (Figure 1D).

PEG-6000 stress consistently and significantly increased soluble protein levels in the *Sg*, *Po*, and *Ps* strains, while it resulted in a significant reduction in the *Pt* strain. The *Sg* strain reached its maximum soluble protein content at 15% PEG-6000, followed by a decreasing trend with increasing PEG-6000 concentrations. Conversely, soluble protein content in the *Po* and *Ps* strains increased significantly with rising PEG-6000 levels, peaking at 35% PEG-6000. In contrast, the *Pt* strain exhibited the lowest soluble protein content at 35% PEG-6000 (Figure 1E).

3.4 Root fungal colonization and growth performance of *P. tabuliformis*

In the non-inoculated treatments, no fungal structure was observed in the roots of *P. tabuliformis* (Figure 2A). The fungal colonization rates showed that, except for the *Po* inoculation under SD treatment, all other inoculation treatments under various drought conditions had colonization rates exceeding 50% (Figure 2A). The colonization rates of *Sg* increased with drought severity, whereas the rates for other treatments first increased and then decreased. The colonization rates for *Pt*, *Po*, and mix treatments peaked under LD conditions. The mix treatment had higher colonization rates than other treatments under WW and LD conditions.

There was a significant interaction between fungal inoculation and drought stress on the mycorrhizal growth response of the seedlings (Supplementary Table S2). With increasing drought stress, the mycorrhizal growth response values for the inoculation treatments initially increased and then decreased (Figure 2B). All inoculation treatments positively affected the growth of *P. tabuliformis* seedlings under drought conditions. The MGR values of the mixed inoculation treatment were higher than those of individual inoculations, indicating the greatest growth promotion effects under drought stress.

The plant height of non-inoculated, *Sg*-, *Po*-, and *Ps*-inoculated seedlings decreased with increasing drought stress (Figure 2C). The plant height of *Pt*- and mix-inoculated seedlings first increased and then decreased, peaking under LD conditions. Shoot and total biomass of non-inoculated seedlings decreased with increasing drought stress, while inoculated seedlings showed an initial increase followed by a decrease, reaching a maximum under LD conditions (Figures 2D, E). Under all drought conditions, mixed inoculation treatments resulted in significantly higher shoot and total biomass compared to single inoculations.

3.5 Effects of different inoculation treatments on the antioxidant enzyme activities of *P. tabuliformis* seedlings under drought stress

With increasing drought stress, the SOD activity of non-inoculated seedlings gradually increased, while that of inoculated seedlings first increased and then decreased (Figure 3A). The SOD activity of all inoculated seedlings peaked under LD conditions. CAT activity in seedlings from all treatments increased first and then decreased with increasing drought stress severity (Figure 3B). Under LD and SD treatments, inoculated seedlings had higher SOD and CAT activities than non-inoculated ones, indicating that inoculation treatments enhanced antioxidant enzyme activities to cope with drought stress.

3.6 Effects of different inoculation treatments on the MDA content of *P. tabuliformis* seedlings under drought stress

Under drought stress, the MDA content of seedlings increased with drought severity, while inoculation treatments reduced MDA accumulation (Figure 3C). There was no significant difference in MDA content between inoculated and control seedlings under WW conditions. Under LD conditions, inoculation treatments significantly reduced MDA content by 22.8%, 35.4%, 12.0%, 32.0%, and 35.9%, respectively. Under SD conditions, the reductions were 30.9%, 40.5%, 27.0%, 33.3%, and 41.2%, respectively. mixed inoculation treatments significantly reduced MDA content under LD and SD conditions.

3.7 Effects of different inoculation treatments on the osmotic adjustment substance contents of *P. tabuliformis* seedlings under drought stress

With increasing drought severity, the proline content of non-inoculated, *Sg*-, *Po*-, and mix-inoculated seedlings gradually increased, while the proline content of *Pt*- and *Ps*-inoculated seedlings first increased and then decreased (Figure 3D). Under SD conditions, the proline content of all inoculation treatments significantly increased compared to the control, except for the *Ps* inoculation. The mix-inoculated treatment had the highest proline content.

Under WW, LD, and SD conditions, all fungal inoculation treatments significantly increased the soluble protein content in the seedlings (Figure 3E). Specifically, under WW conditions, the soluble protein content in the mix and *Pt* treatments was significantly higher than in the *Sg*, *Po*, and *Ps* treatments. Under LD and SD conditions, the soluble protein content of the mixed-inoculated seedings was significantly higher than that of the other four single inoculation treatments.

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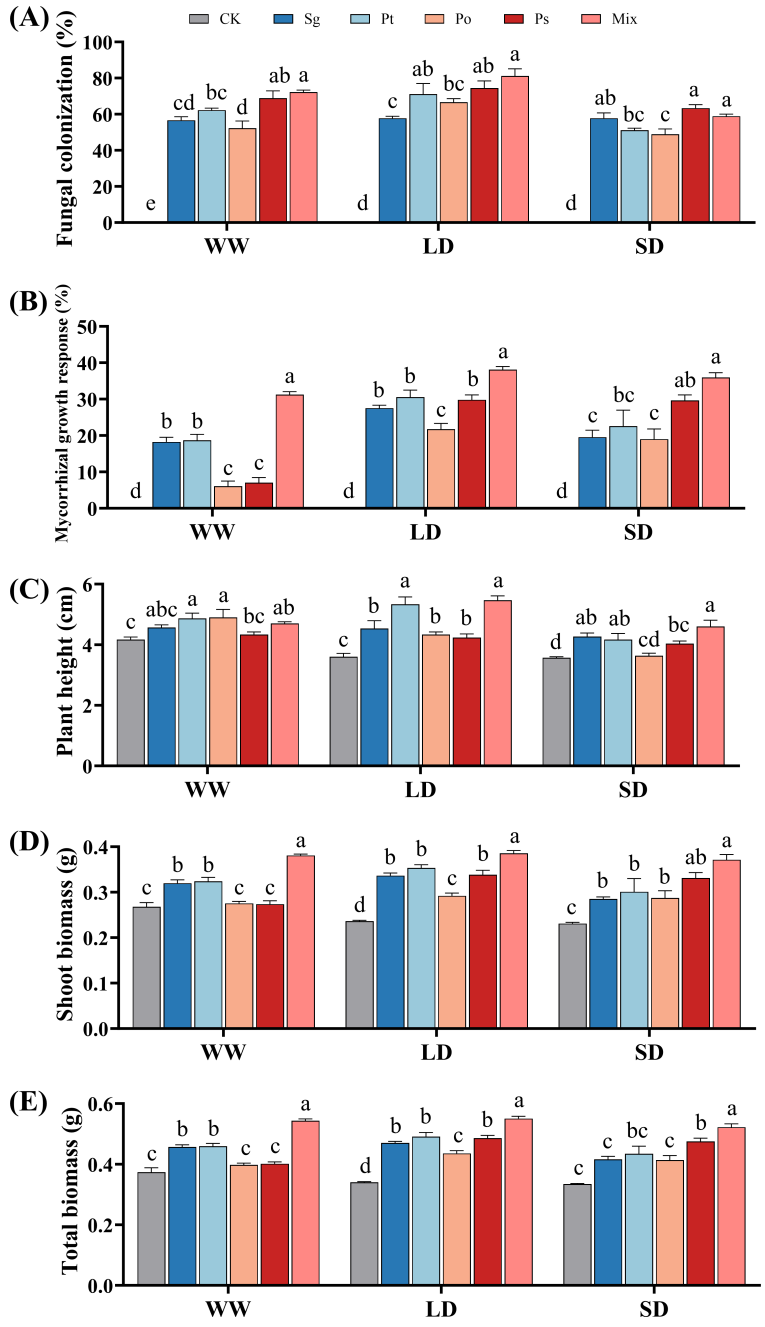

FIGURE 2
Effects of different inoculation treatments on the growth performance of *Pinus tabuliformis* seedlings under drought stress. Data (means ± SD, n = 3) are significantly different ($p < 0.05$) if followed by different letters above the bars. **(A)**, fungal colonization rate; **(B)**, mycorrhizal growth response; **(C)**, plant height; **(D)**, shoot biomass and **(E)**, total biomass. *Sg*, *Suillus granulatus*; *Pt*, *Pisolithus tinctorius*; *Po*, *Pleotrichocladium opacum*; *Ps*, *Pseudopyrenochaeta* sp.; Mix, mixed inoculation of four root symbiotic fungi. WW, well-watered; LD, light drought; SD, severe drought.

3.8 Effects of different inoculation treatments on the nutrient content of *P. tabuliformis* seedlings under drought stress

With increasing drought severity, the N content in the shoots and roots of non-inoculated and *Sg*-inoculated seedlings gradually decreased, while N content in the shoots and roots of *Ps*- and mix-inoculated seedlings first increased and then decreased (Figures 4A, B). The P content in the shoots of non-inoculated seedlings gradually decreased, while the P content in the roots first increased and then decreased (Figures 4C, D). The P content in the shoots and roots of *Sg*-, *Ps*-, and mix-inoculated seedlings gradually decreased, whereas in *Po*-inoculated seedlings, it first increased and then decreased with increasing drought severity (Figures 4C, D).

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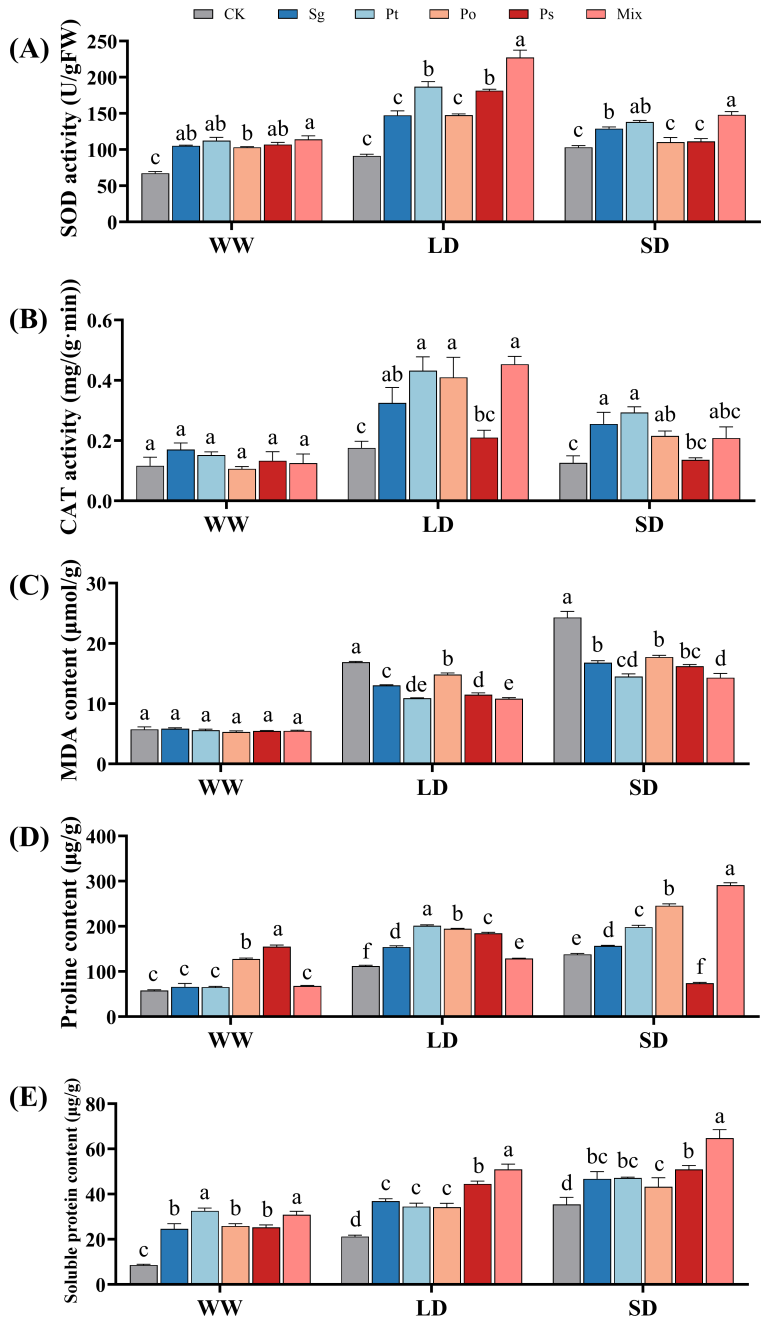

FIGURE 3
Effects of different inoculation treatments on the physiological characteristics of *Pinus tabuliformis* seedlings under drought stress. Data (means ± SD, n = 3) are significantly different ($p < 0.05$) if followed by different letters above the bars. **(A)**, superoxide dismutase activity; **(B)**, catalase activity; **(C)**, malondialdehyde content; **(D)**, proline and **(E)**, soluble protein. *Sg*, *Suillus granulatus*; *Pt*, *Pisolithus tinctorius*; *Po*, *Pleotrichocladium opacum*; *Ps*, *Pseudopyrenochaeta* sp.; Mix, mixed inoculation of four root symbiotic fungi. WW, well-watered; LD, light drought; SD, severe drought.

3.9 Correlation analysis

Correlation analysis of different inoculation treatments on the growth and physiological indexes of *P. tabuliformis* seedlings under drought stress revealed significant positive correlations between fungal colonization rates and various growth and nutrient parameters (Figure 5). The colonization rates of *Pt*-inoculated seedlings were significantly positively correlated with plant height, total biomass, root N content, shoot P content, and root P content ($p < 0.05$) (Figure 5B). *Po*-inoculated seedlings showed significant positive correlations with root biomass, SOD, CAT, and shoot P content ($p < 0.05$) (Figure 5C). Mix-inoculated seedlings exhibited significant positive correlations with total biomass and shoot N content ($p < 0.05$) (Figure 5E). Higher fungal colonization rates of *Pt*-, *Po*-, and mix-inoculated seedlings promoted seedling growth and nutrient absorption.

The root biomass of *Po*-inoculated seedlings was significantly positively correlated with SOD, CAT, and shoot P content, while

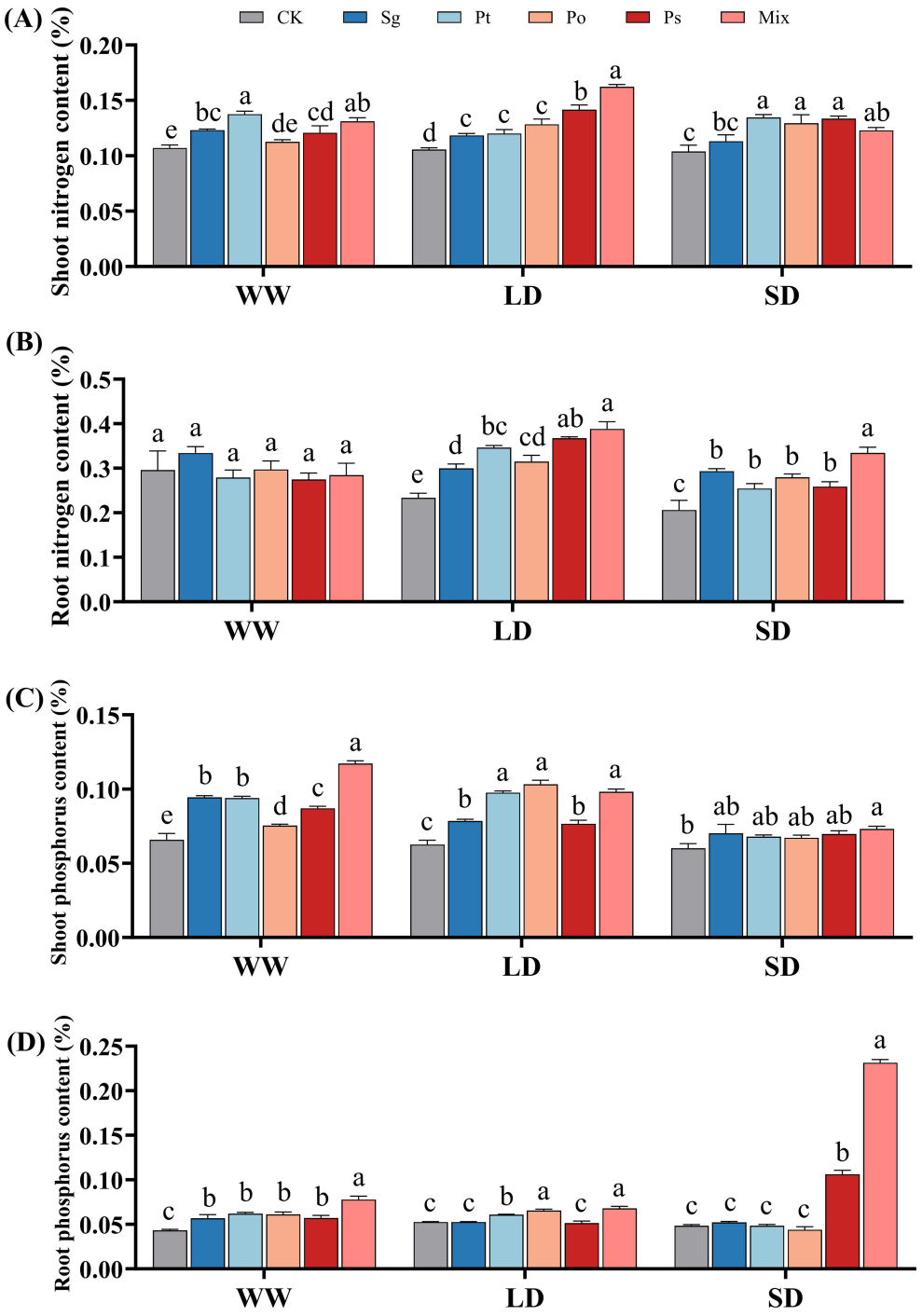

FIGURE 4
Effects of different inoculation treatments on nitrogen and phosphorus content in the shoots and roots of *Pinus tabuliformis* seedlings under drought stress Data (means ± SD, n = 3) are significantly different ($p < 0.05$) if followed by different letters above the bars. **(A)**, shoot nitrogen content; **(B)**, root nitrogen content; **(C)**, shoot nitrogen content and **(D)**, root phosphorus content. *Sg*, *Suillus granulatus*; *Pt*, *Pisolithus tinctorius*; *Po*, *Pleotrichocladium opacum*; *Ps*, *Pseudopyrenochaeta* sp.; Mix, mixed inoculation of four root symbiotic fungi. WW, well-watered; LD, light drought; SD, severe drought.

total biomass was significantly positively correlated with SOD and CAT ($p < 0.05$) (Figure 5C). *Ps*-inoculated seedlings' root biomass was significantly positively correlated with soluble protein and shoot N content ($p < 0.05$) (Figure 5D). The plant height of mix-inoculated seedlings was significantly positively correlated with SOD, CAT, shoot N content, and root N content ($p < 0.05$) (Figure 5E). These results indicate that the strong antioxidant defense and nutrient uptake abilities of *Po*- and mix-inoculated seedlings significantly promote growth and improve drought resistance.

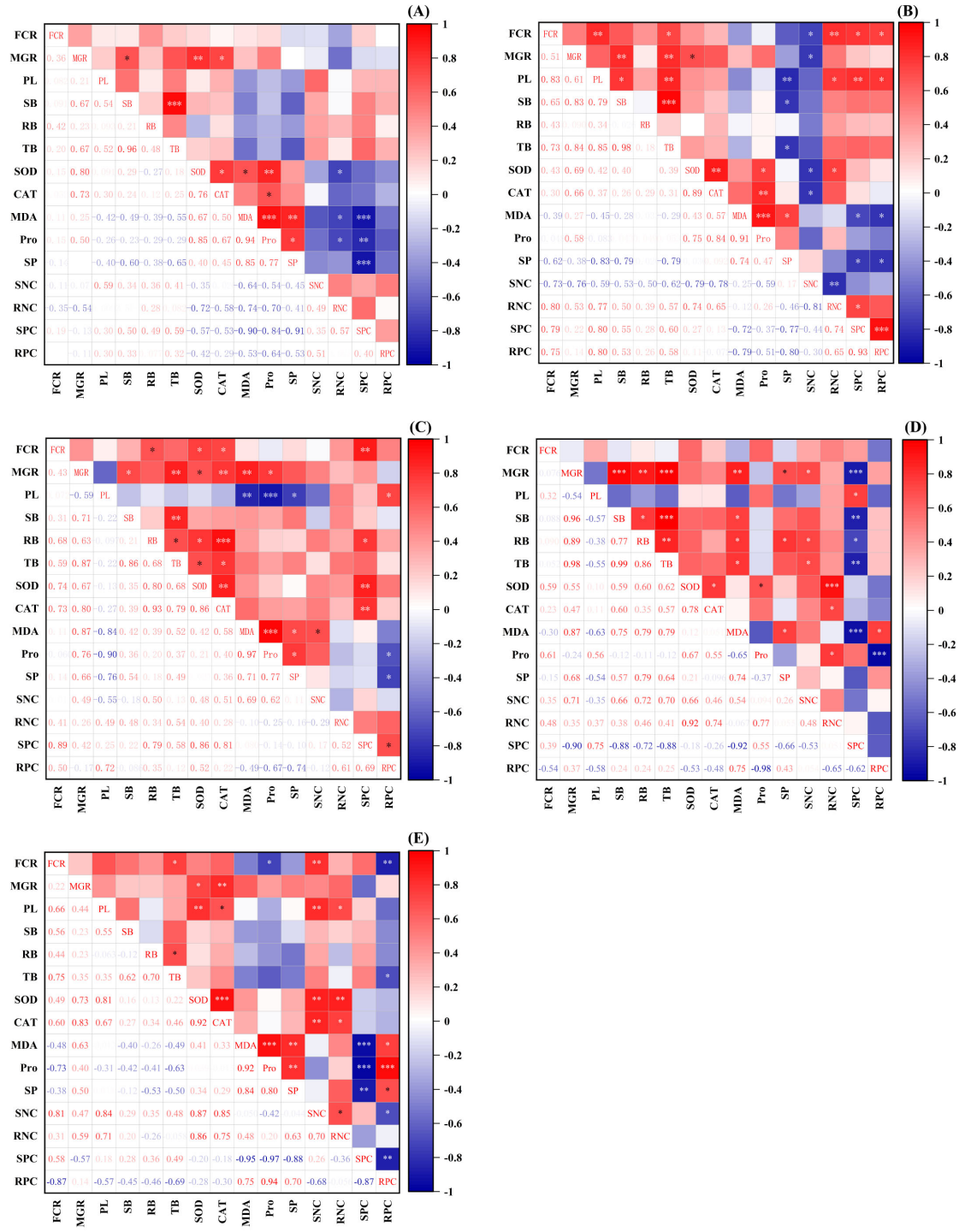

FIGURE 5
Correlations among indicators in the pot experiment. **(A)** *Sg*, **(B)** *Pt*, **(C)** *Po*, **(D)** *Ps*, **(E)** Mix; *: ($p < 0.05$); **: $p < 0.01$; ***: $p < 0.001$; FCR, Fungal colonization rate; MGR, Mycorrhizal growth response; PL, Plant height; SB, Shoot biomass; RB, Root biomass; TB, Total biomass; SOD, Superoxide dismutase activity; CAT, Catalase activity; MDA, Malondialdehyde content; Pro, Proline; SP, Soluble protein; SNC, Shoot N content; RNC, Root N content; SPC, Shoot P content; RPC, Root P content.

3.10 PCA analysis

PCA was used to evaluate the similarity among different inoculation treatments and the relationships between plant height, biomass, enzyme activity, antioxidant capacity, and nutrient composition (Figure 6). The first principal component (PC1) and the second principal component (PC2) explained 46.4% and 19.9% of the variance, respectively. There were significant differences between control and inoculated treatments under drought stress (Figure 6A). The mix inoculation treatment was

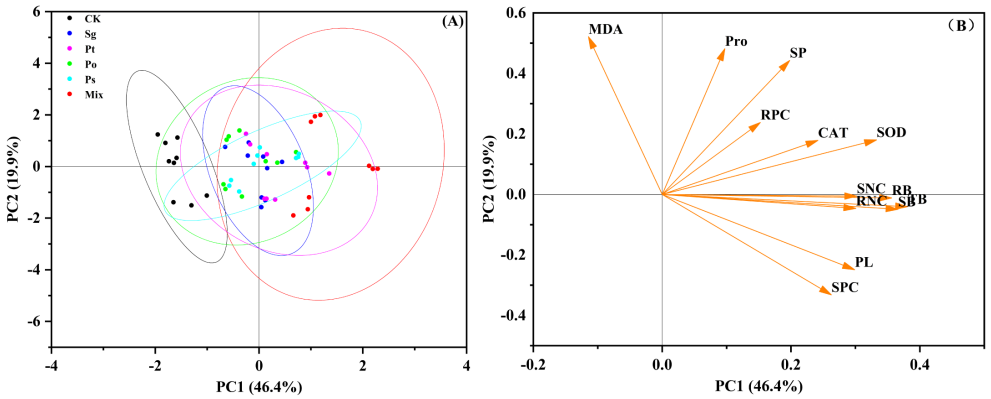

FIGURE 6
Principal component analysis (PCA) of indicators in the pot experiment. **(A)** *Sg* indicates *Suillus granulatus*; *Pt* indicates *Pisolithus tinctorius*; *Po* indicates *Pleotrichocladium opacum*; *Ps* indicates *Pseudopyrenochaeta* sp.; Mix indicates mixed inoculation of four root symbiotic fungi.; **(B)** PL, Plant height; SB, Shoot biomass; RB, Root biomass; TB, Total biomass; SOD, Superoxide dismutase activity; CAT, Catalase activity; MDA, Malondialdehyde content; Pro, Proline; SP, Soluble protein; SNC, Shoot N content; RNC, Root N content; SPC, Shoot P content; RPC, Root P content.

significantly separated from other treatments. Antioxidant enzyme activity, osmotic adjustment substances, and nutrient content were the main factors affecting plant growth and were positively correlated with biomass (Figure 6B). The results indicated that inoculation treatments promoted plant growth and improved antioxidant capacity, enhancing the tolerance of *P. tabuliformis* seedlings to drought stress.

4 Discussion

4.1 Effects of drought stress on the growth and physiological characteristics of root symbiotic fungi

The biomass of fungi is a crucial indicator of their resistance under stressful environments. All four fungi exhibited normal growth under PEG stress, indicating their tolerance to PEG-induced drought stress. Abiotic stresses, such as drought and salt, have been shown to induce an increase in plant peroxisome content (Fahy et al., 2017; Castillo et al., 2008; Sanad et al., 2019). Li et al. (2022b) demonstrated that strains with greater drought resistance exhibit significant increases in antioxidant enzyme activities (SOD, POD, and CAT) under drought stress, indicating that increased antioxidant enzyme activity is a response to drought in sensitive strains. In this study, the biomass of the *Sg*, *Po*, and *Ps* strains, except for the *Pt* strain, was greater under other PEG-6000 treatments compared to the 0% PEG-6000 treatment. This may be related to the higher contents of antioxidant enzymes and osmotic adjustment substances in these strains under PEG-6000 treatments. SOD and CAT are critical protective enzymes in the antioxidant system, responsible for scavenging reactive oxygen species (ROS) and H_2O_2, working together to maintain the balance between ROS production and scavenging (Raja et al., 2017). This study found that the activities of antioxidant enzymes and the contents of osmotic adjustment substances increased in all four fungi under PEG stress, with SOD and CAT activities showing consistent changes. This indicates that all four fungi could alleviate oxidative damage by synthesizing antioxidant enzymes to scavenge ROS produced by PEG-6000 stress. Simultaneously, they improved their accumulation of proline and soluble proteins, promoting cellular osmoregulation to protect mycelial cell membrane structures, thereby enhancing stress tolerance. This study also revealed that DSE strains had greater biomass than ECMF strains, suggesting that DSE strains were less affected by PEG-6000 stress, possibly due to their highly melanized hyphae. Melanin in fungi serves to protect them from harmful environmental conditions (Fernandez and Koide, 2013).

4.2 Effects of root symbiotic fungi on the growth and development of *P. tabuliformis* seedlings under drought stress

Microorganisms are natural partners in plant defense mechanisms under adverse conditions (Meena et al., 2017). In this study, typical mycorrhizal structures and DSE hyphae and microsclerotia were observed in root samples inoculated with ECMF and DSE strains, indicating effective colonization even under drought conditions. Additionally, the mycorrhizal growth response values of inoculation treatments were all positive under drought stress, with mixed inoculation treatments showing greater effects than individual treatments. Among the four individual inoculation treatments, the Ps-inoculated seedlings under SD conditions exhibited higher fungal colonization rates, mycorrhizal growth responses, and biomass compared to those inoculated with the other three fungi. This increased performance is likely due to the reduction in MDA content and the increase in soluble protein content in the Ps-inoculated seedlings, which enhances cell membrane stability and improves dehydration resistance, allowing the plants to better adapt to stressful environments. These findings align with previous pure culture experiments where the Ps strain

demonstrated higher biomass and stronger drought tolerance under the highest PEG concentration stress. Thus, different fungi exhibit varying levels of drought tolerance and influence the drought resistance of plants differently. Studies have shown that inoculation with *Amanita vaginata* significantly promotes the growth of *P. tabuliformis* (Zhang et al., 2011), and the biomass of *Hedysarum scoparium* inoculated with DSE under drought stress was significantly greater than that of non-inoculated treatments (Li et al., 2019). However, few studies have investigated the co-inoculation of ECMF and DSE strains under drought stress. This experiment demonstrated that inoculating ECMF and DSE strains under drought stress promoted increases in plant height, shoot biomass, root biomass, and total biomass of *P. tabuliformis* seedlings. These growth indices were significantly higher in mixed inoculation treatments compared to non-inoculated and individual inoculation treatments. Increased plant biomass indicates more organic matter to support plants, enhancing their survival in arid environments. Our study revealed that inoculating root symbiotic fungi under drought stress effectively mitigated adverse environmental effects on *P. tabuliformis* seedlings, promoting growth and improving drought resistance. Furthermore, mixed inoculation treatments had synergistic rather than competitive effects on the growth of *P. tabuliformis* seedlings under drought conditions.

4.3 Effects of root symbiotic fungi on the physiological characteristics of *P. tabuliformis* seedlings under drought stress

Drought stress leads to the overproduction of ROS in plants, causing degradation of lipids, proteins, and nucleic acids, damaging plant cells, and reducing growth and development (Gill and Tuteja, 2010; Kapoor et al., 2019). Plants produce enzymes such as SOD and CAT to scavenge ROS, protecting cells from oxidative damage and preserving membrane integrity (Wu et al., 2006; Raja et al., 2017). In this study, antioxidant enzyme activities in *P. tabuliformis* seedlings inoculated with root symbiotic fungi increased under drought stress, indicating that these fungi protect host plants from oxidative damage by enhancing antioxidant enzyme activities. Under LD conditions, mixed inoculation treatments significantly increased SOD and CAT activities in seedlings compared to individual treatments, indicating a greater ability to eliminate ROS. MDA content, an indicator of oxidative damage, reflects the degree of stress injury to plants. Excessive MDA accumulation often results from oxidative damage to membrane lipids, with lower MDA levels indicating higher membrane stability (Quiroga et al., 2020). In this study, MDA content in *P. tabuliformis* seedlings decreased after inoculation with root symbiotic fungi, indicating improved cell membrane stability. Under LD and SD conditions, mixed inoculation treatments resulted in significantly lower MDA content compared to control and individual inoculation treatments, consistent with findings in *P. sylvestris* var. *mongolica* seedlings (Zhao et al., 2020). However, under WW conditions, there was no significant difference in MDA content between mixed inoculation and control treatments, indicating no significant synergistic effect of co-inoculation under well-watered conditions.

Osmotic adjustment is a crucial mechanism for plants to regulate water potential under drought stress. Proline and soluble protein are key osmotic adjustment substances that allow plants to adapt to stressful environments by increasing dehydration tolerance through accumulation (Pal et al., 2018; Sharma and Verslues, 2010; Azmat and Moin, 2019). In this study, proline content in inoculated seedlings under LD and SD conditions was significantly higher than in control treatments, with mixed inoculation treatments showing the highest proline content under SD conditions. Rezaei-Chiyaneh et al. (2021) also found that co-inoculated plants under severe water stress had the highest proline content. This study indicates that co-inoculation of ECMF and DSE strains promotes proline biosynthesis, enhancing osmotic regulation under severe water stress. Higher contents of soluble protein in inoculated plants suggest greater osmotic ability, helping seedlings maintain turgor pressure under drought conditions (Liu et al., 2021). Therefore, root symbiotic fungi improve osmotic balance by increasing the content of osmotic adjustment substances, enhancing plant tolerance and alleviating the negative effects of drought.

Mycorrhizal symbionts significantly affect the uptake of nitrogen and phosphorus by plants (Veresoglou et al., 2012). Research has shown that fungal inoculation improves nutrient recovery efficiency, accumulation, and growth in host plants (Surono and Narisawa, 2017; Vergara et al., 2017, 2018; Lu et al., 2016). In this experiment, N contents in the shoots and roots of inoculated plants under LD and SD conditions were significantly higher than in control treatments, with mixed inoculation treatments showing the highest N contents under LD conditions. Mycorrhizal fungi increase nitrogen uptake under drought stress by enhancing root hydraulic conductivity (Graham and Syversen, 1984). Mycorrhizal fungi also promote nitrogen uptake by decomposing organic matter (Hodge et al., 2001; Goussous and Mohammad, 2009), helping maintain water status under water scarcity. Mixed inoculation treatments significantly increased P content in shoots and roots under WW conditions and were more effective than individual treatments under LD and SD conditions. Fungi can explore more soil volume than non-mycorrhizal plants, enhancing P uptake (Evelin et al., 2009). Adding mycorrhizal fungi to soil increases microbial biomass and CO_2 release, forming weak acids that dissolve phosphorus-bearing minerals, and increasing phosphorus availability (Subramanian et al., 2006). Thus, plants inoculated with mycorrhizal fungi exhibit higher productivity even under adverse conditions. In summary, root symbiotic fungi improve the uptake of nutrients such as N and P, promoting growth and enhancing plant resistance. The synergistic effects of ECMF and DSE on nutrient absorption are greater than individual inoculations.

5 Conclusion

Drought stress affects a range of physiological changes in plants, and root symbiotic fungi can help plants withstand the adverse effects of drought. This study demonstrated that root symbiotic fungi (*Sg*,

Po, *Ps*, and *Pt*) enhance the growth and drought resistance of *P. tabuliformis* seedlings. All fungi tolerated PEG-induced drought stress, with increased antioxidant enzyme activities and osmotic adjustment substances. DSE strains showed greater resilience due to their melanized hyphae. Acids produced by root symbiotic fungi under drought stress may help plants to activate elements in the soil that plants fail to take up. Therefore, the determination of the composition and content of metabolites of root symbiotic fungi under drought stress could be carried out in future studies. Inoculation, especially mixed inoculation, significantly improved plant height, biomass, and drought resistance. The fungi increased antioxidant enzymes and osmotic substances, mitigating oxidative damage and enhancing osmotic regulation. Additionally, they improved nutrient uptake, particularly nitrogen and phosphorus. These findings suggest that root symbiotic fungi, particularly in combination, are effective in enhancing drought resistance and growth of *P. tabuliformis*, providing valuable insights for afforestation and forest management in arid regions.

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

LX: Writing – review & editing, Writing – original draft, Methodology, Data curation. JH: Writing – review & editing, Writing – original draft, Visualization. YM: Writing – review & editing. YYZ: Writing – review & editing, Validation, Data curation. BL: Writing – review & editing, Data curation. JZ: Writing – review & editing. YZ: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation.

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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.2024.1446437/full#supplementary-material

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Arbuscular mycorrhizal fungi enhance nitrogen assimilation and drought adaptability in tea plants by promoting amino acid accumulation

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The development and quality of tea plants (*Camellia sinensis* (L.) O. Ktze.) are greatly hampered by drought stress (DS), which affects them in a number of ways, including by interfering with their metabolism of nitrogen (N). Arbuscular mycorrhizal fungi (AMF) are known to enhance water and nutrient absorption in plants, but their specific effects on tea plant N metabolism under DS and the associated regulatory mechanisms remain unclear. This study aimed to evaluate the impact of *Claroideoglomus etunicatum* inoculation on N assimilation in tea plants (*C. sinensis* cv. Fuding Dabaicha) under well-watered (WW) and DS conditions, and to explore potential molecular mechanisms. After 8 weeks of DS treatment, root mycorrhizal colonization was significantly inhibited, and the biomass of tea shoots and roots, as well as the contents of various amino acids (AAs) were reduced. However, AMF inoculation significantly increased the contents of tea polyphenols and catechins in leaves by 13.74%-36.90% under both WW and DS conditions. Additionally, mycorrhizal colonization notably increased N content by 12.65%-35.70%, various AAs by 11.88%-325.42%, and enzymatic activities associated with N metabolism by 3.80%-147.62% in both leaves and roots. Gene expression analysis revealed a universal upregulation of N assimilation-related genes (*CsAMT1;2*, *CsAMT3;1*, *CsGS1*, *CsNADH-GOGAT*, *CsTS2*, *CsGGT1*, and *CsADC*) in AMF-colonized tea roots, regardless of water status. Under DS condition, AMF inoculation significantly upregulated the expressions of *CsNRT1;2*, *CsNRT1;5*, *CsNRT2;5*, *CsNR*, *CsGS1*, *CsGDH1*, *CsGDH2*, *CsTS2*, *CsGGT1*, *CsGGT3*, and *CsSAMDC* in tea leaves. These findings suggest that AMF improved tea plant adaptability to DS by enhancing N absorption and assimilation, accompanied by the synthesis and accumulation of various AAs, such as Glu, Gln, Asp, Lys, Arg, GABA and Pro. This is achieved through the upregulation of N metabolism-related genes and the activation of related enzymes in tea plants under DS condition. These findings provide valuable insights into the role of AMF in regulating tea plant N metabolism and enhancing stress tolerance.

KEYWORDS

amino acid, arbuscular mycorrhizal fungus, drought stress, nitrogen metabolism, tea plant

1 Introduction

Camellia sinensis (L.) O. Ktze., originating from southwestern China, is highly valued economically as it is processed into one of the world's three major non-alcoholic beverages. Consequently, tea has become an important leafy economic crop in the tropical and subtropical regions of Asia (Liu et al., 2020a). Tea plants are highly sensitive to soil moisture, and drought stress (DS) significantly inhibit their growth, affecting both quality and yield. Therefore, DS remains one of the principal challenges in tea production (Guo et al., 2017). Previous study has revealed that DS restricts plant development through inhibiting photosynthesis and respiration, reducing carbohydrate synthesis, accelerating protein hydrolysis, damaging cell membrane permeability, enhancing antioxidant metabolism, and altering the levels and distribution of hormones and amino acid (AA) (Qian et al., 2018). DS also affects the transcriptional levels of drought-responsive functional genes (Qian et al., 2018). Additionally, DS significantly inhibits the absorption and transportation of mineral elements such as nitrogen (N), phosphorus (P), potassium (K), and calcium (Ca) in tea plants (Liu et al., 2024), among which N plays a pivotal role in regulating tea plant growth and the synthesis of AAs and related secondary metabolites, which are crucial for yield and quality (Xie et al., 2023). This makes understanding N metabolism under DS critical.

Ammonium (NH_4^+) and nitrate (NO_3^-) are the primary inorganic N sources absorbed by tea roots from the soil. These compounds are transported through ammonium transporters (AMTs) and nitrate transporters (NRTs) to be utilized by plants (Zhang et al., 2023). AMTs are localized in the cell membrane, and the transcription of genes encoding these proteins is influenced by various factors, including external N levels and mycorrhizal symbiosis (Zhang et al., 2023). In tea plants, the expressions of *CsAMTs* and membrane-bound NRTs exhibit tissue-specific patterns. Specifically, *CsAMT1;2* and *CsNRT1;5* show the highest transcriptional abundance in roots, while *CsAMT1;1*, *CsAMT3;1*, *CsNRT1;2*, and *CsNRT2;5* are primarily expressed in leaves (Zhang et al., 2018, 2023). NO_3^- absorbed by plants must first be reduced to nitrite (NO_2^-) in the cytoplasm by nitrate reductase (NR) and then further reduced to NH_4^+ in the plastids by nitrite reductase (NiR) (Liu et al., 2022b). In vascular plants, more than 95% of NH_4^+/NH_3 is assimilated through the glutamine synthetase-glutamate synthetase (GS-GOGAT) cycle (Thomsen et al., 2014). The expression of GS- and GOGAT-related genes exhibits tissue-specific patterns in tea plants. GS is primarily involved in NH_4^+ assimilation and NO_3^- reduction, whereas GOGAT predominantly facilitates ammonia assimilation (Zhang et al., 2023). Additionally, glutamate dehydrogenase (GDH) catalyzes the conversion of ammonia to glutamic acid (Glu) in plant tissues and deaminates Glu to α-ketoglutarate, which serves as a stress response enzyme to detoxify intracellular high ammonia (Zhang et al., 2023). Furthermore, theanine (Thea) synthesis represents a distinctive N assimilation pathway in tea plants (Lin et al., 2023). In tea roots, Thea can be synthesized by two routes: theanine synthetase (TS) from Glu and ethylamine (EA) (Zhang et al., 2023), and gamma-glutamyl transferase (GGT) from glutamine (Gln) and EA (Yang et al., 2021). In this process, EA is synthesized from alanine (Ala) through alanine decarboxylase (AlaDC) catalysis, while Ala is typically produced from pyruvate via alanine aminotransferase (ALT) (Lin et al., 2023). S-adenosylmethionine decarboxylase (SAMDC) and arginine decarboxylase (ADC) share domains with AlaDC and are often analyzed as alternative enzymes (Shi et al., 2011). Thea synthesized in roots is transported to new buds and leaves via vascular tissues for accumulation and catabolic metabolism, ultimately degrading into Glu and EA, with EA being oxidized into acetaldehyde by amine oxidase (AO), potentially contributing to catechin synthesis (Lin et al., 2023). Thus, understanding N uptake and assimilation in tea plants is essential.

Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) serve as key enzymes for producing other AAs by catalyzing the conversion of Glu in plants (Hildebrandt et al., 2015). Glutamate decarboxylase (GAD) specifically catalyzes the synthesis of gamma-aminobutyric acid (GABA) from Glu (Xu et al., 2017). Asparagine synthase (ASNS) is a crucial enzyme in plant N metabolism, catalyzing the synthesis of asparagine (Asn), an essential carrier for transporting newly synthesized N within the plant and for translocating N from aging organs (Gaufichon et al., 2010). Pyruvate, a crucial intermediate of glycolysis, not only participates in energy metabolism but also closely links to amino acid synthesis through reactions such as transamination, serving as a vital bridge between carbohydrate and amino acid metabolism. Key enzymes, including phosphoenolpyruvate carboxylase (PEPC), pyruvate kinase (PK), pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH), and pyruvate dehydrogenase kinase (PDK), play significant roles in pyruvate production, consumption, and regulation (Liu et al., 2020b). Recent studies have shown that under DS, N absorption in plants decreases, and the activity of N metabolism-related enzymes weakens, leading to lower N metabolism efficiency (Dong et al., 2022; Du et al., 2020). Therefore, enhancing drought resistance and improving tea quality by optimizing N metabolism in tea plants is particularly important.

Arbuscular mycorrhizal fungi (AMF), beneficial microorganisms in soil, infect approximately 80% of terrestrial plant roots, establishing a mutualistic symbiosis (Parniske, 2008). Research has found that AMF promote plant growth and development by rapidly supplying water and nutrients through its extensive external and intraradical hyphae (Parniske, 2008). Numerous studies have revealed that AMF enhances plant drought tolerance by accelerating nutrient acquisition, promoting leaf gas exchange, activating antioxidant defense systems, improving osmotic adjustment, and regulating the expression of drought-tolerant functional genes (Wang et al., 2023). Additionally, AMF has been shown to increase root system development, enhancing water and nutrient uptake, which contributes to better physiological performance under drought conditions (Nader et al., 2024). Biochemically, AMF enhances the activities of antioxidant enzymes like catalase and peroxidase, mitigating oxidative stress and reducing lipid peroxidation (He et al., 2020; Sheteiwy et al., 2021). On a molecular level, AMF modulates the expression of key genes involved in proline metabolism and sugar transport, as well as

aquaporin genes, which are crucial for maintaining water homeostasis, further improving the plant's resilience to drought stress (Sheteiwy et al., 2021). Regarding N metabolism, previous study has reported that AMF inoculation facilitates N absorption and utilization in white clover (*Trifolium repens*), evidenced by increased N content, N metabolic enzymes activities, and AA concentrations in both leaves and roots (Xie et al., 2021). Similar results were obtained in *Catalpa bungei*. Inoculation with AMF could promote N uptake and assimilation by comprehensively regulating the expression of key enzyme genes of root nitrogen metabolism and nitrate transporter genes (Chen et al., 2023). In tea plants, the synthesis and accumulation of free AAs in AMF-colonized seedlings were notably promoted, possibly related to the upregulation of AA synthesis genes *CsGDH* and *CsGOGAT* (Shao et al., 2019; Wang et al., 2020). These processes emphasize the importance of AA synthesis under stress. These findings suggest a significant role for AMF in enhancing N metabolism.

Acknowledging the challenge posed by the conflict between tea plants' preference for acidic soils (pH 4.5-6.0) and the optimal pH range (6.0-8.0) for N assimilation (Li et al., 2016a), which is crucial for tea growth and development, our study endeavors to fill this research gap. While AMF are known to enhance water and nutrient uptake by plants, the specific effect on N metabolism of tea plants under DS condition and the underlying regulatory mechanism remain unclear. We hypothesize that mycorrhizal colonization with *Claroideoglomus etunicatum* (*C. etunicatum*) can optimize AA accumulation and distribution in tea plants, thereby promoting their growth and development through improved N metabolism and transport under DS condition. To test this hypothesis, we inoculated tea seedlings and exposed them to both well-watered (WW) and DS conditions. The present study investigated mycorrhizal development, biomass accumulation, N and phenolic contents, amino acid composition, as well as the activity and gene expression of enzymes related to AA synthesis. Our objectives were to evaluate the effect of *C. etunicatum* inoculation on the N assimilation process of *C. sinensis* cv. Fuding Dabaicha, and to explore the potential molecular mechanism involved, providing new insights into how mycorrhizal colonization can enhance tea growth and stress resistance under drought condition.

2 Materials and methods

2.1 Experimental materials

According to Shao et al. (2018), *Claroideoglomus etunicatum* was selected as the AMF strain, provided by the Chinese Arbuscular Mycorrhizal Fungi Germplasm Bank (BGC), and propagated for 16 weeks with white clover (*Trifolium repens*). Tea seeds of *C. sinensis* cv. Fuding Dabaicha were provided by the Tea Research Institute of Guizhou Academy of Agricultural Sciences. The seeds were disinfected and germinated for 45 days following the method described by Liu et al. (2022a). Two 3-leaf-old tea seedlings of uniform size were selected and transplanted into 1.8 L plastic pots (top inner diameter: 12 cm, bottom inner diameter: 9.0 cm, height: 14 cm) filled with 1.5 kg of pre-autoclaved (0.11 MPa, 121°C, 2h) mixed substrate (soil: sand = 1:1, v/v). At the time of transplantation, 80 g of mycorrhizal inoculum (approximately 3440 spores) were added to each pot as the AMF treatment. The inoculum contained spores (43 spores/g), river sand, fungal mycelium, and root fragments. For the non-AMF treatment, an equal volume of autoclaved (0.11 MPa, 121°C, 2h) inoculum was added, along with a 2 mL filtrate (25 μm) of the inoculum to maintain a similar microbial population, except for the AM fungus. All the seedlings were placed in a greenhouse at Yangtze University (Jingzhou) with a photon flux density of 900 μmol/m^2/s, a diurnal temperature range of 28/20°C, and a relative humidity of 80%. The pots were rearranged weekly to mitigate environmental effects.

2.2 Experimental design

A completely randomized design with two factors was implemented: inoculation with (+AMF) or without (-AMF) AMF, and water treatments: well-watered (WW, 75% of soil maximum water holding capacity) and drought-stressed (DS, 55% of soil maximum water holding capacity), resulting in four treatment combinations. Each treatment was replicated six times, resulting in a total of 24 pots arranged randomly.

Tea seedlings were cultivated for 4 weeks in WW condition to ensure proper mycorrhizal colonization. Then, half of the AMF and non-AMF seedlings were randomly assigned to DS condition for 8 weeks, while the others remained under WW setting. The DS-treated pots were weighed daily at 18:00, and any water lost was restored to keep the soil water content constant.

2.3 Biomass and mycorrhizal development

The roots and leaves of the tea plants were randomly divided into two parts at harvest. One part was oven-dried at 75°C for 48 hours after an initial treatment at 105°C and weighed, while the other part was washed and stored at −80°C. Additionally, twenty 1-cm long root segments from each seedling were cleared with 10% KOH at 95°C for 90 minutes and examined under a biological microscope after staining with 0.05% trypan blue in lactoglycerol for three minutes, as modified from Phillips and Hayman (1970). Mycorrhizal dependency, a difference in plant growth between mycorrhizal and nonmycorrhizal treatments, was calculated using plant dry matter content data following the formula described by Ma et al. (2021).

2.4 N content and phenolic substances content

The oven-dried root and leaf samples were ground into 0.5 mm powder. Samples were extracted using H_2SO_4-H_2O_2 solution and the total N content was determined using the SmartChem® 200 Wet Chemistry Analyzer according to Guo et al. (2023). Tea polyphenols and catechins play important roles in the response of tea plants to the DS (Lv et al., 2021). The tea polyphenol content in

the leaves was determined using the ferrous tartrate method, as described by de la Rosa et al. (2011), while the catechin content was quantified through the vanillin method, with catechin serving as the standard (Cao et al., 2021).

2.5 Amino acids content

The composition and content of various AAs in tea leaves and roots, including alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), valine (Val), ornithine (Orn), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr), gamma-aminobutyric acid (GABA), serine (Ser), tryptophan (Trp), glycine (Gly), homocysteine (Hcy), and methionine (Met), were extracted as previously described by Virág et al. (2020), and determined using ultra-performance liquid chromatography (UPLC). The UPLC system consisted of an Eksigent Expert Ultra LC 100 (Eksigent, Netherlands) coupled to an AB Sciex QTrap 4500 series triple quadrupole linear ion trap mass spectrometer (Sciex, USA) in electron spray ionization (ESI) mode. Chromatographic separation was achieved using gradient elution on an Agilent Zorbax Eclipse C18 (1.7 μm, 2.1 mm × 100 mm) with a mixture of 10% formic acid methanol-H_2O_2 (1:1, v/v) as the eluant. Mobile phases A and B were 10% and 50% methanol water (containing 0.1% formic acid), respectively. The sample size was 5 μL, with a flow rate of 0.3 mL/min and a column oven temperature of 40°C. The ESI parameters were set as follows: turbo spray ion source at 500°C, ion spray voltage at 5500 V, collision gas at 6 psi, curtain gas at 30 psi, and atomization and auxiliary gases at 50 psi each.

Thea was extracted from the leaves and roots using 6 mol/L hydrochloric acid. One milliliter of homogenate was filled with nitrogen gas, hydrolyzed at 110°C for 18 hours, centrifuged at 12,000 rpm for five minutes, vacuum dried, dissolved in 0.5 mL of 0.1 mol/L hydrochloric acid, and centrifuged again at 12,000 rpm for five minutes. Thea content was detected using high-performance liquid chromatography (HPLC) equipped with a 2489 ultraviolet (UV)-visible detector and a reverse phase C18 column (5 μm, 250 mm × 4.6 mm, Phenomenex, Los Angeles, USA). The column oven temperature was set at 35°C and the wavelength at 254 nm. The mobile phase consisted of 0.05 mol/L sodium acetate aqueous solution (A) and a mixture of methanol, acetonitrile, and water (B, 1:3:1, v/v/v). The sample size was 10 μL with a flow rate of 1 mL/min.

2.6 N metabolism related enzymes activities

The activities of NR, GS, GOGAT, GDH, GOT, and GPT were determined following the method established by Dong et al. (2022). Crude enzyme extractions for GAD, PDC, PEPC, ASNS, PDH, PK, and PDK were prepared according to the instructions provided by the kit (Ke Ming Biotech Co., Ltd., Suzhou, China) and determined by enzyme-linked immunosorbent assay (ELISA) at 540, 340, 340, 540, and 505 nm. Enzymes activities were calculated based on fresh weight (FW) using the corresponding formulas.

2.7 Expression analysis of genes related to nitrogen assimilation

For N assimilation-related gene expression analysis, total RNA was isolated and purified from tea leaves and roots using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China). First-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser according to the supplier's manual. The relative expression levels of N transporter genes (*CsAMT1;1*, *CsAMT1;2*, *CsAMT3;1*, *CsNRT1;2*, *CsNRT1;5*, *CsNRT2;5*) and AA synthesis and metabolism genes (*CsNR*, *CsNiR*, *CsGS1*, *CsGS2*, *CsNADH-GOGAT*, *CsFd-GOGAT*, *CsGDH1*, *CsGDH2*, *CsTS1*, *CsTS2*, *CsGGT1*, *CsGGT3*, *CsALT*, *CsSAMD*, *CsADC*, *CsCuAO*, *CsPAO*) were determined by qRT-PCR. Relative expression levels were measured using the CFX96 Real Time PCR Detection System (BIO-RAD, Berkeley, CA, USA) with TBP as the internal reference gene. qRT-PCR was run on a Bio-Rad CFX96 with SYBR Green I dye (Vazyme, China), with a reaction mixture of 10 μL SYBR qPCR Master Mix, 0.4 μL each of forward and reverse primers, 2 μL cDNA, and 7.2 μL ddH_2O. Data were analyzed with Opticon monitor software (Bio-Rad). *CsTBP* was used as an internal control. All primers used for qRT-PCR are listed in Table 1. Each sample was analyzed in triplicate (biological replicates), and quantitative results were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8 Statistical analysis

Data processing and graph creation were performed using Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA) and SigmaPlot version 10.0 software (Systat Software Inc., San Jose, CA, USA). Statistical analyses were conducted using SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was employed to determine the significance of differences among treatments. Multiple comparisons were performed using Duncan's multiple range test at a significance level of $p < 0.05$.

3 Results

3.1 Mycorrhizal development and biomass production

The presence of typical mycorrhizal structures, such as vesicles, arbuscules, and mycelium, indicated successful colonization of tea roots by AMF (Figure 1) under both WW and DS conditions. In

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TABLE 1 Gene-specific primer sequences used in this study.

Gene	Sequence of Forward Primer (5′→3′)	Sequence of Reverse Primer (5′→3′)
CsAMT1;1	GGTTGTACGGCGCTTTCATC	GCTTCCGCTGGTGTATGGAT
CsAMT1;2	TATTTTCGGGTGGGTGTCGG	TTCTGAGGCTCGACCTTCCT
CsAMT3;1	ATCACCGGTCTCGTTTGCAT	TGTGTCGTCGATTTGCTGGA
CsNRT1;2	TTCATCCTCCCCATTGTGC	TGGATTGAATTGGTCGGCTC
CsNRT1;5	CCAGGTTCTGCCAAGTGATAGT	GCTCCCCTTTTCATTCACTACA
CsNRT2;5	GACAATCGACAACATTATAGCGC	AGTCTGAACCACCCACAAAGTCC
CsNR	TTGATGCTTGGGCTGACA	ACGGACCAGGGATGTGCT
CsNiR	GGACAGGCTGCCCAAATAG	TCACTCCCAATCCTCCCTC
CsGS1	ATCAGTTGTGGATGGCTCG	CACTTCGCATGGACTTGGTAC
CsGS2	GTGGCACCAACGGAGAAGT	CAAGGATGTATCTAGCGCACC
CsNADH-GOGAT	GCAGCGAGGAGATGATTGA	CACCTTCCACATTGGTTGAG
CsFd-GOGAT	TGCTGGTATGACTGGAGGTT	CAACTGCCAGAATAGCGGTA
CsGDH1	GAGCTGAAGACATACATGACCA	GCACGAGCAACACGATTAA
CsGDH2	ATGTGGGACGAAGAGAAGGTG	GCAACACGATTCACTCCCAG
CsTS1	AGACCGCCGACATCAACAC	ATGGCTTCCACAGCAGAGT
CsTS2	CCTAAACCTATTGAGGGTGACTG	TCCTGTAAGCCGACGCTCATT
CsGGT1	GATGAATCTTGGTGATCCTGAT	TTCCACCGTCCACCATAGT
CsGGT3	GGAGTCAGCTTCAAGATCACG	CAGTAGGCGTCGAGAAGTCAC
CsALT	CGAGTCCTACGAGTCTTATTATGC	GGAGGCGTTGACAATAGAATG
CsSAMDC	TTCCAGCCAAGCGAGTTC	AACCTCCCTCCTTGCCGA
CsADC	GGGCTTATGAGGAGGCAC	GCAAGAGGGTCCTGGCAT
CsCuAO	CAGGTGTTGAGGTGAATGTTA	AATCCAGTGGCGAGCAGA
CsPAO	GTCGGGGTGACGATACCTTAG	CACCACTTAGCGTCGACATTAT
CsTBP	GGCGGATCAAGTGTTGGAAGGGAG	ACGCTTGGGATTGTATTCGGCATTA

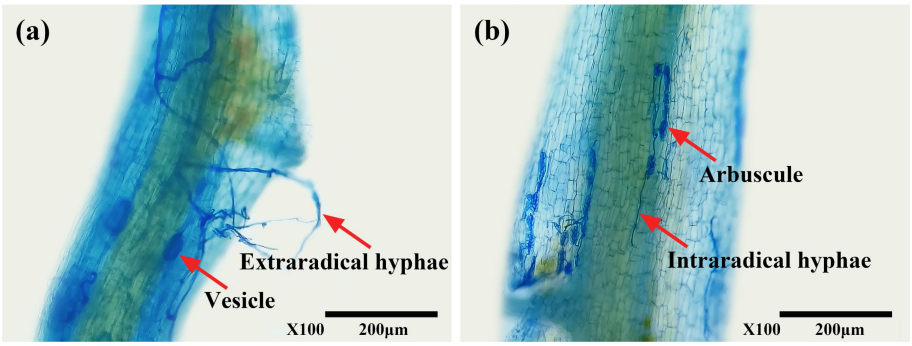

FIGURE 1
Root colonization of tea (*Camellia sinensis* cv. Fuding Dabaicha) seedlings by *C. etunicatum*. **(A)** External hyphae (external hyphae of *C. etunicatum* adhering to the root surface) and Vesicle (spherical or oval, indicating a developmental stage where nutrients and energy are stored, potentially for further colonization or reproduction); **(B)** Intraradical hyphae (intraradical hyphae of *C. etunicatum* branching within the root cortex, forming a symbiotic relationship with the host plant) and arbuscule (characterized by its highly branched structure, enhances nutrient exchange between the fungus and plant).

contrast, non-AMF seedlings exhibited no mycorrhizal formation. Compared to WW treatment, DS treatment significantly reduced mycorrhizal colonization by 41.99% but increased mycorrhizal dependency of tea seedlings by 38.97% (Table 2).

Under DS treatment, the dry biomass of both leaves and roots were significantly decreased in tea seedlings compared to WW treatment (Figure 2). However, AMF treatment significantly increased biomass production in leaves and roots by 25.00% and 34.88% under WW condition, and 42.22% and 50.00% under DS condition, respectively (Table 2).

These findings indicated that AMF colonization effectively enhanced biomass production in tea seedlings, particularly under DS conditions.

3.2 Leaf phenolic substances

Relative to WW, DS did not affect tea polyphenol concentration (Figure 3A) but significantly increased catechin content by 45.45% and 10.52% (Figure 3B) in both AMF and non-AMF treatments. Compared to the non-AMF treatment, AMF inoculation significantly increased tea polyphenol content by 13.74% and 17.20% under WW and DS conditions, respectively, and increased catechin content by 36.90% under WW condition, with no significant effect under DS condition. This suggested that AMF inoculation selectively enhanced specific phenolic compounds in tea leaves, contributing to potential variations in tea quality under different water conditions.

3.3 Leaf and root nitrogen content

Total N concentrations in tea seedlings were influenced by AMF inoculation and soil water status. DS treatment generally decreased leaf N content by 9.52% and 12.54% (Figure 3C) but significantly increased root N content by 21.30% and 4.80% in non-AMF and AMF seedlings, respectively (Figure 3D). Additionally, AM symbiosis notably increased N content by 16.54% and 12.65% in leaves and by 35.70% and 17.24% in roots under WW and DS conditions, respectively. Overall, AMF inoculation improved N uptake and distribution in tea seedlings, especially under DS, which enhanced the plant's adaptive capacity.

3.4 Leaf and root amino acids content

DS treatment reduced the contents of Thea, Ala, Arg, Asn, Asp, His, Leu, Phe, Pro, Thr, GABA, Ser, Trp, Gly, and Met while increasing the content of Hcy in leaves, regardless of mycorrhizal colonization (Figure 4A). DS treatment significantly decreased Ile and Val contents under non-AMF treatment and Gln, Glu, Lys, Orn, and Tyr contents under AMF treatment. However, AMF colonization increased the contents of Thea, Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Lys, Val, Orn, Phe, Pro, Thr, Tyr, Ser, Trp, Gly, and Met but decreased Hcy content in leaves regardless of water conditions. Additionally, under DS condition, GABA accumulation was significantly enhanced by AMF inoculation.

In roots, DS treatment generally reduced the concentrations of Thea, Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Lys, Val, Orn, Phe, Pro, Thr, Tyr, Ser, Trp, Gly, and Met regardless of mycorrhizal colonization (Figure 4B). DS treatment also significantly reduced GABA content in AMF-colonized roots and Hcy content in non-AMF-colonized roots. Compared to non-AMF treatment, AMF colonization significantly increased root contents of Thea, Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Lys, Orn, Phe, Pro, Thr, Tyr, Ser, and Gly under WW and DS conditions. Additionally, AMF-colonized seedlings exhibited significantly higher GABA and Met concentrations under WW condition and Val concentrations under DS condition. AMF symbiosis slightly reduced Hcy content under WW condition.

These results suggested that AMF symbiosis mitigated the negative effects of DS on AA profiles in tea seedlings, potentially aiding in stress tolerance.

3.5 Leaf and root N metabolism-related enzymes activities

In leaves, DS treatment significantly reduced the activities of NR, GS, GOGAT, GDH, GOT, GPT, ASNS, and PDH in non-AMF seedlings; and it also reduced the activities of NR, GOGAT, GDH, GOT, GPT, ASNS, PDK, PDH, PDC, and GAD in AMF-colonized seedlings. However, DS treatment significantly increased the activities of PK, PDK, and GAD in non-AMF seedlings, and the activities of GS and PK in AMF-colonized seedlings. Compared to non-AMF treatment, AMF-treated seedlings recorded significantly

TABLE 2 Effects of *C. etunicatum* on root AMF colonization and biomass of tea (*Camellia sinensis* cv. Fuding Dabaicha) leaves and roots under well-watered and drought stress.

Treatment	Mycorrhizal colonization (%)	Mycorrhizal dependence (%)	Biomass (g. DW/Plant)	
			Leaf	Root
WW-AMF	0 ± 0c	0 ± 0c	0.76 ± 0.06b	0.43 ± 0.04b
WW+AMF	29.58 ± 2.01a	22.22 ± 1.05b	0.95 ± 0.10a	0.58 ± 0.05a
DS-AMF	0 ± 0c	0 ± 0c	0.45 ± 0.05d	0.24 ± 0.01d
DS+AMF	17.16 ± 1.30b	30.88 ± 2.64a	0.64 ± 0.07c	0.36 ± 0.03c

Data (means ± SE, n = 6) followed by different lowercase letters among treatments indicate significant differences at $p < 0.05$.
WW, well-watered; DS, drought stress; -AMF, non-AMF inoculation; +AMF, AMF inoculation; DW, dry weight.

FIGURE 2
Effects of *C. etunicatum* inoculation on the growth of tea (*Camellia sinensis* cv. Fuding Dabaicha) under well-watered and drought stress. WW, well-watered; DS, drought stress; -AMF, non-AMF inoculation; +AMF, AMF inoculation.

higher activities of NR (13.96%), GS (49.85%), GOGAT (39.33%), GDH (58.82%), GOT (115.28%), GPT (27.78%), PDK (23.24%), PDC (10.92%), and GAD (23.76%) under WW condition, and considerably higher activities of NR (8.27%), GS (123.83%), GOGAT (147.62%), GDH (73.86%), GOT (79.25%), ASNS (16.14%), PK (8.85%), and GAD (4.30%) under DS condition, respectively (Figure 5A).

In roots, DS treatment significantly reduced the activities of NR, GOGAT, GPT, PDK, PDH, PDC, and GAD compared to WW, regardless of mycorrhizal colonization status. Additionally, DS treatment decreased GOT activity in AMF-colonized seedlings, whereas it significantly increased PK activity in non-AMF seedlings and ASNS, PK, and PEPC activities in AMF-colonized seedlings. Nevertheless, AMF symbiosis significantly enhanced the activities of NR, GOGAT, GDH, GOT, GPT, ASNS, PK, PDK, PDC, PEPC, and GAD. Specifically, the activities increased by 41.52%, 12.05%, 21.24%, 70.51%, 69.18%, 4.08%, 11.53%, 8.57%, 12.42%, 22.05%, and 7.92% under WW condition, and by 71.11%, 54.24%, 30.48%, 46.15%, 116.67%, 14.95%, 11.11%, 23.42%, 15.04%, 26.82%, and 8.30% under DS condition, respectively. Meanwhile, AMF

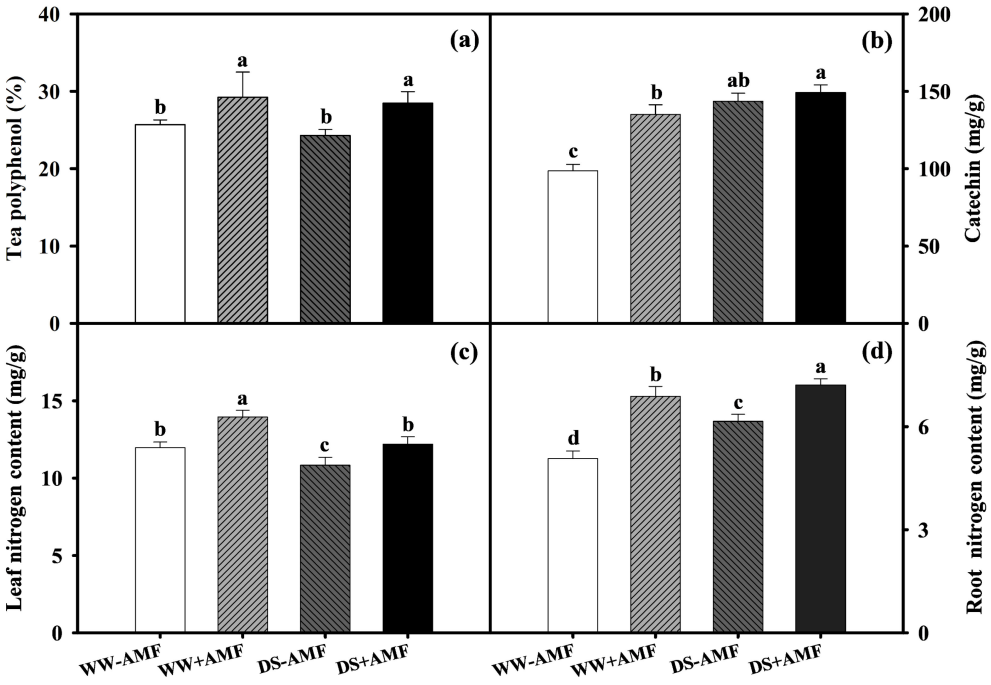

FIGURE 3
Effects of *C. etunicatum* inoculation on the phenolic compounds and nitrogen content of tea (*Camellia sinensis* cv. Fuding Dabaicha) leaves and roots under well-watered and drought stress. **(A)** Leaf tea polyphenol content. **(B)** Leaf catechin content. **(C)** Leaf nitrogen content. **(D)** Root nitrogen content. Different lowercase letters indicate significant difference within the same column at 0.05 level by LSD.

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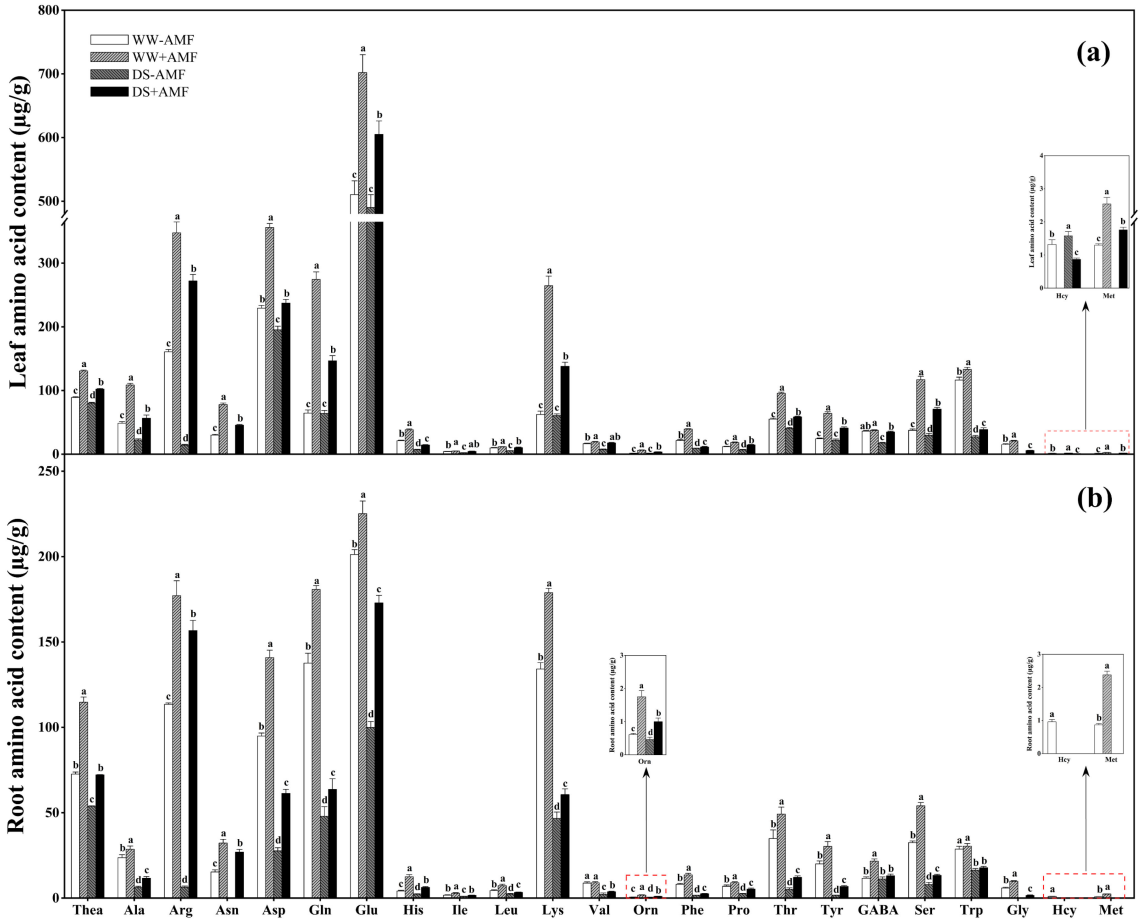

FIGURE 4
Effect of *C. etunicatum* inoculation on amino acid content (μg/g) of tea (*Camellia sinensis* cv. Fuding Dabaicha) leaves and roots under well-watered and drought stress. **(A)** Leaf amino acid content. **(B)** Root amino acid content. Different lowercase letters indicate significant difference within the same column at 0.05 level by LSD. Blank spaces indicate not detected. Thea, theanine; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Val, valine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Thr, threonine; Tyr, tyrosine; GABA, gamma-aminobutyric acid; Ser, serine; Trp, tryptophan; Gly, glycine; Hcy, homocysteine; Met, methionine.

symbiosis also significantly enhanced PDH activity by 3.80% under DS condition but did not significantly affect GS activity, irrespective of water conditions (Figure 5B).

Thus, AMF colonization significantly modulated N metabolism-related enzyme activities, which may have contributed to enhanced N utilization and stress resilience in tea plants.

3.6 Relative expression of N transporter genes

Compared with WW treatment, DS treatment significantly downregulated the expressions of *CsAMT1;1*, *CsAMT3;1*, *CsNRT1;2*, *CsNRT1;5*, and *CsNRT2;5* in leaves of non-AMF seedlings and *CsAMT1;1* and *CsAMT3;1* in leaves of AMF-colonized seedlings (Figures 6A, B). DS treatment significantly upregulated the expressions of leaf *CsAMT1;2* in non-AMF seedlings and *CsNRT1;2*, *CsNRT1;5*, and *CsNRT2;5* in AMF-colonized seedlings by 1.14-fold, 2.62-fold, 13.33-fold, and 2.30-fold, respectively. Compared to non-AMF treatment, AMF treatment significantly upregulated the expressions of leaf *CsNRT1;2*, *CsNRT1;5*, and *CsNRT2;5* by 4.90-fold, 161.00-fold, and 12.78-fold under DS condition, respectively. However, the expressions of leaf *CsAMT1;2*, *CsAMT3;1*, *CsNRT1;2*, and *CsNRT2;5* under WW condition and *CsAMT1;2* under DS condition was dramatically downregulated (Figures 6A, B).

In roots, DS treatment notably upregulated the expressions of *CsAMT1;1*, *CsAMT1;2*, *CsAMT3;1*, *CsNRT1;5*, and *CsNRT2;5* by 1.28-fold, 1.38-fold, 1.55-fold, 3.40-fold, and 1.77-fold under non-AMF treatment, and *CsAMT1;2*, *CsNRT1;2*, and *CsNRT1;5* by 1.27-fold, 1.17-fold, and 2.96-fold under AMF treatment, respectively. However, the expressions of *CsNRT1;2* in non-AMF treatment and *CsAMT1;1*, *CsAMT3;1*, and *CsNRT2;5* in AMF treatment seedlings were evidently decreased under DS treatment compared to WW treatment (Figures 6C, D). Compared to non-AMF treatment, AMF inoculation significantly upregulated the expressions of *CsAMT1;1*, *CsAMT1;2*, *CsAMT3;1*, and *CsNRT2;5* by 1.31-fold, 1.45-fold, 1.98-fold, and 6.58-fold, while significantly downregulating the expressions of *CsNRT1;2* and *CsNRT1;5* by 2.89-fold and 2.60-

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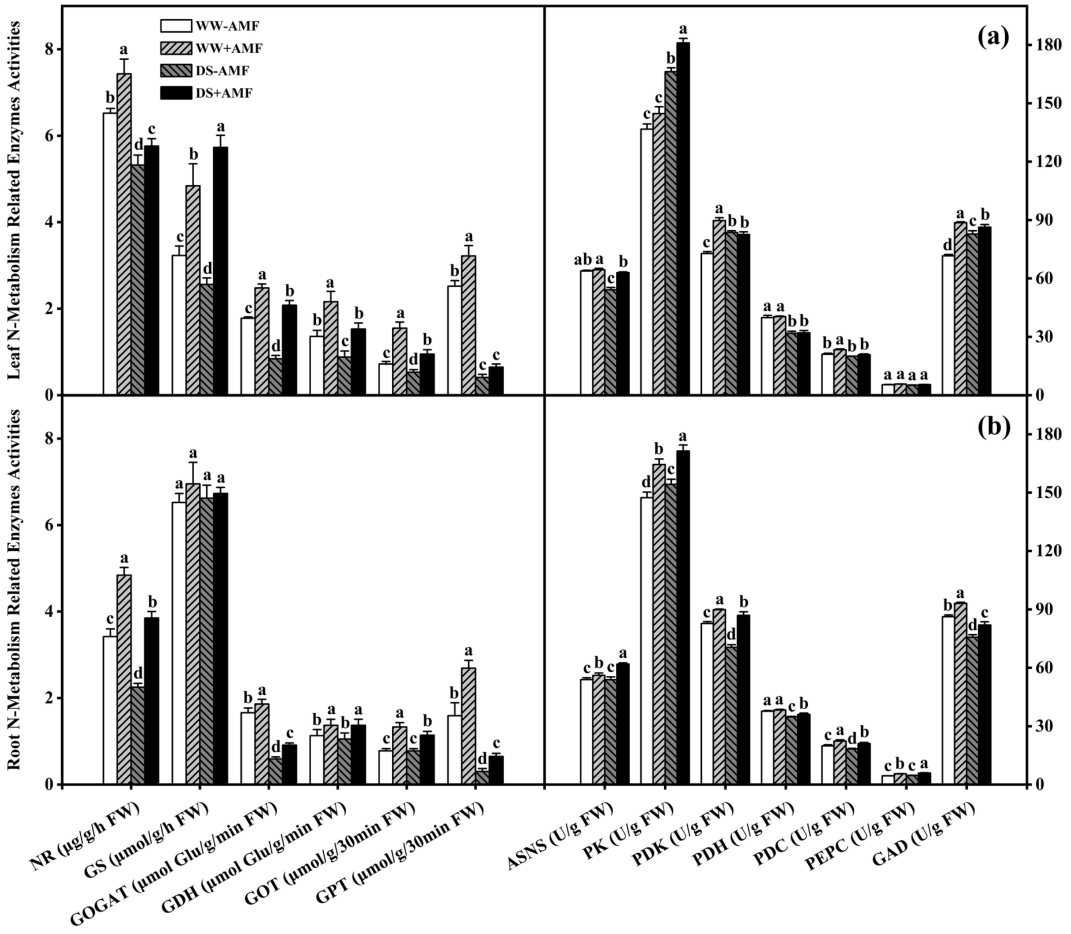

FIGURE 5
Effect of *C. etunicatum* inoculation on various N metabolism-related enzymes activities of tea (*Camellia sinensis* cv. Fuding Dabaicha) plants under well-watered and drought stress. **(A)** Leaf N metabolism-related enzymes activities. **(B)** Leaf N metabolism-related enzymes activities. Different lowercase letters indicate significant difference within the same column at 0.05 level by LSD. NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate Synthase; GDH, glutamate dehydrogenase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ASNS, asparagine synthetase; PK, pyruvate kinase; PDK, pyruvate dehydrogenase kinase; PDH, pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; GAD, glutamate decarboxylase; FW, fresh weight. The unit of measurement for each enzyme activity varies according to the method.

fold under WW condition. Under DS condition, AMF inoculation significantly upregulated the expressions of *CsAMT1;2*, *CsAMT3;1*, and *CsNRT1;2* by 1.34-fold, 1.16-fold, and 2.38-fold, but downregulated the expressions of *CsAMT1;1*, *CsNRT1;5*, and *CsNRT2;5* by 1.26-fold, 2.99-fold, and 1.31-fold, respectively.

These changes in gene expression highlighted the role of AMF in regulating N transport under varying water conditions, potentially optimizing nutrient uptake efficiency.

3.7 Relative expression of N metabolism related enzyme genes

The expressions of N metabolism-related enzyme genes in leaves and roots significantly changed after AMF inoculation, regardless of water status (Figures 7A, B).

In leaves, DS treatment significantly downregulated the expressions of *CsNR*, *CsGS1*, *CsGDH2*, *CsTS1*, *CsTS2*, *CsSAMDC*, *CsADC*, and *CsCuAO* by 1.54-, 1.68-, 1.24-, 1.16-, 1.67-, 2.86-, 1.95-, and 25.62-fold in non-AMF seedlings and *CsGS1*, *CsGS2*, *CsNADH-GOGAT*, *CsTS1*, *CsADC*, *CsCuAO*, and *CsPAO* by 1.16-, 6.96-, 1.16-, 1.49-, 2.18-, 7.39-, and 1.80-fold in AMF seedlings, respectively. Simultaneously, DS treatment dramatically upregulated the expressions of *CsNiR*, *CsNADH-GOGAT*, *CsFd-GOGAT*, *CsGDH1*, *CsGGT1*, *CsGGT3*, *CsALT*, and *CsPAO* by 6.00-, 1.16-, 1.97-, 1.33-, 7.76-, 1.48-, 1.85-, and 1.25-fold in non-AMF treatment and *CsNR*, *CsNiR*, *CsGDH1*, *CsGDH2*, *CsGGT1*, *CsGGT3*, *CsALT*, and *CsSAMDC* by 2.09-, 2.53-, 1.09-, 4.69-, 2.12-, 1.38-, 1.58-, and 1.91-fold in AMF treatment, respectively (Figure 7A).

Compared with the uninoculated treatment, AMF inoculation increased the expressions of *CsNiR*, *CsFd-GOGAT*, *CsGDH1*, *CsGGT1*, *CsGGT3*, and *CsADC* by 1.70-, 1.46-, 1.98-, 6.91-, 2.42-, and 1.28-fold, respectively, and decreased the expressions of *CsNR*, *CsGS1*, *CsNADH-GOGAT*, *CsGDH2*, *CsTS1*, *CsTS2*, *CsSAMDC*, *CsCuAO* and *CsPAO* by 2.34-, 1.17-, 1.49-, 2.44-, 1.34-, 1.26-, 2.22-, 1.38-, and 1.16-fold under WW condition. Simultaneously, AMF colonization significantly upregulated the expressions of *CsNR*, *CsGS1*, *CsGDH1*, *CsGDH2*, *CsTS2*, *CsGGT1*, *CsGGT3*, and

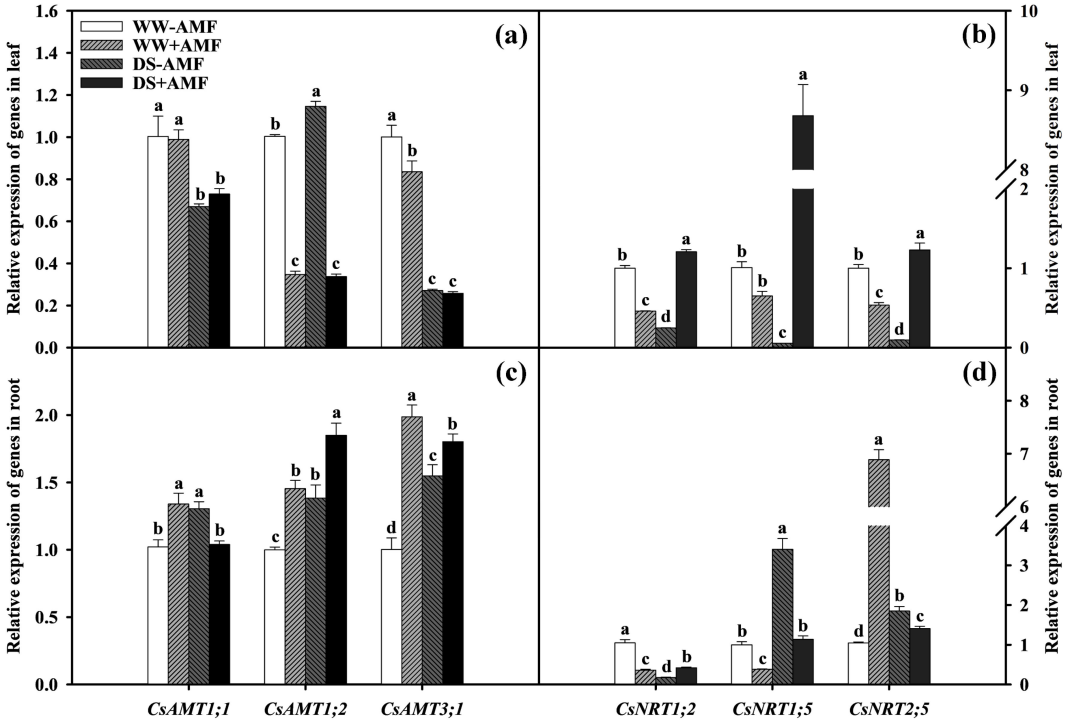

FIGURE 6
Effect of inoculation with *C. etunicatum* under well-watered and drought stress on the relative expression of *CsAMTs* **(A)** and *CsNRTs* **(B)** in leaves and *CsAMTs* **(C)** and *CsNRTs* **(D)** in roots of tea (*Camellia sinensis* cv. Fuding Dabaicha) plants. Different lowercase letters indicate significant difference within the same column at 0.05 level by LSD.

CsSAMDC by 1.38-, 1.24-, 1.63-, 2.39-, 1.33-, 1.89-, 2.24-, and 2.45-fold while downregulating the expressions of *CsNiR*, *CsGS2*, *CsNADH-GOGAT*, *CsFd-GOGAT*, *CsTS1*, *CsALT*, and *CsPAO* by 1.39-, 7.23-, 2.00-, 1.39-, 1.72-, 1.22-, and 2.61-fold under DS condition, respectively (Figure 7A).

In the roots of plants not inoculated with AMF, DS treatment versus WW treatment significantly decreased the expressions of *CsNR*, *CsNiR*, *CsGS1*, *CsGS2*, *CsFd-GOGAT*, *CsGDH1*, *CsGDH2*, *CsTS1*, *CsTS2*, *CsGGT1*, *CsGGT3*, *CsALT*, *CsSAMDC*, and *CsCuAO* by 1.79-, 3.66-, 1.19-, 1.63-, 1.26-, 2.04-, 1.77-, 1.90-, 1.42-, 2.35-, 1.59-, 1.28-, 2.78-, and 1.77-fold, respectively, whereas the expression of *CsADC* increased by 1.45-fold. In roots of AMF-inoculated plants, DS treatment versus WW treatment upregulated the expressions of *CsGS2*, *CsNADH-GOGAT*, *CsFd-GOGAT*, *CsGDH2*, *CsTS1*, *CsGGT3*, *CsALT*, *CsSAMDC*, *CsADC*, and *CsCuAO* by 1.95-, 2.37-, 1.45-, 1.45-, 1.22-, 1.46-, 1.68-, 3.19-, 7.00-, and 1.98-fold, respectively, while downregulating the expressions of *CsNR*, *CsNiR*, *CsGS1*, *CsTS2*, and *CsPAO* by 2.79-, 2.12-, 1.30-, 1.24-, and 5.94-fold, respectively (Figure 7B).

Additionally, AMF inoculation upregulated the expression levels of *CsNR*, *CsGS1*, *CsNADH-GOGAT*, *CsTS2*, *CsGGT1*, and *CsADC* under WW condition by 1.13-, 1.33-, 2.01-, 1.21-, 1.74-, and 2.40-fold, respectively, whereas the expression levels of *CsNiR*, *CsGS2*, *CsFd-GOGAT*, *CsGDH1*, *CsGDH2*, *CsTS1*, *CsGGT3*, *CsALT*, *CsSAMDC*, *CsCuAO*, and *CsPAO* were downregulated by 1.32-, 1.28-, 2.29-, 1.42-, 2.17-, 1.43-, 2.23-, 1.16-, 1.50-, 2.01-, and 1.34-fold, respectively. Under DS condition, AMF inoculation upregulated the expressions of *CsNiR*, *CsGS1*, *CsGS2*, *CsNADH-GOGAT*, *CsGDH1*, *CsGDH2*, *CsTS1*, *CsTS2*, *CsGGT1*, *CsALT*, *CsSAMDC*, *CsADC*, and *CsCuAO* by 1.31-, 1.23-, 2.49-, 5.69-, 1.46-, 1.19-, 1.63-, 1.39-, 4.05-, 1.86-, 5.94-, 11.59-, and 1.74-fold, respectively, while downregulating the expressions of *CsNR*, *CsFd-GOGAT*, and *CsPAO* by 1.38-, 1.25-, and 8.15-fold, respectively (Figure 7B).

The differential expression of these genes underscored the impact of AMF on N metabolism pathways, enhancing the adaptability of tea plants to DS.

3.8 Principal component analysis

Utilizing Principal Component Analysis (PCA), this study examined the correlations between N metabolism-related genes and major factors (biomass, N content, phenolic content, AAs levels, and enzymes activities) in both tea leaves and roots. For leaves (Figure 8), the cumulative contribution of the two principal components (PC1 and PC2) was 79.5% (55.7% and 23.7%, respectively), displaying good intra-group consistency and substantial inter-group variations. The expressions of certain genes (*CsAMT1;1*, *CsAMT3;1*, *CsTS2*, *CsGS1*, *CsADC*, *CsCuAO*), leaf biomass, N content, tea polyphenol content, most AAs (excluding Hcy), and enzymes activities (NR, GOGAT, GDH, GOT, GPT, ASNS, PEPC, PDC, PDH) had significant positive impacts on PC1. Notably, the expressions of some genes (*CsFd-*

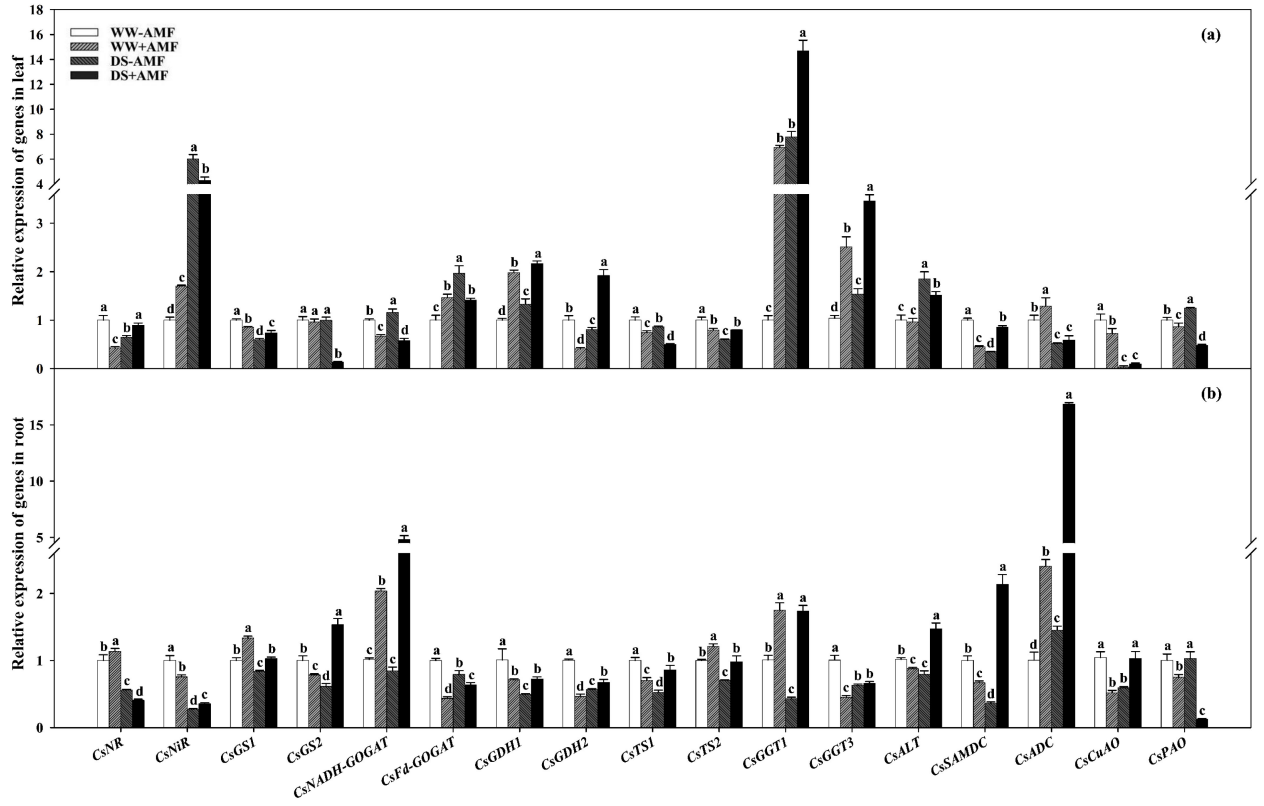

FIGURE 7
Effect of *C. etunicatum* inoculation on gene expression of enzymes related to nitrogen metabolism in leaves **(A)** and roots **(B)** of tea (*C. sinensis* cv. Fuding Dabaicha) under well-watered and drought stress. Different lowercase letters indicate significant difference within the same column at 0.05 level by LSD.

GOGAT, *CsNiR*, *CsALT*, *CsNR*, *CsNADH-GOGAT*, *CsAMT1;2*) showed negative correlations with these variables. Additionally, catechin content, the activities of specific enzymes (GS, GAD, PK, PDK), and the expressions of genes (*CsNRT1;5*, *CsGDH1*, *CsGDH2*, *CsGGT1*, *CsGGT3*) had substantial positive effects on PC2, while the expressions of *CsGS2* and *CsTS1* exhibited negative correlations.

In roots (Figure 9), the cumulative contribution of the two principal components was 77.9% (53.4% for PC1 and 24.5% for PC2), also showing good intra-group reproducibility and inter-group differences. The expressions of genes (*CsTS2*, *CsGS1*, *CsGGT1*, *CsNRT2;5*, *CsNR*, *CsNiR*), root biomass, most AAs (excluding Hcy), and enzymes activities (NR, GOGAT, GDH, GOT, GPT, PDC, PDH, PDK, GAD) positively influenced PC1. Notably, the expressions of CsFd-GOGAT and CsNRT1;5 negatively correlated with these variables. Furthermore, root nitrogen content, activities of enzymes (ASNS, PEPC, PK), and the expressions of genes (*CsAMT1;2*, *CsAMT3;1*, *CsNADH-GOGAT*, *CsADC*) had pronounced positive effects on PC2, whereas the expressions of *CsNRT1;2*, *CsFd-GOGAT*, *CsGDH2*, *CsGGT3*, and *CsPAO* showed negative correlations.

Overall, the PCA results emphasized the complex interplay between gene expression, enzyme activities, and N metabolism, further illustrating the beneficial effects of AMF on tea plant physiology under stress conditions.

4 Discussion

4.1 Mycorrhizal development and plant growth physiology

The present study demonstrated that DS treatment significantly inhibited mycorrhizal colonization in tea seedlings (Figure 1; Table 2), consistent with previous findings (Liu et al., 2020a). This suggests that soil water scarcity may suppress spore germination and extraradical hyphae extension, thereby limiting mycorrhizal development (Huang et al., 2017). However, the mycorrhizal dependency of tea seedlings increased significantly under DS condition (Table 2), similar to results reported in barley by Li et al. (2014). This could be due to the hindered nutrient absorption capacity of tea roots under DS condition, making them more reliant on AMF for the acquisition of essential minerals like P, K, Ca, and Mg (Liu et al., 2024). Additionally, AMF inoculation effectively mitigated the inhibitory effects of DS treatment on biomass accumulation in tea leaves and roots (Figure 2; Table 2), aligning with the findings of Liu et al. (2024). These results reinforce the role of AMF in promoting plant growth and enhancing drought resistance (Wang et al., 2023).

In the present study, DS treatment markedly increased catechin accumulation in tea leaves (Figure 3B), regardless of mycorrhizal

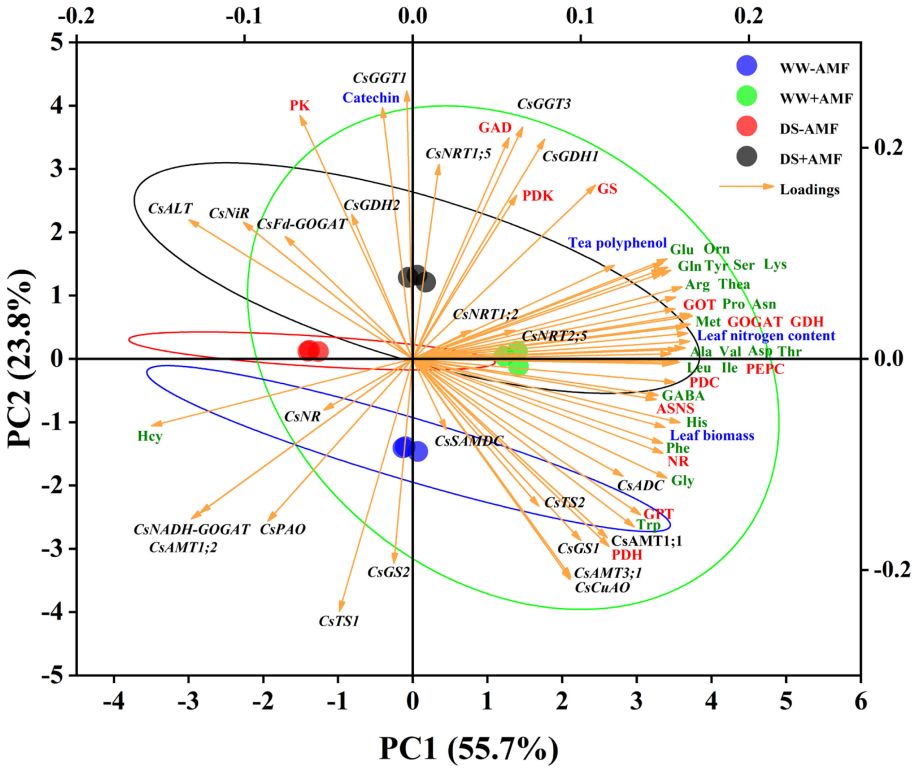

FIGURE 8
Unsupervised principal component analysis of physiological and molecular parameters of selected leaves of tea (*C. sinensis* cv. Fuding Dabaicha) inoculated with *C. etunicatum* under well-watered and drought stress.

colonization, suggesting that plants may enhance drought resistance by increasing catechin content to eliminate excessive reactive oxygen species (ROS) under DS condition (Lv et al., 2021). Furthermore, AMF symbiosis notably increased tea polyphenol and catechin levels in leaves under both water conditions (Figures 3A, B), consistent with findings by Wang et al. (2020). This suggests that AMF may mitigate ROS-induced damage by promoting phenolic compound accumulation, thereby enhancing drought resistance and tea quality.

4.2 Changes in N absorption and transportation

N is a critical nutrient and limiting factor in plant growth and development. In tea plants, N content directly affects quality. Our study showed that DS treatment significantly reduced N content in tea leaves while increasing it in roots, regardless of mycorrhizal inoculation (Figures 3C, D). This may be due to a higher N absorption rate by the roots under DS treatment compared to the transfer rate from roots to leaves, resulting in more N concentration in the roots (He et al., 2022). The distribution of N in leaves and roots might be related to the expression of N transporter genes. The results of PCA showed that *CsAMT1;1*, *CsAMT3;1*, *CsNRT1;2*, *CsNRT2;5* were positively correlated with leaf N content (Figure 8), and *CsAMT1;2*, *CsAMT3;1* were positively correlated with root N content (Figure 9). DS treatment markedly downregulated the expressions of leaf *CsAMT1;1* and *CsAMT3;1* in both mycorrhizal and non-mycorrhizal seedlings, and leaf *CsNRT1;2*, *CsNRT1;5*, and *CsNRT2;5* in AMF-colonized seedlings (Figures 6A, B), potentially suppressing photorespiratory ammonia metabolism in leaves (Zhang et al., 2018) and impeding NO_3^- transport from roots to xylem (Lin et al., 2008), ultimately leading to reduced leaf N content. Meanwhile, DS treatment upregulated the expressions of root *CsAMT1;2* (Figure 6C), enhancing the absorption of NH_4^+ from the soil (Zhang et al., 2023). Notably, AMF inoculation increased N content in both leaves and roots of tea seedlings regardless of water status, consistent with previous studies (Shao et al., 2018; Liu et al., 2024). This increase was accompanied by the upregulated expressions of *CsAMT1;2* and *CsAMT3;1* in roots (Figure 6C), indicating enhanced root absorption and transportation of NH_4^+ due to these genes' upregulation (Zhang et al., 2023). However, mycorrhizal colonization significantly downregulated *CsNRT1;5* expression in tea roots (Figure 6D), similar to observations in *Catalpa bungei* seedlings, suggesting that mycorrhizal symbiosis may inhibit the direct absorption of NO_3^- by plant roots (Chen et al., 2023).

4.3 Mycorrhizal colonization promote the accumulation of AAs

AAs are crucial for tea quality, balancing the astringency and bitterness of catechins and caffeine (Li et al., 2019). Previous studies have shown varying effects of DS treatment on AA contents in tea

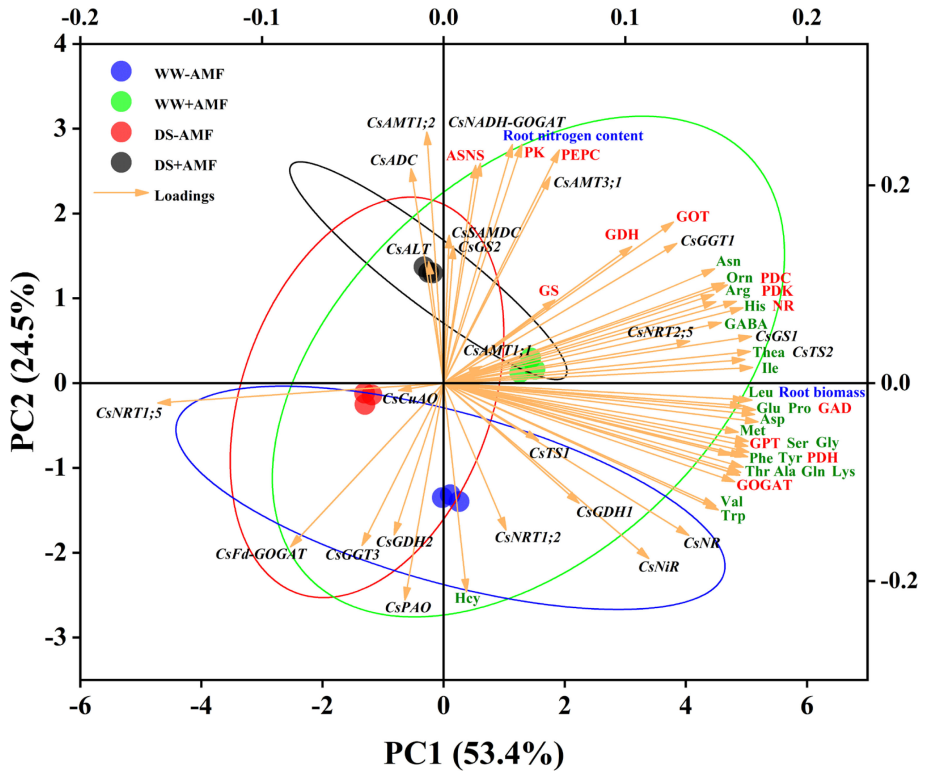

FIGURE 9
Unsupervised principal component analysis of physiological and molecular parameters of selected roots of tea (*C. sinensis* cv. Fuding Dabaicha) inoculated with *C. etunicatum* under well-watered and drought stress.

plants (Wang et al., 2016). This study found that DS treatment decreased the contents of 22 kinds of AAs (except Hcy) in both leaves and roots (Figure 4), which may be associated with differing drought tolerance among tea varieties (Qian et al., 2018). AMF colonization increased AAs contents in both leaves and roots under both water conditions (Figure 4), consistent with findings in white clover (Xie et al., 2020). Notably, GABA and Pro contents increased more under DS condition than WW condition (Figure 4), suggesting that AMF might enhance drought resistance by promoting GABA and Pro accumulation under DS condition (Mekonnen et al., 2016; Qian et al., 2018). Furthermore, AMF colonization increased Glu, Gln, Asp, Lys, and Arg contents (Figure 4), potentially enhancing N assimilation and storage within plants, thus promoting the synthesis of other AAs (Yang et al., 2020; Hildebrandt et al., 2015; Winter et al., 2015). The increase in Thea content, directly associated with tea quality (Lin et al., 2023), in AMF-colonized seedlings further indicates the beneficial effect of AMF inoculation on tea quality.

4.4 Changes in N metabolism-related enzymes activities induced by AMF

NR activity in tea plants is a key determinant of NO_3- reduction ability (Liu et al., 2022b). DS treatment significantly suppressed NR activity, consistent with previous findings (Du et al., 2020; Zahoor et al., 2017). However, after AMF inoculation, NR activity was significantly increased in both leaves and roots (Figure 5), while PCA results showed a positive correlation between NR activity and N content in both leaves and roots (Figures 8, 9), suggesting that AMF can enhance NO_3^- reduction by increasing NR activity, thereby promoting N absorption by roots. Additionally, DS treatment inhibited GOGAT and GDH activities, but these were enhanced after AMF inoculation (Figure 5). As a key enzyme in the GS-GOGAT cycle, GS activity was significantly reduced in non-AMF seedlings under DS condition but increased in AMF-colonized seedlings regardless of water status (Figure 5). AMF inoculation had no significant impact on GS activity in roots (Figure 5B), possibly due to the weak influence of a single AMF (Xie et al., 2021). Nevertheless, AMF plays a significant role in enhancing N assimilation in tea seedlings.

DS treatment significantly inhibited GOT and GPT activities in leaves and roots, whereas AMF inoculation significantly alleviated this inhibition (Figure 5), similar to findings on exogenous spermidine effects on maize GOT and GPT under DS condition (Dong et al., 2022). This suggests that AMF may alleviate N metabolic disorders caused by DS by enhancing transaminase activity (Dong et al., 2022). DS treatment also significantly reduced root GAD activity and leaf GAD activity in AMF-colonized seedlings (Figure 5). However, AMF inoculation significantly enhanced GAD activity in both leaves and roots, consistent with GABA content changes (Figure 5). Meanwhile, PCA results indicated that root GAD activity was positively correlated with GABA content (Figure 9), suggesting that AMF

may promote GABA accumulation by enhancing GAD activity, thereby enhancing drought resistance (Mekonnen et al., 2016). Furthermore, AMF inoculation alleviated the inhibitory effect of DS treatment on ASNS activity (Figure 5), consistent with Asn content changes (Figure 4), while the results of PCA also indicated a positive correlation between ASNS activity and Asn content in both leaves and roots (Figure 8; Figure 9), indicating that AMF may enhance Asn content by enhancing ASNS activity, mitigating DS's inhibitory effect on N transportation (Gaufichon et al., 2010; Li et al., 2016b). The increase in PK activity and suppression of PDH and PDC activities induced by DS treatment might increase pyruvate content (Figure 5), potentially inducing increased respiratory activity as a physiological response to DS treatment (Zabalza et al., 2009). Additionally, the activities of PEPC, PK, PDC, PDH, and PDK (except PDK under DS treatment) in both leaves and roots were significantly enhanced by AMF treatment (Figure 5), potentially influencing other metabolic reactions (Jardine et al., 2010).

4.5 N metabolism-related enzyme genes expression pattern in mycorrhizal colonized seedlings

Regarding the expressions of N assimilation-related genes in tea roots, DS treatment significantly downregulated the expressions of *CsNR*, *CsNiR*, *CsGs1*, *CsGs2*, *CsFd-GOGAT*, *CsGDH1*, and *CsGDH2* in non-AMF seedlings (Figure 7B), consistent with previous findings (Xia et al., 2020). However, DS treatment had varying effects on these genes in AMF-colonized seedlings (Figure 7B), suggesting AMF involvement in multiple pathways. Under WW condition, AMF inoculation significantly upregulated the expressions of *CsNR*, *CsGS1*, and *CsNADH-GOGAT*, while under DS condition, AMF colonization upregulated the expressions of *CsNiR*, *CsGS1*, *CsGS2*, *CsNADH-GOGAT*, *CsGDH1*, and *CsGDH2*, consistent with changes in N metabolism-related enzymes activities (Figure 7B). Meanwhile, PCA results also showed that the expression of *CsNR* was positively correlated with NR activity, and the expressions of *CsGS1* and *CsGS2* were positively correlated with GS activity (Figure 9). These genes and enzymes are closely related to N assimilation, indicating that AMF may alleviate DS's inhibitory effect on N assimilation by regulating the expressions of N assimilation-related genes and promoting the activities of N metabolism enzymes. Notably, AMF inoculation downregulated *CsGDH1* and *CsGDH2* expressions under WW condition (Figure 7B), possibly due to the synergistic role of *CsGSs* and *CsGDHs* in ammonium assimilation (Tang et al., 2021). This study also showed that AMF inoculation upregulated the expressions of *CsGSs* and *CsNADH-GOGAT* (Figure 7B), potentially promoting NH_4^+ assimilation while weakening *CsGDHs*' function (Tang et al., 2021). In tea leaves, DS induced overexpression of *CsNiR* (Figure 7A), potentially a stress response to alleviate NO_2^- accumulation toxicity under DS condition (Ezzine and Ghorbel, 2006). Alternatively, the downregulation of *CsGSs* under DS treatment could lead to NH_4^+ accumulation and upregulation of *CsGDHs*, promoting NH_4^+ assimilation and positive regulation of *CsNiR* expression to meet N requirements (Xia et al., 2020; Tang et al., 2021). The regulation of AMF inoculation on the expression of leaf N metabolism-related genes is relatively complex (Figure 7A). Under WW condition, AMF inoculation significantly upregulated *CsNiR*, *CsFd-GOGAT*, and *CsGDH1* expressions while downregulating *CsNR*, *CsGS1*, *CsNADH-GOGAT*, and *CsGDH2* expressions (Figure 7A). Under DS condition, AMF colonization upregulated *CsNR*, *CsGS1*, *CsGDH1*, and *CsGDH2* expressions, while downregulating *CsNiR*, *CsGS2*, *CsNADH-GOGAT*, and *CsFd-GOGAT* expressions (Figure 7A). These changes suggest that AMF influences N metabolism comprehensively, potentially regulating multiple genes to modulate enzymes activities (Chen et al., 2023).

The expressions of Thea metabolism-related genes are crucial for its accumulation (Liu et al., 2020b). This study revealed that DS treatment significantly downregulated the expressions of *CsTS1*, *CsTS2*, *CsGGT1*, *CsGGT3*, and *CsSAMDC* in the roots of tea seedlings (Figure 7B), which corresponded with reduced Thea content, consistent with Wang et al. (2016). This indicates that DS treatment negatively affects Thea accumulation. Notably, AMF inoculation significantly upregulated the expressions of *CsTS2*, *CsGGT1*, and *CsADC* regardless of water conditions (Figure 7B), aligning with the increase in Thea content in roots (Figure 4B). Meanwhile, the results of PCA showed strong correlation between CsTS2, CsGGT1 and Thea content (Figure 9), indicating that these genes are crucial for Thea synthesis and that AMF symbiosis can enhance Thea synthesis in roots.

In leaves, DS significantly downregulated the expressions of *CsTS1*, *CsTS2*, *CsSAMDC*, *CsADC*, and *CsCuAO* while upregulating the expressions of *CsALT* and *CsGGT1* (Figure 7A). The significant downregulation of *CsSAMDC* and *CsADC*, which function similarly to *CsAlaDC*, could hinder EA synthesis, a precursor for Thea (Lin et al., 2023; Shi et al., 2011). Consequently, Thea content in leaves decreased despite the upregulation of *CsGGT1* expression (Figure 4A), suggesting that the synergistic action of *CsAlaDC* and *CsTS* is crucial for high-concentration Thea synthesis in tea plants (Zhu et al., 2021). AMF colonization reasonably upregulated the expressions of *CsGGT1*, *CsGGT3*, and *CsADC* to varying degrees (Figure 7A), while PCA results also showed that CsGGT3 and CsADC were positively correlated with Thea content (Figure 8), confirming AMF's stimulatory role in Thea synthesis. However, AMF inoculation significantly downregulated *CsPAO* expression, despite its crucial role in plant growth, development, and stress resistance (Yu et al., 2019), warranting further research.

5 Conclusion

In conclusion, our comprehensive study of the interplay between DS and AMF inoculation in tea seedlings has yielded insightful findings. DS was found to significantly hinder mycorrhizal infection, increasing mycorrhizal dependency and negatively impacting key physiological processes, including biomass accumulation, AAs content, and N metabolism enzyme

activities in both roots and leaves. This ultimately led to a reduction in tea quality attributes such as tea polyphenol content and Thea content. Conversely, AMF inoculation emerged as an effective countermeasure, significantly boosting leaf and root biomass, N content, and AAs content, while enhancing the accumulation of tea polyphenols and catechins. Furthermore, AMF promoted the expression of genes and activities of enzymes involved in N metabolism, facilitating efficient nitrogen assimilation and alleviating the adverse effects of DS on the growth and development of tea plants. Our results underscore the potential of AMF as a biological tool to enhance tea plant resilience against DS, with implications for sustainable tea production under changing climatic conditions. Further research is warranted to elucidate the underlying molecular mechanisms and to optimize AMF inoculation strategies for wide-scale application in tea cultivation.

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

X-LW: Data curation, Methodology, Software, Writing – original draft. YH: Writing – review & editing. WL: Data curation, Writing – review & editing. C-YL: Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. J-DH: Supervision, Writing – original draft, 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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Bacillus velezensis GH1-13 enhances drought tolerance in rice by reducing the accumulation of reactive oxygen species

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Plant growth-promoting rhizobacteria colonize the rhizosphere through dynamic and intricate interactions with plants, thereby providing various benefits and contributing to plant growth. Moreover, increasing evidence suggests that plant growth-promoting rhizobacteria affect plant tolerance to abiotic stress, but the underlying molecular mechanisms remain largely unknown. In this study, we investigated the effect of *Bacillus velezensis* strain GH1-13 on drought stress tolerance in rice. Phenotypical analysis, including the measurement of chlorophyll content and survival rate, showed that *B. velezensis* GH1-13 enhances rice tolerance to drought stress. Additionally, visualizing ROS levels and quantifying the expression of ROS-scavenging genes revealed that GH1-13 treatment reduces ROS accumulation under drought stress by activating the expression of antioxidant genes. Furthermore, the GH1-13 treatment stimulated the jasmonic acid response, which is a key phytohormone that mediates plant stress tolerance. Together with the result that jasmonic acid treatment promotes the expression of antioxidant genes, these findings indicate that *B. velezensis* GH1-13 improves drought tolerance in rice by reducing ROS accumulation and suggest that activation of the jasmonic acid response is deeply involved in this process.

KEYWORDS

abiotic stress, *Bacillus velezensis*, drought, jasmonic acid, reactive oxygen species, rice

Introduction

Drought is a major abiotic stress that impedes crop growth and productivity. Global climate change is increasing the frequency and intensity of drought stress, and interactions with other abiotic stresses such as salinity and heat stress exacerbate the harmful effect on crops (Panda et al., 2021; Ramegowda et al., 2024). Rice cultivated in paddy fields serves as a

staple food for more than half of the world's population and is highly susceptible to drought stress (Seck et al., 2012; Seo et al., 2020; Zargar et al., 2022). Recent reports indicate that drought stress can reduce rice yields by approximately 13%–75% (Bhutta et al., 2019; Zargar et al., 2022). Therefore, various strategies to improve drought tolerance in rice have been investigated, including the utilization of plant microbiomes.

Plant growth-promoting rhizobacteria (PGPRs) are microorganisms that inhabit the rhizosphere and enhance plant growth through processes such as nutrient solubilization, nitrogen fixation, and hormone production (Lugtenberg and Kamilova, 2009; Murali et al., 2021a; Gowtham et al., 2022; Sujkowska-Rybkowska et al., 2022; Al-Turki et al., 2023). *Bacillus thuringiensis* S7, *Herbaspirillum seropedicae* ZA15, and *Klebsiella oxytoca* LCK121 have been found to enhance the growth and productivity of cereal crops, such as rice and barley, by promoting phosphorus solubilization (Estrada et al., 2013; Xiao et al., 2020; Khalifa and Aldayel, 2022). *Bacillus pumilus* JM52 and *Rhizobium daejeonense* JR5 enhanced shoot and root growth in rice by increasing soil ammonium levels, and *Arthrobacter chlorophenolicus* BHU3 improved plant height and grain yield in wheat by enhancing nitrogen uptake (Habibi et al., 2014; Kumar et al., 2014). Additionally, it was reported that *Bacillus flexus* P4 and *Azospirillum brasilense* RA-17 stimulate crop growth by promoting the production of phytohormones such as auxin and cytokinin (Ahmed and Hasnain, 2010; Zaheer et al., 2022). Furthermore, increasing research suggests that PGPRs are involved in enhancing plant response and tolerance to abiotic stresses, including drought (Gowtham et al., 2020; Murali et al., 2021b). For example, *Bacillus paramycoides* DT-85 and *Bacillus licheniformis* K11 were found to enhance drought tolerance in wheat and peppers, and *Bacillus aryabhattai* B8W22 and *Bacillus halotolerans* MSR-H4 improved salt stress tolerance in rice and wheat (Lim and Kim, 2013; El-Akhdar et al., 2020; Shultana et al., 2021; Yadav et al., 2022). Moreover, it has been reported that *Achromobacter* strains, including *Achromobacter xylosoxidans* Cm4 and *Achromobacter piechaudii* ARV8, as well as *Pseudomonas* strains, including *Pseudomonas psychrotolerans* CS51 and *Pseudomonas stutzeri* C4, exhibit the ability to enhance abiotic stress tolerance in various crops such as potato, peppers, maize, and tomato (Mayak et al., 2004; Tank and Saraf, 2010; Belimov et al., 2015; Kubi et al., 2021).

Reactive oxygen species (ROS) are highly reactive due to the presence of unpaired electrons, and they are naturally produced as by-products of cellular metabolic processes, such as in the photosynthetic system in chloroplasts and the cellular respiration system in mitochondria (Dat et al., 2000; Mittler, 2002; Sharma et al., 2012). When ROS levels are excessively increased in cells, it can directly cause lipid peroxidation, protein carbonylation, and DNA damage, which leads to loss of membrane integrity, enzyme activity inhibition, and cell death (Tripathy and Oelmüller, 2012; Schieber and Chandel, 2014; Nisa et al., 2019). Therefore, ROS-scavenging mechanisms, including the expression of antioxidant genes such as *ascorbate peroxidase* (*APX*), *catalase* (*CAT*), *glutathione peroxidase* (*GPX*), and *superoxide dismutase* (*SOD*), are crucial for defending against oxidative stress. ROS levels in plants are extensively increased by abiotic stresses, such as drought, cold, and salinity, which promote the accumulation of ROS in plants (Moran et al., 1994; Agnihotri and Seth, 2017; Huang et al., 2019; Wu et al., 2019; Mariyam et al., 2023a, b; Choudhary et al., 2024; Kumar et al., 2024a). This suggests that ROS accumulation is involved in plant response and tolerance to abiotic stresses, and experimental results using knock-out mutations and the overexpression of antioxidant genes have demonstrated this. Zhang et al. (2013) showed that *OsAPX2* mutant and *OsAPX2*-overexpressing transgenic rice exhibited elevated and reduced ROS levels, respectively, compared with wild-type rice under abiotic stress conditions (Zhang et al., 2013). Correspondingly, *OsAPX2* mutant rice exhibited hypersensitivity to drought stress, whereas *OsAPX2*-overexpressing rice displayed enhanced tolerance. Similarly, plants that overexpress *CAT* enzymes, such as *PtCAT2*, *OsCATa*, and *OsCATc*, displayed enhanced tolerance to drought stress (Joo et al., 2014; Xu et al., 2023). These results indicate the crucial role of antioxidant gene expression in managing ROS accumulation and enhancing plant tolerance under abiotic stress conditions. This is further supported by previous research, which revealed that the expression of antioxidant genes and ROS accumulation are regulated by jasmonic acid (JA), a key phytohormone that mediates plant defense against both abiotic and biotic stresses (Abdelgawad et al., 2014; Brenya et al., 2020; Kim et al., 2021; Sheteiwy et al., 2021).

Despite growing evidence that PGPRs, including *Bacillus velezensis*, improve plant tolerance to abiotic stress, our understanding of the underlying mechanisms remains limited. In this study, we show that the *B. velezensis* strain GH1-13 enhances drought tolerance by promoting the expression of ROS-scavenging genes and suppressing ROS accumulation. Furthermore, our findings suggest that the activation of the JA response is deeply involved in this process.

Materials and methods

Plant materials and growth

Two varieties of japonica rice, *Oryza sativa* cv. *Shindongjin* and cv. *Saechungmu*, were used in this study. Rice seeds were sterilized with 2.5% sodium hypochlorite (v/v) for 1 h and then washed five times with sterile distilled water. The sterilized seeds were germinated on half-strength Murashige and Skoog ($^1/_2$ MS) solid medium for 3 days. They were then transplanted and grown in soil under normal growth chamber conditions (16 h/8 h = light/dark, 32°C).

Microbe materials, growth, and treatment

B. velezensis GH1-13 has been previously described (Kim et al., 2017, 2019; Lee et al., 2023), and for this study, GH1-13 was

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; APX, ascorbate peroxidase; bHLH, basic helix-loop-helix; CAT, catalase; COI1, coronatine-insensitive 1; DAB, diaminobenzidine; JA, jasmonic acid; LOX, lipoxygenase; MeJA, methyl jasmonate; NBT, nitroblue tetrazolium; PGPR, plant growth-promoting rhizobacteria; ROS, reactive oxygen species; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SOD, superoxide dismutase.

obtained from the Center for Industrialization of Agricultural and Livestock Microorganisms. For the GH1-13 treatment of rice, GH1-13 was cultivated in a tryptic soy broth (glucose 0.5%, soybean flour 0.8%, NaCl 0.15%, K_2HPO_4 0.25%, Na_2CO_3 0.05%, and $MgSO_4{\cdot}7H_2O$ 0.1%) at 30°C for 36 h in a shaking incubator and collected by centrifugation. The rice was treated with GH1-13 at a final concentration of 1×10^7 CFU/mL.

Drought and JA treatment

For drought treatment, the plants were exposed to dehydration conditions for 3 days. Following this 3-day drought treatment, the plants were re-watered and cultivated under normal growth conditions (16 h/8 h = light/dark, 32°C). The survival rate was calculated by dividing the number of plants that survived by the total number of plants that were tested. For JA treatment, a methyl jasmonate (MeJA) solution [100 µM MeJA in double-distilled water (ddH_2O) and 0.1% Tween-20 (v/v)] was applied to the rice by spraying, and the plants were incubated under normal growth conditions for 12 h.

Measurement of chlorophyll contents

The chlorophyll content was measured as previously described by Wellburn (1994) with slight modifications (Wellburn, 1994). To extract the chlorophyll, fresh leaves (0.3 g) were collected from the indicated plants and incubated with 10 mL of 80% acetone (v/v) in the dark for 24 h. After incubation, the samples were centrifuged at 12,000 ×*g* for 5 min, and the supernatant was diluted 10-fold with 80% acetone. The chlorophyll contents were measured spectrophotometrically at wavelengths of 663 and 646 nm (Molecular Devices VersaMax Microplate Reader) and calculated according to the method described by Wellburn (1994).

Visualization of ROS levels by DAB and NBT staining

To visualize the ROS levels in rice, the histochemical detection of H_2O_2 and $O_2^{\cdot-}$ was performed using diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining. For DAB and NBT staining, leaves collected from the indicated plants were incubated in DAB staining solution [1 mg/mL 10 mM Na_2HPO_4 (pH 3.0)] and NBT staining solution [0.1% (w/v) nitroblue tetrazolium in 10 mM sodium azide and 50 mM potassium phosphate buffer (pH 6.4)] at room temperature overnight in the dark. After incubation, the leaves were incubated in a bleaching solution (ethanol:glycerol:acetic acid at a ratio of 3:1:1, v/v/v) to bleach out the chlorophyll. To quantify NBT staining intensity, the formazan contents were measured in the NBT-stained samples. The NBT-stained samples (0.3 g) were ground with liquid nitrogen and then the formazans were extracted from the ground samples using an extraction solution (2 M potassium hydroxide:DMSO at a ratio of 1:1.6, v/v). The formazan contents were measured with a spectrophotometer at a wavelength of 630 nm (Molecular Devices VersaMax Microplate Reader) and calculated as previously described by Grellet Bournonville and Díaz-Ricci (2011).

Total RNA extraction and quantitative RT-PCR analysis

To analyze the relative transcript levels, total RNA was extracted from the indicated plants using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The first complementary DNA (cDNA) strand was synthesized using 1 µg of total RNA, oligo dT primers, and GoScript Reverse Transcriptase (Promega). For quantitative RT-PCR (RT-qPCR), a master mix was prepared using the AccuPower GreenStar qPCR Master Mix (Bioneer). The PCR reaction and fluorescence detection were performed with a CFX Connect Real-Time System (Bio-Rad). Three technical replicates of the RT-qPCR were performed using three biological replicates. The RT-qPCR conditions included an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 10 s. *OsACTIN1* was used as the internal control. Primer sequence information is available in Supplementary Table 1.

Embedding, sectioning, and toluidine blue staining

To observe the internal anatomy of the rice roots, Technovit embedding and physical sectioning were performed as previously described by Seo et al. (2021) with slight modifications. Rice root samples were collected from the indicated plants and fixed in 4% paraformaldehyde solution (w/v) for 2 h, and then washed five times with ddH_2O. The fixed samples were dehydrated in an ethanol series [25%, 50%, 75%, and 100% (v/v) in ddH_2O] for 2 h at each step. The dehydrated samples were incubated in a series of Technovit 7100 cold-polymerizing resin solutions [25%, 50%, 75%, and 100% (v/v) in ethyl alcohol] for 2 h at each step. Samples were further incubated in a 100% Technovit 7100 solution for 1 day and solidified in a 15:1 (v/v) mixture of Technovit 7100 and hardener solution II at room temperature in a mold for 1 day. Sections (3 µm) were obtained from solidified samples using an RM 2145 microtome (Leica). The sections were stained with 0.05% toluidine blue solution (w/v, pH 4.5) for 1 min and observed with a light microscope (DM-2500, Leica).

Statistical analysis

Quantification data for the RT-PCR and chlorophyll contents are the averages of three biological replicates with three technical replicates each. The survival rate results are presented as the mean values of at least three biological replications. Statistical analysis was performed using Microsoft Excel 365 MSO (version 2406, Build 16.0.1726.20078). Statistical differences between the samples and their respective controls were determined using a two-tailed Student's *t*-test with $p < 0.01$.

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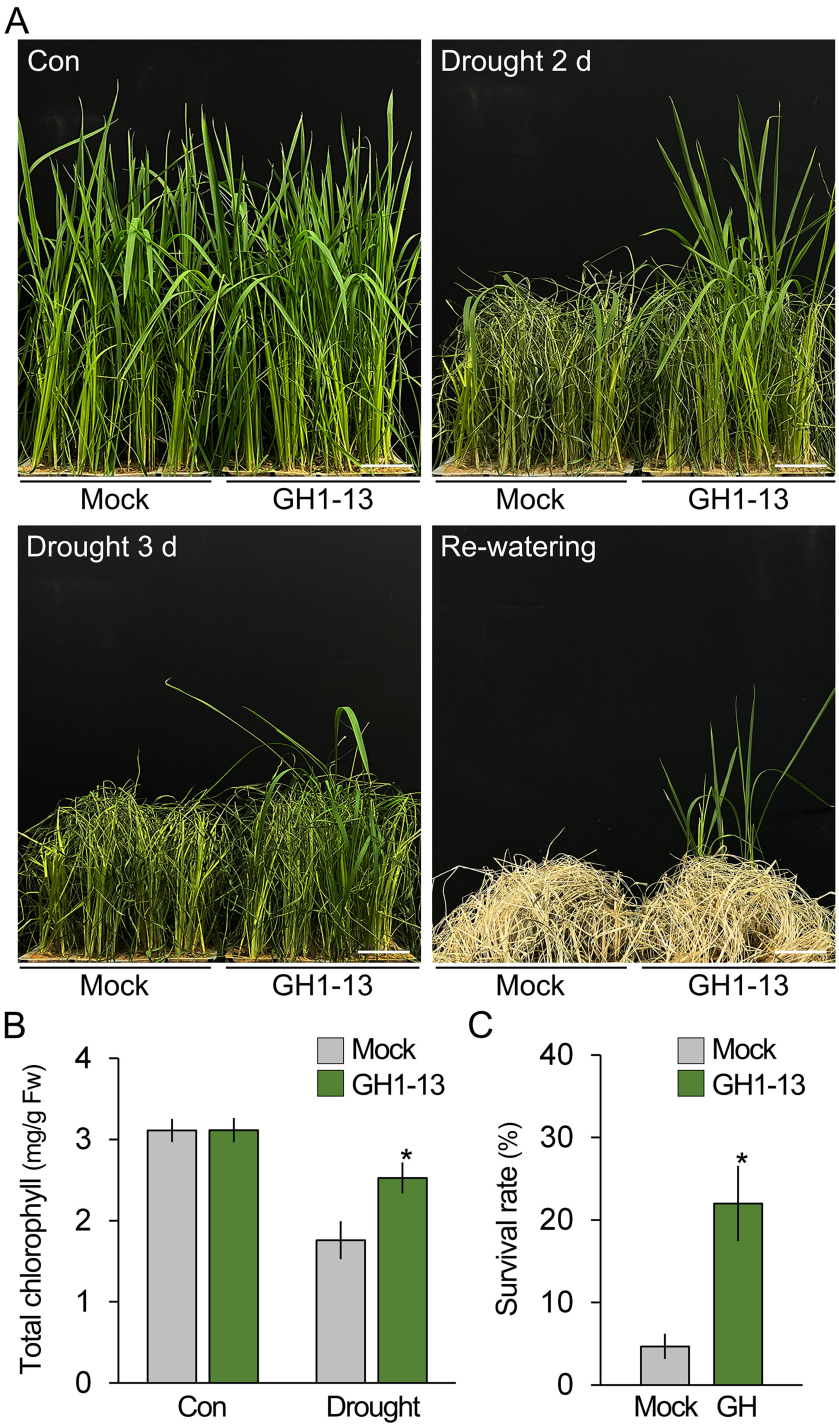

FIGURE 1
Bacillus velezensis GH1-13 improves drought stress tolerance in rice. **(A)** Phenotypic analysis of drought stress tolerance in 8-week-old rice (cv. *Shindongjin*) treated with and without *B. velezensis* GH1-13. Rice was exposed to drought stress for 3 days and subsequently re-watered and cultivated under normal growth conditions for 10 days. **(B)** Effects of GH1-13 treatment on the chlorophyll content of rice treated and untreated with drought stress for 2 days. Mock and GH1-13 indicate *B. velezensis* GH1-13-untreated and -treated rice for 7 days, respectively. **(C)** Survival rates of the re-watered rice plants ($n > 250$). The survival rate was calculated by dividing the number of surviving plants by the total number of plants tested. Error bars indicate SD. Asterisks indicate statistically significant differences between the corresponding samples and their control ($p < 0.01$, *t*-test). Scale bars = 5 cm.

Results

Bacillus velezensis GH1-13 improves drought tolerance in rice

To investigate the effect of *B. velezensis* GH1-13 on drought stress tolerance in rice, 8-week-old rice (cv. *Shindongjin*) plants were treated and untreated with GH1-13 and exposed to drought conditions, and their phenotypical and physiological changes were monitored over time (Figure 1). After 2 days of drought stress, both the GH1-13-treated and -untreated plants exhibited symptoms of drought stress, including chlorosis, leaf rolling, and wilting. However, the visual symptoms in the GH1-13-treated rice were milder compared to those of the untreated rice, and the quantification results of total chlorophyll contents supported this observation (Table 1). Unlike the GH1-13-untreated rice, in which drought stress reduced the total chlorophyll contents by 43.5%, the GH1-13-treated rice exhibited an 18.9% total chlorophyll content decrease under drought stress conditions. This suggests that GH1-13 treatment enhances rice tolerance to drought stress, and the survival rates of the re-watered rice supported this. When these plants were re-watered and grown for 10 days, the rice treated with GH1-13 exhibited an approximately 4.7-fold increase in survival rate compared to that of untreated rice. The effect of GH1-13 on rice drought tolerance was also evident in another rice cultivar, *Saechungmu* (Supplementary Figure 1; Table 1). Similar to *Shindongjin* rice, GH1-13 treatment significantly improved drought stress tolerance in *Saechungmu* rice, which highlights the role of GH1-13 in enhancing rice tolerance to drought stress.

Bacillus velezensis GH1-13 reduces ROS accumulation

The regulation of ROS accumulation is closely linked to plant tolerance to abiotic stress (Moran et al., 1994; Mittler, 2002; Sharma et al., 2012; Huang et al., 2019; Wu et al., 2019; Kim et al., 2021; Sun et al., 2021). Since GH1-13 improves drought stress tolerance in rice, we expected that GH1-13 treatment would affect ROS accumulation under drought stress conditions. To investigate this, we visualized the accumulated ROS using DAB staining (Figure 2A). Under normal growth conditions, there was no difference in DAB staining intensity between the untreated and treated rice with GH1-13. However, under drought conditions, the GH1-13-treated rice exhibited reduced intensity compared to the untreated rice, which suggests that the GH1-13 treatment diminishes drought-induced ROS accumulation. To explore this further, the ROS levels in these plants were visualized and quantified using NBT staining (Figures 2B, C). Consistent with the DAB staining results, the intensity of NBT staining in the GH1-13-treated rice was markedly lower than that in the untreated rice under drought stress conditions. Quantification of NBT staining intensity by measuring the formazan content revealed that the NBT staining intensity in the GH1-13-treated rice was 48.88% lower than that in the GH1-13-untreated rice, thus indicating that GH1-13 treatment reduces ROS accumulation under drought stress conditions.

GH1-13 promotes expressions of ROS-scavenging and JA-responsive genes

Since *B. velezensis* GH1-13 reduces ROS accumulation and enhances drought stress tolerance, we hypothesized that GH1-13 treatment influences the expression of antioxidant genes such as rice *ascorbate peroxidase* (*OsAPX*), *catalase* (*OsCAT*), *glutathione peroxidase* (*OsGPX*), and *superoxide dismutase* (*OsSOD*). To test this hypothesis, we quantitatively analyzed the transcript levels of ROS-scavenging genes in rice treated with and without GH1-13 using RT-qPCR (Figure 3A). Our finding revealed that the GH1-13-treated rice exhibited an increased expression of ROS-scavenging genes compared to the untreated rice, which explains the reduced ROS levels in the rice treated with *B. velezensis* GH1-13.

Since JA is deeply involved in plant–microbe communication and regulates the expression of antioxidant genes (Van der Ent et al., 2009; Carvalhais et al., 2013; Fan et al., 2016; Farooq et al., 2016; Liu et al., 2017; Sheteiwy et al., 2021; Zhu et al., 2022), it was hypothesized that GH1-13 possibly affects JA response. To address

TABLE 1 The effect of GH1-13 on chlorophyll content and survival rate in rice.

Rice cultivar	Conditions		Total chlorophyll (mg/g Fw)	Survival rate (%)
Shindongjin	Con	Mock	3.11 ± 0.14	–
		GH1-13	3.12 ± 0.17	–
	Drought	Mock	1.76 ± 0.23	4.67 ± 1.53
		GH1-13	2.53 ± 0.17*	22.00 ± 4.58*
Saechungmu	Con	Mock	3.07 ± 0.14	–
		GH1-13	3.13 ± 0.15	–
	Drought	Mock	1.65 ± 0.19	9.67 ± 3.05
		GH1-13	2.37 ± 0.18*	36.67 ± 5.13*

Asterisks indicate statistically significant differences between the corresponding samples and their control ($p < 0.01$, *t*-test).

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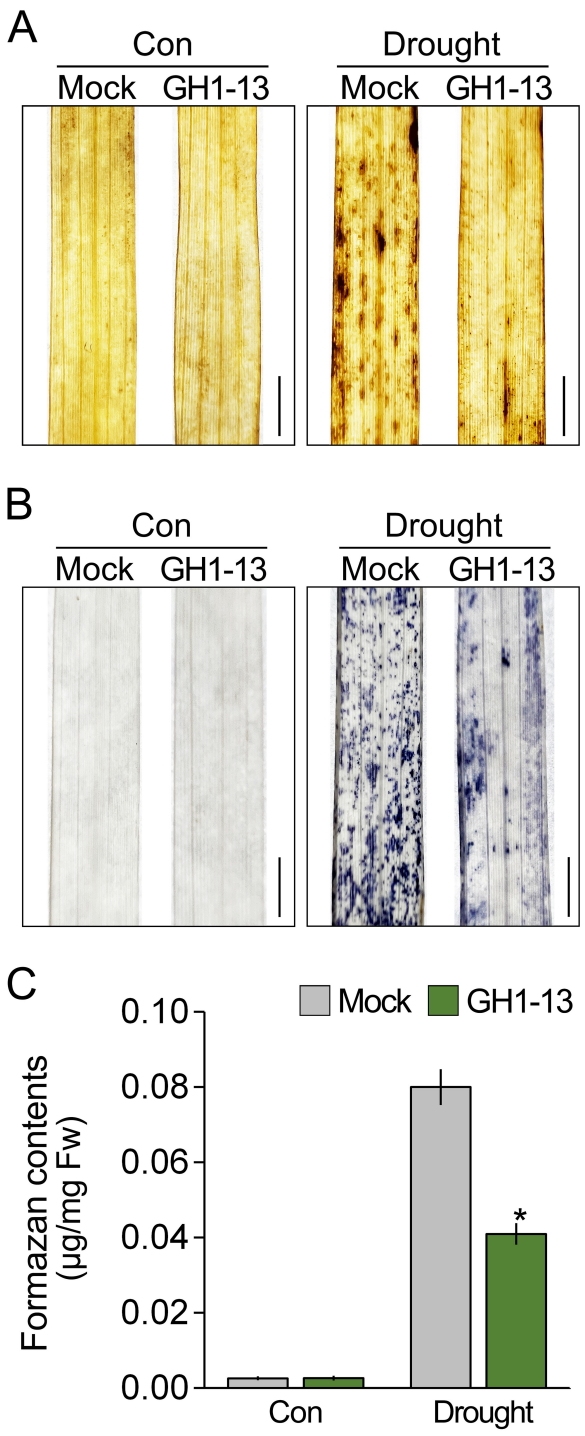

FIGURE 2
B. velezensis GH1-13 reduces drought-induced ROS accumulation. DAB **(A)** and NBT staining analysis **(B)** visualized the effect of GH1-13 treatment on ROS accumulation under drought stress. Rice was exposed to drought stress for 2 days. Mock and GH1-13 indicate *B. velezensis* GH1-13-untreated and -treated rice for 7 days, respectively. **(C)** Quantification of formazan contents to quantify the NBT staining intensity in these plants. Error bars indicate SD. An asterisk indicates statistically significant differences between the corresponding samples and their control ($p < 0.01$, *t*-test). Scale bars = 0.5 cm.

this, the JA response was examined in GH1-13-treated and -untreated rice by analyzing the transcript levels of JA-responsive genes responsible for JA signaling (*OsbHLH148* and *OsJAMYB*) and for JA biosynthesis (*OsLOX2*, *OsAOS2*, and *OsAOC1*) (Seo et al., 2011; Yang et al., 2020; Qiu et al., 2022) (Figures 3B, C). The expression level of JA-responsive genes in the GH1-13-treated rice was significantly higher than that of the untreated rice, which suggests that *B. velezensis* GH1-13 activates the JA response in rice.

Jasmonic acid treatment enhances drought tolerance in rice

Since JA response is deeply involved in abiotic stress tolerance and ROS homeostasis in plants, it was expected that JA affects ROS accumulation in rice under drought stress conditions. To test this, we treated 7-week-old rice with JA for 12 h and then analyzed the ROS levels under both normal and drought conditions using DAB and NBT staining (Figures 4A-C). Although drought stress increased the intensity of the DAB and NBT staining in both the JA-treated and -untreated rice, the intensity in the JA-untreated rice was significantly higher than that in the JA-treated rice under drought conditions. These findings indicated that the ROS levels in JA-treated rice are lower than those in the untreated rice under drought stress conditions, which suggests that JA diminished the accumulation of drought-induced ROS. In addition, we found that transcript levels of ROS-scavenging genes in the JA-treated rice were higher than those in the JA-untreated rice (Figure 4D). These results indicate that JA activates the expression of ROS-scavenging genes and suggest that JA reduces ROS accumulation by activating the expression of ROS-scavenging genes.

Because JA, like *B. velezensis* GH1-13, promoted the expression of antioxidant genes and reduced the accumulation of ROS, it was hypothesized that JA treatment would be sufficient to enhance the drought stress tolerance of rice. To test this, JA-treated and -untreated 7-week-old rice plants were exposed to drought stress, and their phenotypical and physiological changes were analyzed (Figure 5). Although both sets of plants exhibited obvious visual symptoms of drought stress, such as leaf rolling and wilting, these symptoms were less severe in the JA-treated rice compared to the untreated rice. In addition, these observations were supported by the results showing that the chlorophyll content and survival rates were higher in the JA-treated rice compared to the untreated rice. Together with the finding that *B. velezensis* GH1-13 activates the JA response, these results suggest that *B. velezensis* GH1-13 enhances drought stress tolerance in rice by reducing ROS accumulation, and the activation of the JA response is deeply involved in this process.

Analysis of root growth in rice treated with GH1-13

Roots are essential organs that interact with soil microbes, and drought stress tolerance is influenced by root growth (Ekanayake

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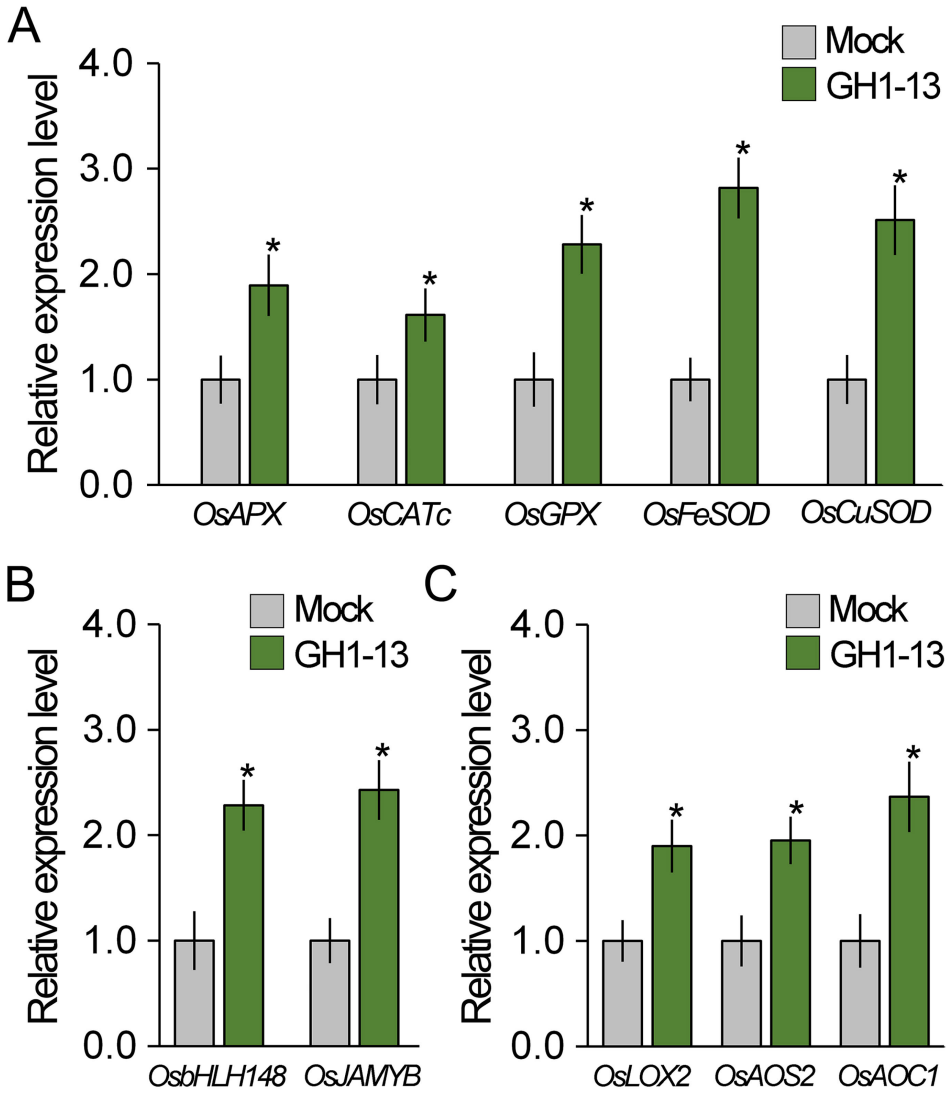

FIGURE 3
GH1-13 treatment activates the expression of ROS-scavenging and JA-responsive genes. **(A)** Quantitative RT-PCR results showing the expression of ROS-scavenging genes in *B. velezensis* GH1-13-treated and -untreated 8-week-old rice. **(B, C)** Expression levels of JA signaling **(B)** and biosynthesis genes **(C)** in *B. velezensis* GH1-13-treated and -untreated rice. Mock and GH1-13 indicate *B. velezensis* GH1-13-untreated and -treated rice for 7 days, respectively. *OsACTIN1* was used as the reference gene to normalize the RT-qPCR results. Error bars indicate SD. Asterisks indicates statistically significant differences between the corresponding samples and their control ($p < 0.01$, *t*-test).

et al., 1985; Seo et al., 2020; Grover et al., 2021; Khoso et al., 2023). To determine whether root development contributes to the enhanced tolerance by *B. velezensis* GH1-13 treatment, we analyzed the root morphology of rice plants treated with and without GH1-13. When comparing the phenotypes of 8-week-old roots grown under GH1-13-treated and -untreated conditions, no significant differences were observed in root growth, including root length (Figure 6A). In addition, experimental results obtained from 2-week-old roots were consistent with those from 8-week-old roots (Supplementary Figure 2). To further investigate this, we analyzed the internal anatomy of the roots through physical sectioning and microscopic observation (Figure 6B). No significant differences in root development, including root radial patterning, were observed between the treated and -untreated roots in the root meristem and maturation zones. This suggests that GH1-13 treatment improves drought stress tolerance in rice by regulating physiological processes, such as inhibiting ROS accumulation, rather than influencing root development.

Discussion

In this study, we showed that *B. velezensis* GH1-13 enhances drought stress tolerance in rice by activating the expression of antioxidant genes and suppressing ROS accumulation. Previous studies have revealed that ROS levels are extensively increased by stresses. For example, tomatoes treated with drought conditions and *Botrytis cinerea* exhibited approximately 2.5- and 3-fold increased ROS levels, respectively, compared to the untreated control plants (Pietrowska et al., 2015; Gowtham et al., 2020). This suggests that

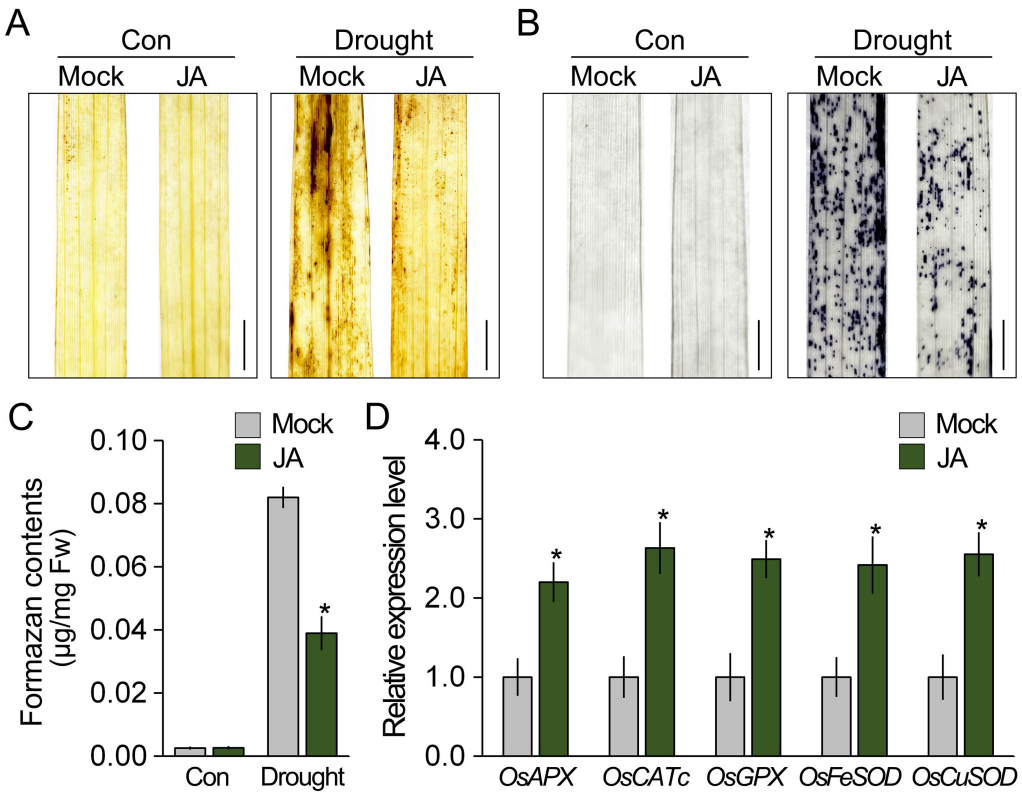

FIGURE 4
JA reduces the accumulation of ROS in rice. The effect of JA on ROS accumulation was visualized using DAB **(A)** and NBT staining **(B)**. Visualization of ROS accumulation. Rice treated and untreated with JA for 12 h was exposed to drought stress for 2 days. **(C)** Measurement of formazan contents to quantify the NBT staining intensity in these plants. **(D)** Changes in the expression of ROS-scavenging genes by JA treatment. Total RNA was extracted from 7-week-old rice treated and untreated with JA for 12 h, and *OsACTIN1* was used as the reference gene to normalize the RT-qPCR results. Error bars indicate SD. Asterisks indicates statistically significant differences between the corresponding samples and their control ($p < 0.01$, *t*-test). Scale bars = 0.5 cm.

plant responses and tolerance to stress are closely linked to the accumulation of ROS, and studies investigating ROS-scavenging genes, such as *APXs*, *CATs*, and *SODs*, further support this (Zhang et al., 2013; Xu et al., 2023). The expression level and the activity of ROS-scavenging genes were affected by abiotic and biotic stresses, such as drought and *Pseudomonas syringae*, and knock-out mutations of antioxidant genes, such as *APXs*, *CATs*, and *SODs*, resulted in the increased accumulation of ROS and reduced tolerance to abiotic stresses (Moran et al., 1994; Mittler et al., 1999; Bueso et al., 2007; Dvořák et al., 2021). In contrast, the overexpression of these antioxidant genes led to a decrease in ROS accumulation and enhanced tolerance in plants, such as Arabidopsis and rice (Joo et al., 2014; Li et al., 2015, 2017; Xu et al., 2023). Furthermore, the overexpression of drought-responsive *IbMYB116* and *VaNAC17* genes decreased ROS levels by promoting the transcriptional expression of ROS scavenging genes and enhanced drought stress tolerance (Zhou et al., 2019; Su et al., 2020). These results indicate that ROS accumulation plays a crucial role in plant tolerance to abiotic stresses and support our finding that *B. velezensis* GH1-13 enhances drought stress tolerance in rice by activating the expression of ROS-scavenging genes and suppressing the accumulation of ROS.

JA is a phytohormone that plays a key role in mediating the plant's defense against abiotic stresses, as demonstrated by previous studies involving the endogenous modulation of the JA response (Seo et al., 2011; Grebner et al., 2013; Wu et al., 2015; Fu et al., 2017; Xing et al., 2020; Zhang et al., 2023; Kumar et al., 2024b). A study by Seo et al. (2011) showed that the overexpression of *OsbHLH148*, a key transcription factor in JA signaling, enhanced drought tolerance by activating the JA response in rice (Seo et al., 2011). A study by Fu et al. (2017) revealed that a knock-out mutation of *OsJAZ1*, a negative regulator of JA signaling, improved drought tolerance in rice, whereas the overexpression of *OsJAZ1* reduced drought tolerance compared to wild-type rice (Fu et al., 2017). Consistently, modulation of the JA response through knock-out mutations and overexpression of JA biosynthetic genes, such as *LOXs* and *AOSs*, affects plant tolerance to drought stress. For example, a knock-out mutation of *AtLOX6* decreased JA levels, which led to hypersensitivity to drought stress in *Arabidopsis thaliana* (Grebner et al., 2013). In contrast, the overexpression of *CmLOX10* and *BoAOS* increased JA levels and improved drought stress tolerance (Wu et al., 2015; Xing et al., 2020). These findings indicate that activation of the JA response is closely associated with plant tolerance to drought stress.

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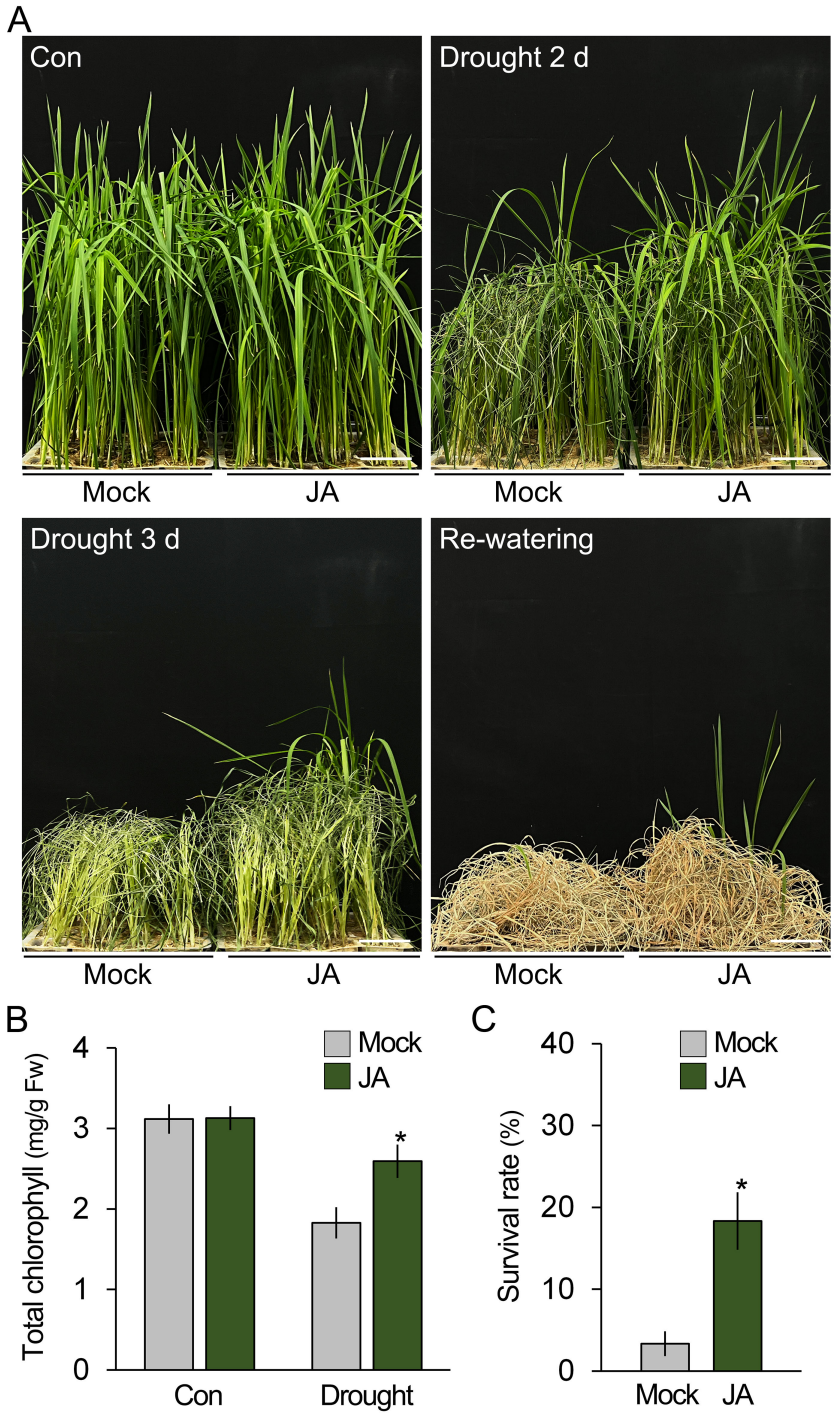

FIGURE 5
Drought tolerance is enhanced by JA treatment. **(A)** Phenotypic analysis of drought stress tolerance in 7-week-old rice (cv. *Shindongjin*) treated and untreated with JA. Rice, both treated and untreated with JA, was exposed to drought stress for 3 days and then cultivated under re-watered conditions for 10 days. **(B)** Effects of exogenous JA treatment on the chlorophyll content of rice treated and untreated with drought stress for 2 days. Mock and JA indicate exogenous JA-untreated and -treated rice, respectively. **(C)** Survival rates of the re-watered rice ($n > 250$). The survival rate was calculated by dividing the number of surviving plants by the total number of plants tested. Error bars indicate SD. Asterisks indicate statistically significant differences between the corresponding samples and their control ($p < 0.01$, *t*-test). Scale bars = 5 cm.

Furthermore, the crucial role of JA in plant tolerance to drought stress is supported by its impact on the expression of antioxidant genes and the suppression of ROS accumulation. In various crops, such as Indian mustard, wheat, maize, tobacco, millet, and soybeans, JA treatment upregulated the expression of ROS-scavenging genes, such as *APXs*, *CATs*, and SODs, while reducing the accumulation of ROS (Örvar et al., 1997; Abdelgawad et al., 2014; Anjum et al., 2016; Agnihotri and Seth, 2020; Awan et al., 2021; Sheteiwy et al., 2021).

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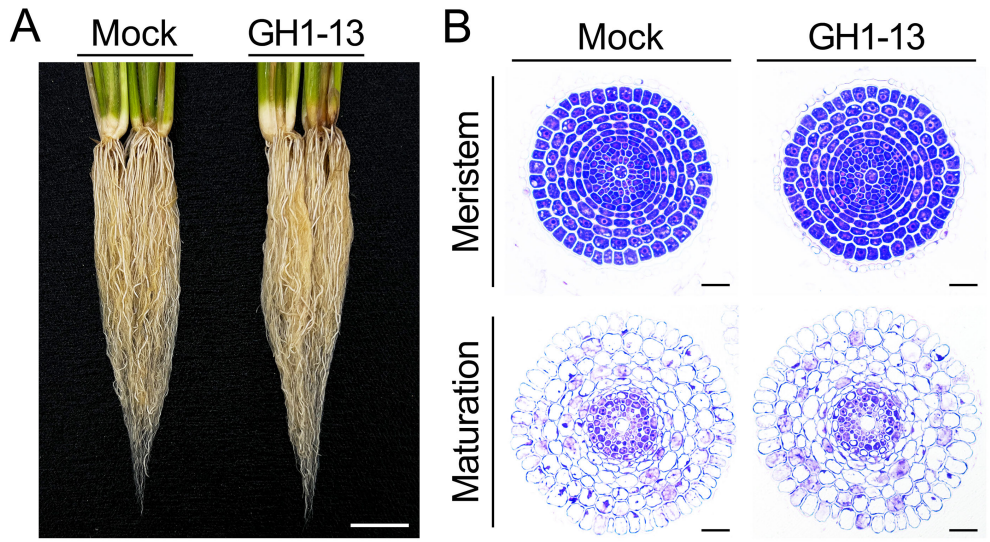

FIGURE 6
Effect of GH1-13 on rice root growth. **(A)** Root development of *B. velezensis* GH1-13-treated and -untreated 8-week-old rice. **(B)** Transverse section images of the roots. Mock and GH1-13 indicate *B. velezensis* GH1-13-untreated and -treated rice for 7 days, respectively. Scale bars = 2 cm in **(A)** and 50 μm in **(B)**.

Similarly, the activation of the JA response through the overexpression of *SlCOI1* or *MdLOX3* reduced ROS accumulation by promoting the expression of ROS-scavenging genes (Chen et al., 2024; Kadam and Barvkar, 2024). Conversely, suppression of the JA response through *SlCOI1* knockdown reduced the JA response, which resulted in a decreased expression of ROS-scavenging genes and increased ROS accumulation (Kadam and Barvkar, 2024). In this study, we showed that GH1-13-treated rice exhibited an increased expression of JA-responsive and ROS-scavenging genes compared to the untreated control plants. Taken together with the tight correlation of JA, ROS accumulation, and drought tolerance, our findings suggest that *B. velezensis* GH1-13 enhances rice drought tolerance by activating the expression of antioxidant genes and reducing ROS accumulation, and activation of the JA response is involved in this process. Accumulating evidence, including the findings from this study, suggests that PGPRs could serve as a key strategy to enhance crop drought tolerance. However, challenges remain, such as optimizing field application conditions and accurately predicting PGPR responses within field environments. Further studies on the mechanisms underlying PGPR-induced tolerance and the practical aspects of their field application will be crucial for unlocking the full potential of PGPRs as effective microbial fertilizers.

Conclusion

This study highlights the role of *B. velezensis* GH1-13 in enhancing drought stress tolerance in rice. Molecular characterization, including ROS accumulation and antioxidant gene expression assessments, indicates that GH1-13 reduces ROS accumulation under drought stress by increasing the expression of antioxidant genes. Furthermore, this finding is supported by evidence that GH1-13 treatment enhances the JA response, which activates the expression of antioxidant genes. Collectively, this study concludes that the ROS-scavenging process mediated by antioxidant genes is a key mechanism that underlies the improved drought stress tolerance in rice conferred by *B. velezensis* GH1-13.

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

DP: Writing – original draft, Writing – review & editing. JJ: Writing – original draft, Writing – review & editing. DS: Writing – original draft, Writing – review & editing. YK: Writing – original draft, Writing – review & editing. GJ: Writing – original draft, 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.2024.1432494/full#supplementary-material

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Enhancing cold tolerance in tobacco through endophytic symbiosis with *Piriformospora indica*

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Tobacco, a warm-season crop originating from the Americas, is highly susceptible to cold stress. The utilization of symbiotic fungi as a means to bolster crops' resilience against abiotic stresses has been proven to be a potent strategy. In this study, we investigated the effect of endophytic fungus *Piriformospora indica* on the cold resistance of tobacco. When exposed to cold stress, the colonization of *P.indica* in tobacco roots effectively stimulates the activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX). This, in turn, reduces the accumulation of reactive oxygen species (ROS), thereby mitigating oxidative damage. Additionally, *P. indica* elevates the levels of osmolytes, such as soluble sugars, proline, and soluble proteins, thus facilitating the restoration of osmotic balance. Under cold stress conditions, *P. indica* also induces the expression of cold-responsive genes. Furthermore, this fungus not only enhances photosynthesis in tobacco by stimulating the synthesis of photosynthetic pigments, strengthening Rubisco activity, and elevating PSII efficiency, but also fortifies tobacco's nitrogen assimilation by inducing the expression of nitrate transporter gene and activating enzymes related to nitrogen assimilation. Consequently, this synergistic optimization of nitrogen and carbon assimilation provides a solid material and energetic foundation for tobacco plants to withstand cold stress. Our study demonstrates that a mycorrhizal association between *P. indica* and tobacco seedlings provides multifaceted protection to tobacco plants against low-temperature stress and offers a valuable insight into how *P. indica* enhances the cold tolerance of tobacco.

KEYWORDS

***Piriformospora indica*, tobacco cold resistance, reactive oxygen species, osmolytes, photosynthesis, N assimilation**

1 Introduction

Temperature is a core environmental factor that shapes the life cycle and yield of plants, and its variations have profound effects on plant growth (Gray and Brady, 2016). It is widely recognized that plant productivity experiences a significant and disproportionate decrease when exposed to cold stress (Pearce, 2001). In such stressful environments, plants tend to generate a heightened quantity of reactive oxygen species, which are capable of causing oxidative damage to cells (You and Chan, 2015; Karami-Moalem et al., 2018). Concurrently, low temperatures hinder the synthesis of chlorophyll, which in turn compromises photosynthetic efficiency (Hajihashemi et al., 2018; Li et al., 2024). Moreover, exposure to low temperatures results in plant dehydration, decreased membrane fluidity, and disturbances in intracellular calcium homeostasis (Conde et al., 2011; Shi et al., 2014; Nechaeva et al., 2017; Agurla et al., 2018; Yuan et al., 2018). Most importantly, cold stress disrupts enzymatic activities within plants, negatively impacting respiratory processes and material metabolism, ultimately affecting the overall health and yield of the plants (Thakur et al., 2010).

To adapt to low-temperature stress, plants employ various defensive mechanisms. When subjected to chilly environments, plants accumulate osmolytes such as proline, soluble sugars, and soluble proteins (Kontunen-Soppela et al., 2002; Hayat et al., 2012; Jogawat, 2019; Ghosh et al., 2021; Xu et al., 2022). These substances are crucial for maintaining normal osmotic pressure within cells, thus effectively preventing cell damage due to dehydration. Concurrently, plants boost the activity of antioxidant enzymes, such as SOD and POD, to eliminate excess ROS (Gill and Tuteja, 2010; Adhikari et al., 2022; Wei et al., 2022). This action mitigates the oxidative damage caused by cold stress to plant cells. Additionally, elevated calcium ion levels regulate protein phosphorylation, which in turn stimulates the expression of cold resistance-related genes and facilitates the synthesis of specific proteins, ultimately bolstering the plant's tolerance to cold stress (Saijo et al., 2000; Ma et al., 2015; Yuan et al., 2018). It is noteworthy that the ICE-CBF-COR pathway mediates plant cold tolerance (Zhao et al., 2016; Shi et al., 2018; Liu et al., 2019). Under freezing conditions, this pathway swiftly activates *CBF* (C-repeat-binding factor) expression, and the encoded protein directly modulates a set of *COR* (cold-regulated) genes, significantly bolstering the plant's frost resistance. The transport and metabolism of inorganic ions, carbohydrates, and lipids also undergo changes in response to cold stress, collectively forming an effective defense against low temperatures in plants (Hurry et al., 2000; Trischuk et al., 2014; Barrero-Sicilia et al., 2017; Adhikari et al., 2022).

Multiple studies have consistently demonstrated that symbiotic fungi exhibit a pronounced beneficial effect on plants' cold resistance or tolerance. Arbuscular mycorrhizal fungi have been shown to play a vital role in augmenting the photosynthesis and antioxidant capabilities of plants such as cucumber, rice, and corn (Charest et al., 1993; Chen et al., 2013; Liu et al., 2013). This, in turn, endows these plants with the resilience to withstand low temperatures. Meanwhile, *Piriformospora indica*, a symbiotic fungus with remarkable growth-promoting potential, has garnered attention for its performance in aiding host plants to withstand abiotic stress (Gill et al., 2016; Aslam et al., 2019). Under low-temperature conditions, *P. indica* is able to elevate the antioxidant capacity of *Phaseolus vulgaris* and barley, thereby fostering their growth (Murphy et al., 2014; Alizadeh Frutan et al., 2016). Li et al. (2021) pointed out that *P. indica* can enhance the cold tolerance of bananas by stimulating the antioxidant capacity of leaves, facilitating soluble sugar accumulation, and activating the expression of cold-responsive genes. Furthermore, Jiang et al. (2020) also noted that *P. indica* can reinforce the frost resistance of *Arabidopsis thaliana* by upregulating the expression of genes in the CBF-dependent pathway, specifically *ICE1* and *CBF1*.

Tobacco, a warm-season crop originating from the Americas, not only serves as an important model plant in biological research but also stands as a significant economic crop. In high-latitude regions, tobacco seedlings are first cultivated in greenhouses and then transplanted to the field in early spring. However, due to tobacco's sensitivity to ambient temperature, exposure to cold stress during seedling cultivation and transplantation can significantly reduce germination rates, retard growth rates, and lower survival rates. These adverse effects severely hinder the normal growth and development of tobacco. Therefore, enhancing tobacco's tolerance to cold stress is crucial. This study reveals that *P. indica* can significantly improve tobacco's tolerance to cold stress. We delve into the multifaceted effects of *P. indica* on tobacco under low-temperature, including its promotional effect on photosynthesis, regulatory role in antioxidant enzyme activity and the accumulation of osmolytes, enhancement of nitrogen absorption and assimilation, and influence on the expression of cold-responsive genes. The findings of this study not only provide new insights into how endophytic fungi regulate plant cold resistance under low-temperature but also have important practical implications for applying these beneficial fungi as inoculants to enhance the cold resistance of crops.

2 Materials and methods

2.1 Plant materials and inoculation of *P. indica*

The inoculation of tobacco with *P. indica* was conducted based on the methodology outlined by Li et al. (2022). The mycelium of *P. indica*, which had been cultivated in Potato Dextrose Broth for a duration of four days, was harvested using high-speed centrifugation at 10,000 rpm. The harvested mycelium was then resuspended in ddH_2O, and the fungal pellet suspension was adjusted to an OD_{600} value of 0.1. Subsequently, this suspension was employed to irrigate uniformly grown, two-month-old tobacco seedlings. Meanwhile, the control seedlings underwent irrigation with an equivalent volume of ddH_2O. Fifteen days post-inoculation, the tobacco roots were stained with 0.05% trypan blue under an optical microscope, according to the method described by Li et al. (2022). The establishment of a symbiotic relationship was ascertained through the observation of stained chlamydospores in the tobacco roots using a 10x objective lens.

2.2 Cold stress treatment of plant materials

Both inoculated and uninoculated tobacco plants with *P. indica* were cultivated in incubators maintained at temperatures of 25°C and 4°C, respectively. Both incubators were kept at a relative humidity of 60-80% and followed a photoperiod of 16-hour light/8-hour dark. Each treatment group consisted of six replicates. After two days of incubation, physiological and biochemical parameters, enzyme activity, as well as photosynthesis and chlorophyll fluorescence parameters were measured in roots and leaves. Additionally, RNA was extracted from samples for subsequent qRT-PCR analysis.

2.3 Determination of the physiological and biochemical parameters

Samples were ground into powder and used for the determination of physiological and biochemical parameters and enzyme activity. Commercial assay kits (Comin Biotechnology Co., Ltd., China) were utilized to determine the levels of superoxide anion ($O_2^{\cdot-}$), H_2O_2, malondialdehyde, starch, soluble protein, soluble sugar, proline, relative electrical conductivity, as well as the activities of Rubisco, nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), NADH-glutamate oxaloacetate transaminase (NADH-GOGAT), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD). Chlorophyll and carotenoid content were assayed spectrophotometrically, referring to a previously established protocol by Lichtenthaler (1987). Additionally, Total nitrogen content was determined using the micro-Kjeldahl method after digesting the samples in H_2SO_4-H_2O_2. The obtained data were derived from four biological replicates.

2.4 Detection of key photosynthetic and chlorophyll fluorescence parameters

The photosynthetic and chlorophyll fluorescence parameters of the samples were measured using a LI-6400 portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA). The photosynthetic parameters encompassed net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular carbon dioxide concentration (Ci), and transpiration rate (Tr). Prior to the measurement of photosynthetic parameters, tobacco leaves were illuminated with the instrument's built-in light source for 30 minutes at a photosynthetic photon flux density of 1000 $\mu molm^{-2}{\cdot}s^{-1}$. Chlorophyll fluorescence parameters consisted of the maximum photochemical efficiency of photosystem II (Fv/Fm), actual photochemical quantum efficiency of photosystem II (ΦPSII), photosynthetic electron transport rate (ETR), photochemical quenching coefficient (qP), and non-photochemical quenching coefficient (NPQ). Before measuring chlorophyll fluorescence parameters, the potted tobacco seedlings were kept in darkness for 40 minutes.

2.5 RNA extraction and qRT-PCR analysis

RNA was extracted from samples using the RNAprep Pure Plant Plus Kit (TIANGEN BIOTECH (BEIJING) CO., LTD). Subsequently, 1 μg of RNA was utilized for the synthesis of the first strand complementary DNA (cDNA) (PrimeScript™ RT reagent Kit with gDNA Eraser, Takara). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the method described previously, employing the fluorescent quantitative PCR kit (TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), Bulk) on the Applied Biosystems ViiA™ 7 real-time PCR instrument. The housekeeping gene *NtACTIN* served as an internal reference gene. The primers for qRT-PCR are listed in Supplementary Table S1.

2.6 Statistical analysis of data

Data were analyzed using Tukey's test ($p < 0.05$) in GraphPad Prism version 4.

3 Results

3.1 *P. indica* enhances the tolerance of tobacco to cold stress

The *P. indica*-colonized tobacco and the uncolonized tobacco plants (control group) were placed in a 4°C incubator with a photoperiod of 16-hour light/8-hour dark for a two-day low-temperature treatment. As shown in Figure 1A, after cold stress treatment, there is a significant phenotype difference between the *P. indica*-inoculated tobacco and the control group. The control group showed obvious wilting symptoms and water-soaked spots, while the *P. indica*-inoculated tobacco exhibited much slighter injury symptoms. To gain a deeper understanding of the physiological disparities, we conducted further measurements of ROS and MDA levels in the leaves of both groups. The findings revealed that the control plants exhibited significantly higher levels of hydrogen peroxide and superoxide anion compared to the *P. indica*-inoculated tobacco (Figures 1B, C). Concurrently, the MDA content in the leaves of the control plants was also significantly higher than that of the *P. indica*-colonized tobacco, indicating that the control group suffered more severe oxidative damage under cold stress (Figure 1D). In addition, we also found that the electrolyte leakage rate in the control group was notably elevated compared to the *P. indica*-colonized tobacco, implying enhanced cell membrane stability in the *P. indica*-colonized plants under cold stress (Figure 1E).

3.2 Effect of *P. indica* on tobacco photosynthesis under cold stress

Cold stress is typically accompanied by detrimental impacts on photosynthesis. In this study, we examined photosynthetic

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pigments, net photosynthetic rate, stomatal conductance, intercellular carbon dioxide concentration, transpiration rate, and Rubisco activity in tobacco leaves under a 4°C environment. The results indicated that cold stress reduced the accumulation of chlorophyll a, chlorophyll b, and carotenoids in leaves of control plants, while inoculation with *P. indica* mitigated the loss of chlorophyll a, chlorophyll b, and carotenoids (Figures 2A-C). In comparison to the control group, tobacco inoculated with *P. indica* demonstrated elevated Pn, Gs, and Tr during cold stress, accompanied by a comparatively lower Ci (Figures 2D-G). Furthermore, the activity of the Rubisco enzyme was also significantly increased in tobacco plants colonized by *P. indica* (Figure 2H). These findings suggest that *P. indica* effectively enhances the photosynthetic capacity of tobacco under cold stress conditions.

To further evaluate the positive role of *P. indica* in maintaining photosynthesis under cold stress, we measured the photosynthetic fluorescence parameters of tobacco leaves under cold stress, including Fv/Fm, ΦPSII, ETR, qP, and NPQ. As illustrated in Figure 3, after cold stress treatment, the Fv/Fm, Φ PSII, ETR, qP, and NPQ values of the control plants were significantly lower than those of *P. indica*-colonized tobacco (Figures 3A-E). These findings indicate that *P. indica* can effectively enhance the efficiency of the photosystem II in tobacco leaves under cold stress.

3.3 Effects of *P. indica* on the accumulation of osmolytes and the activity of antioxidant enzymes in tobacco under cold stress

Plants adapt to cold stress by accumulating osmolytes such as soluble sugars, soluble proteins, and proline. In this study, we observed that the content of soluble sugars, soluble proteins, and proline in leaves of *P. indica*-colonized tobacco was significantly higher than that in the control group after cold stress treatment (Figures 4A, C). Plants can enhance the activity of antioxidant enzymes to eliminate excess ROS, thereby mitigating oxidative damage to plant cells. As illustrated in Figure 5, the activities of SOD, POD, CAT, and APX in tobacco leaves colonized by *P. indica* exhibited a significant increase compared to the control group following exposure to cold stress. These findings suggest that *P. indica* possesses the capability to stimulate the accumulation of osmolytes and enhance the activity of antioxidant enzymes in tobacco leaves under cold stress.

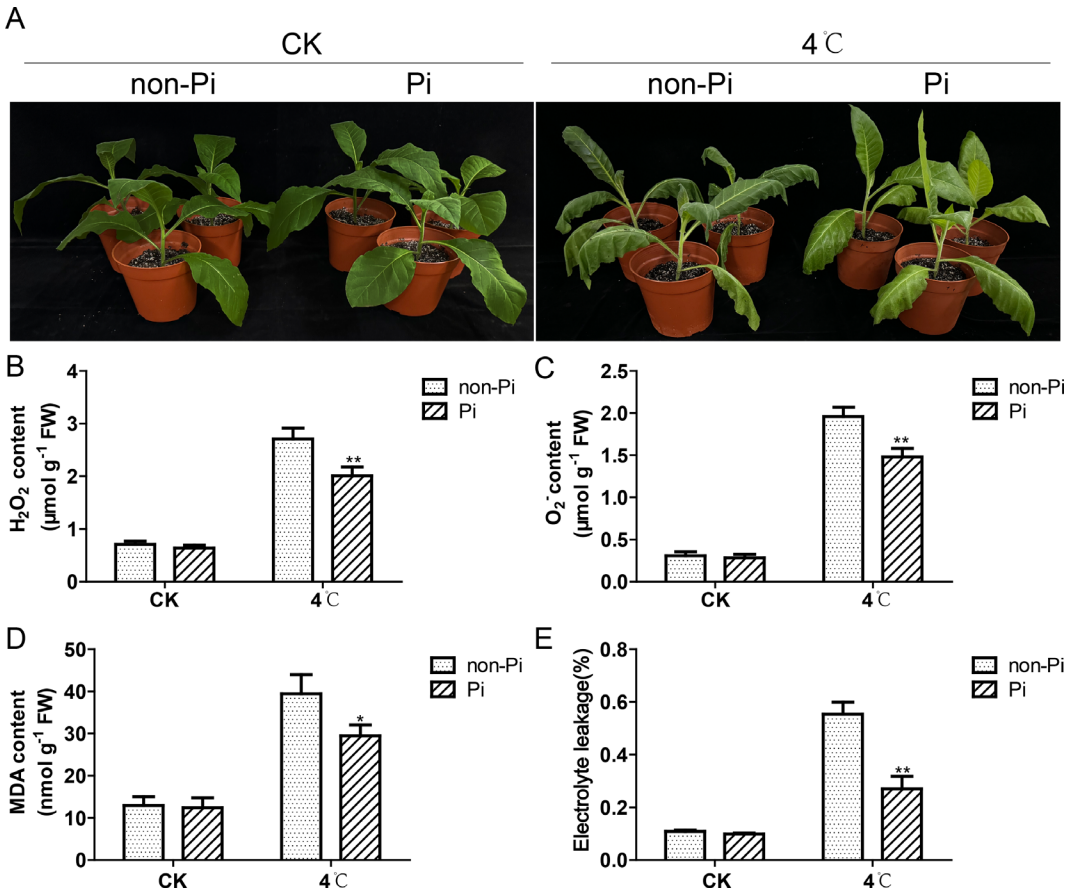

FIGURE 1
Effect of *P. indica* on phenotypes of tobacco leaves and its ability to mitigate oxidative stress under cold stress. **(A)** Effect of *P. indica* on phenotypes of tobacco leaves under cold stress; **(B)** H_2O_2 content in tobacco leaves; **(C)** O^{-2} content in tobacco leaves; **(D)** MDA content in tobacco leaves; **(E)** The relative electrolyte leakage rate of tobacco leaves; CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi, control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

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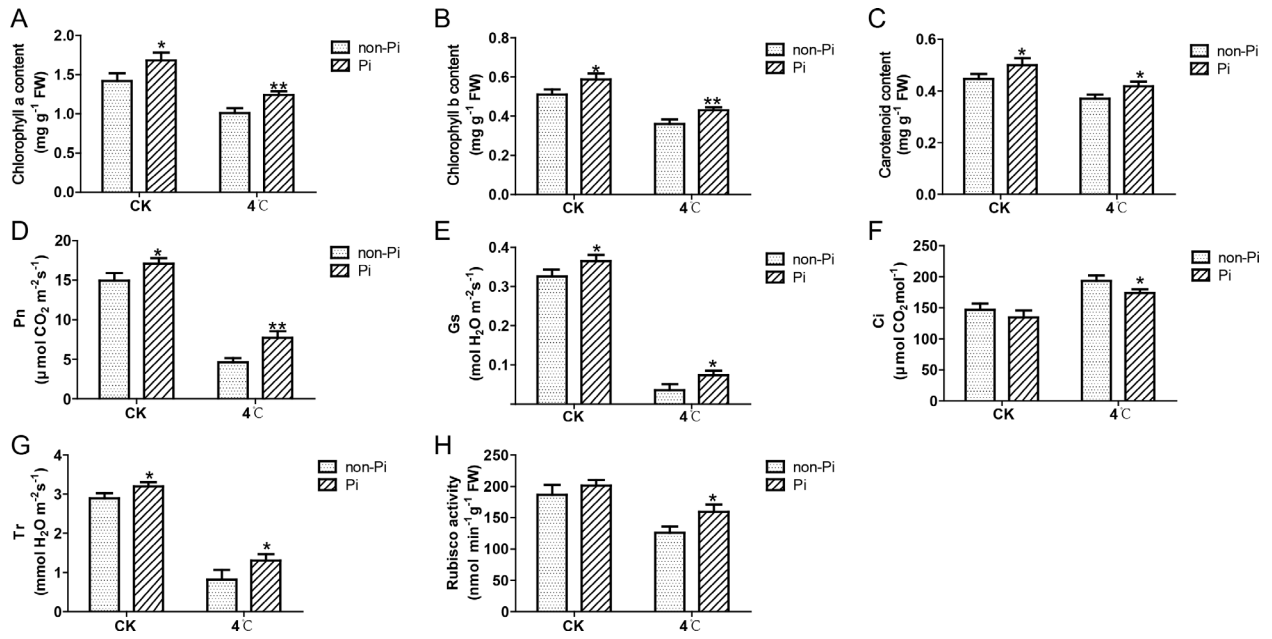

FIGURE 2
Effects of *P. indica* on photosynthesis in tobacco leaves under cold stress. **(A)** Chlorophyll a content in tobacco leaves; **(B)** Chlorophyll b content in tobacco leaves; **(C)** Carotenoid content in tobacco leaves; **(D)** Net photosynthetic rate (Pn) of tobacco leaves; **(E)** Stomatal conductance (Gs) of tobacco leaves; **(F)** Intercellular CO2 concentration (Ci) of tobacco leaves; **(G)** Transpiration rate (Tr) of tobacco leaves; **(H)** Rubisco activity in tobacco leaves; CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi, control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

3.4 Effects of *P. indica* on nitrogen absorption and assimilation in tobacco under cold stress

It is well known that *P. indica* has the ability to enhance plants' efficiency in absorbing external nutrients (Franken, 2012). In this study, we found that under normal temperature, colonization by *P. indica* can induce the expression of nitrate transporter genes (*NtNRT1.1*, *NtNRT1.2*, *NtNRT2.2*), and enhance the activity of nitrate reductase (NR), thereby increasing nitrogen content in the roots (Figures 6A–H; Figures 7A, B). Under cold stress conditions, tobacco plants colonized by *P. indica* exhibited significantly higher expression of nitrate transporter genes, including *NtNRT1.1*, *NtNRT1.2*, *NtNRT2.1*, and *NtNRT2.2*, compared to control plants (Figures 6A–H). Furthermore, the activities of nitrate reductase, nitrite reductase (NIR), glutamine synthetase (GS), and glutamate

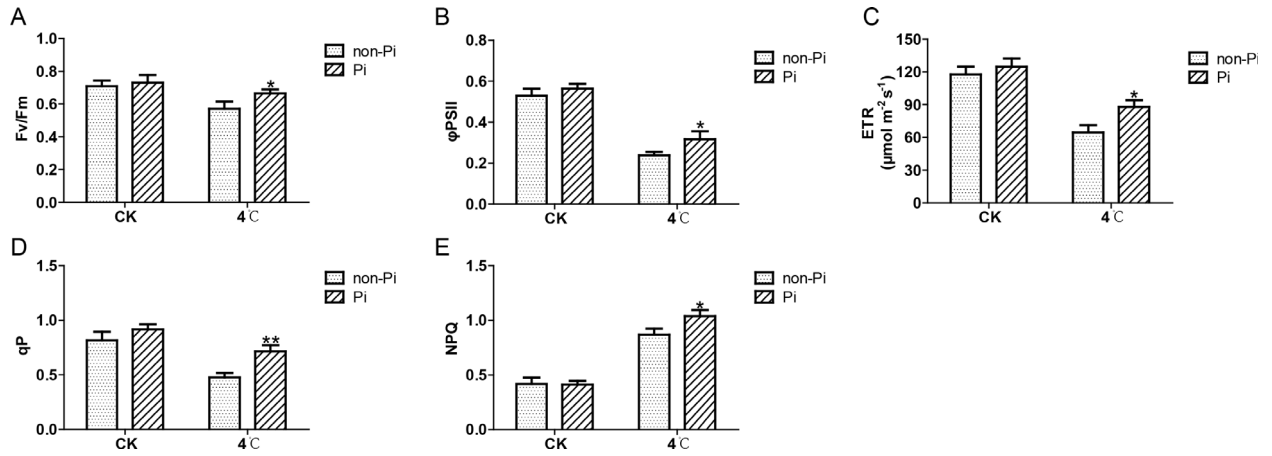

FIGURE 3
Effects of *P. indica* on fluorescence parameters in tobacco leaves under low cold stress. **(A)** Maximum photochemical efficiency of photosystem II (Fv/Fm); **(B)** Actual photochemical quantum efficiency of photosystem II (φPSII); **(C)** Photosynthetic electron transport rate (ETR); **(D)** Photochemical quenching coefficient (qP); **(E)** Non-photochemical quenching coefficient (NPQ); CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi: control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

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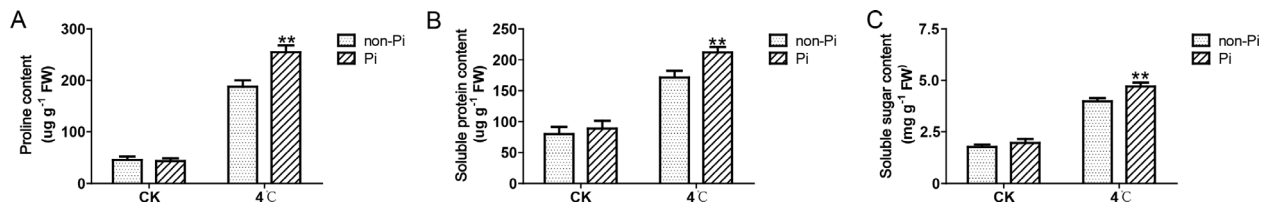

FIGURE 4
Effects of *P. indica* on the content of osmolytes in tobacco leaves under cold stress. **(A)** Proline content in tobacco leaves; **(B)** Soluble protein content in tobacco leaves; **(C)** Soluble sugar content in tobacco leaves; CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi, control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

synthetase (GOGAT) were notably higher in the *P. indica*-colonized plants (Figures 7B-E). More importantly, the nitrogen content in the roots of these colonized plants was also significantly elevated compared to the control group (Figure 7A). These findings suggest that *P. indica* can enhance nitrogen absorption and assimilation processes in tobacco, even under cold stress.

3.5 Effects of *P. indica* on the expression of cold-responsive genes in tobacco

The ICE-CBF-COR pathway plays a pivotal role in plant cold stress tolerance. Therefore, we investigated whether *P. indica* affects the expression of genes in the ICE-CBF-COR pathway in tobacco under cold stress. After low-temperature treatment, apart from *ICE1*, which showed no significant difference in expression between *P. indica*-colonized and non-colonized plants, the genes *NtCBF1*, *NtCBF3*, *NtDREB2B*, *NtERD10B*, and *NtERD10C* exhibited higher expression in *P. indica*-colonized tobacco (Figures 8B-F). This suggests that the cold stress tolerance induced by *P. indica* may be associated with the upregulation of *CBF* and *COR* expression.

4 Discussion

Cold stress, a significant factor limiting plant growth and yield, has profound negative impacts on up to 15% of agricultural land worldwide, leading to plant cell membrane rupture, loss of turgor, metabolic disorders, and even cell death (Thakur et al., 2010; Chaudhry and Sidhu, 2022). Recent extensive studies have revealed that cold stress disrupts the cellular homeostasis, disturbing the balance between the generation and scavenging of ROS, such as O_2^- and H_2O_2, leading to an excessive buildup of intracellular ROS (You and Chan, 2015; Karami-Moalem et al.,

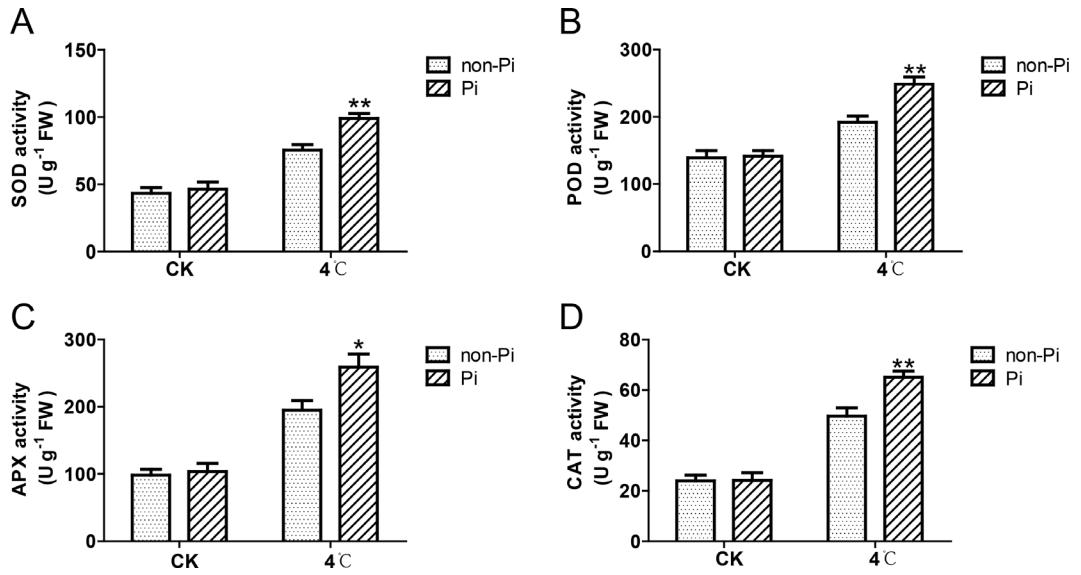

FIGURE 5
Effects of *P. indica* on the activity of antioxidant enzymes in tobacco leaves under cold stress. **(A)** Superoxide dismutase (SOD) activity in tobacco leaves; **(B)** Peroxidase (POD) activity in tobacco leaves; **(C)** Ascorbate peroxidase (APX) activity in tobacco leaves; **(D)** Catalase (CAT) activity in tobacco leaves; CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi, control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

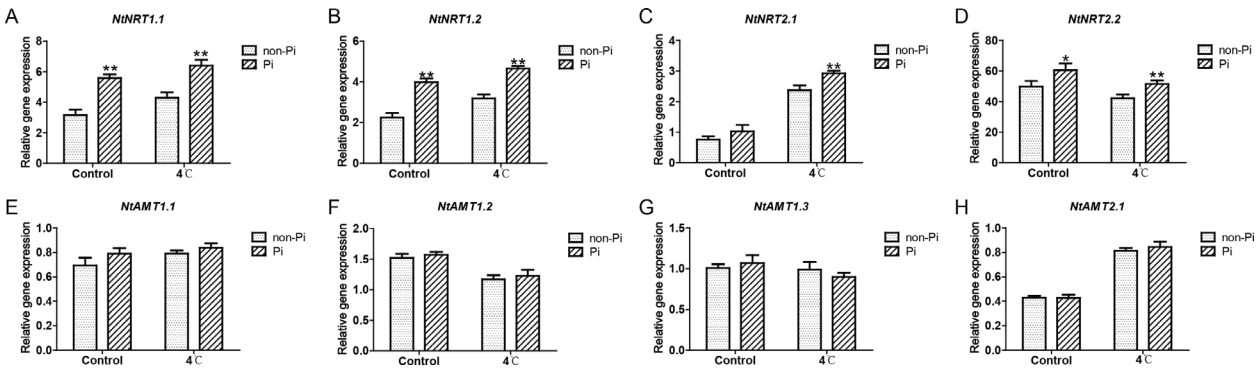

FIGURE 6
Effects of *P. indica* on the expression of ammonium and nitrate transporter genes in tobacco roots under cold stress. **(A-D)** The expression of nitrate transporter genes in tobacco roots; **(E-H)** The expression of ammonium transporter genes in tobacco roots; CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi, control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

2018). Such ROS surge can cause oxidative damage to lipids, proteins, and nucleic acids in plant tissues, further exacerbating the damage caused by cold stress to plants (Pennycooke et al., 2005). The antioxidant enzyme system is considered a crucial defense mechanism for plants to scavenge ROS under environmental stress (Navrot et al., 2007; Rajput et al., 2021). Among these enzymes, SOD specifically converts superoxide radicals into hydrogen peroxide and oxygen. Subsequently, hydrogen peroxide is effectively scavenged under the activity of APX, CAT, and POD (Gill and Tuteja, 2010). Current studies suggest that colonization by symbiotic fungi can enhance the antioxidant enzyme system of host plants, effectively maintaining ROS homeostasis, and thus mitigating the inhibitory effects caused by elevated ROS levels that accumulate during plant cellular processes, growth, and survival under stressful environment. For instance, AMF upregulates the activities of multiple antioxidant enzymes, including SOD, POD, CAT, and APX, and reduces the concentration of MDA, providing enhanced stability to the plasma membrane (Latef and Chaoxing, 2011; Huang et al., 2014; Li et al., 2019; He et al., 2020). In this study, we observed that cold stress triggers an outburst of ROS in tobacco leaves, leading to the generation of MDA and an increase in electrolyte leakage. However, inoculation with *P. indica* significantly enhances the activities of SOD, POD, CAT, and APX, thereby reducing MDA accumulation and alleviating electrolyte leakage. The lower MDA level and electrolyte leakage rate in these plants confirm that colonized plants suffer less from the cold stress. Similarly, in banana, colonization by *P. indica* significantly stimulates the activities of SOD, CAT, APX, and glutathione reductase (GR), effectively reducing the excessive accumulation of ROS under cold stress (Li

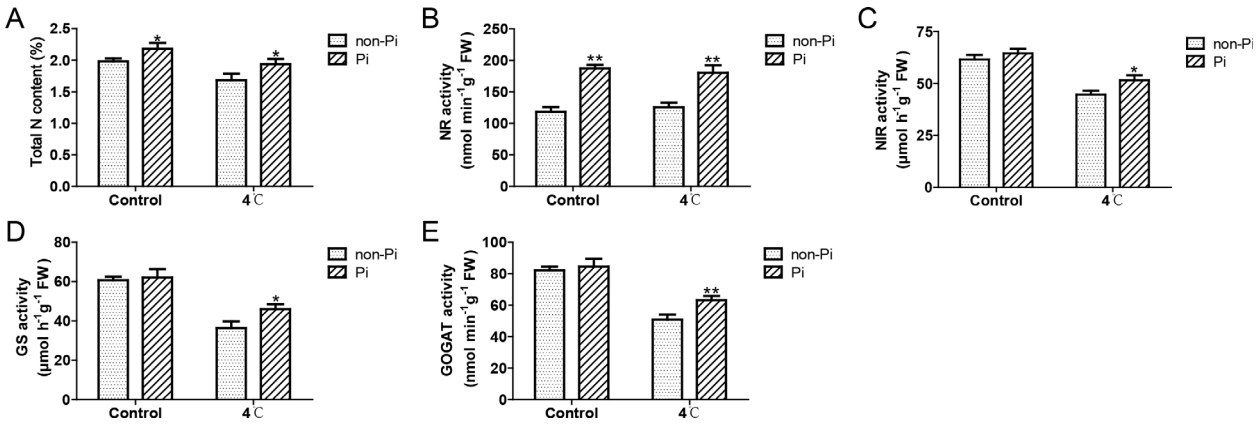

FIGURE 7
Effects of *P. indica* on the activity of key enzymes involved in nitrogen assimilation in tobacco under cold stress. **(A)** Total nitrogen content in tobacco roots; **(B)** Nitrate reductase (NR) activity in tobacco roots; **(C)** Nitrite reductase (NIR) activity in tobacco roots; **(D)** Glutamine synthetase (GS) activity in tobacco roots; **(E)** Glutamate synthase (GOGAT) activity in tobacco roots; CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi, control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

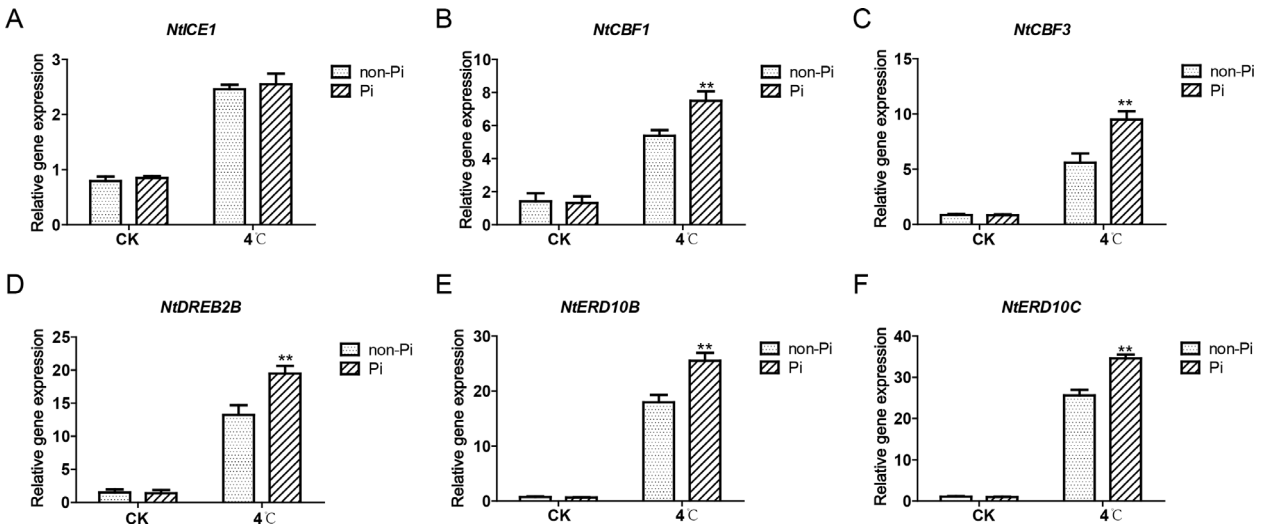

FIGURE 8
Effects of *P. indica* on the expression of cold-responsive genes in tobacco leaves under cold stress. **(A–F)** represent the qRT-PCR analysis results of NtICE1, NtCBF1, NtCBF3, NtDREB2B, NtERD10B, and NtERD10C in control plants and tobacco plants colonized by P. indica, respectively. CK, control group; 4°C, treatment at 4°C; Pi, *P. indica*-colonized tobacco plants; non-Pi, control plants. Data are means (± SD), n=3. Significant differences between means were determined using Student's t-test: *P<0.05, **P<0.01.

et al., 2021). These results indicated that colonization by *P. indica* can enhance the antioxidant enzyme activities of host plants, thereby maintaining cell membrane stability under cold stress.

Cold stress often exerts detrimental effects through osmotic stress, and such damaging effects can be counteracted by the accumulation of osmolytes (such as soluble sugars, proline, and soluble proteins), thereby restoring osmotic balance (Kontunen-Soppela et al., 2002; Hayat et al., 2012; Jogawat, 2019; Ghosh et al., 2021; Xu et al., 2022). Furthermore, these osmolytes can preserve the integrity of cell membranes by directly participating in the scavenging of ROS, as well as by lowering the freezing point of plants, enabling them to better adapt to cold stress (Kontunen-Soppela et al., 2002; Hayat et al., 2012; Jogawat, 2019; Ghosh et al., 2021; Xu et al., 2022). This, in turn, enhances the oxidative tolerance of plants. Among them, proline enhances the stress tolerance of plants by maintaining osmotic balance, cell turgor, and indirectly regulating the metabolism of ROS (Hayat et al., 2012). Soluble sugars serve as an energy source, providing a basis for cellular metabolism, thereby enhancing the cold resistance of plants (Xu et al., 2022). Soluble proteins can bind with water to maintain cellular water content, thus reducing physiological water loss under cold stress (Kontunen-Soppela et al., 2002). In this study, the endophytic fungus *P. indica* significantly increased the contents of proline, soluble sugars, and soluble proteins in tobacco under low-temperature stress. This phenomenon has also been observed in *Arabidopsis thaliana* and banana, where *P. indica* enhances the contents of soluble proteins and proline in *Arabidopsis thaliana* (Jiang et al., 2020), and similarly, increases the levels of soluble sugars and proline in banana (Li et al., 2021). These findings indicate that *P. indica* stimulates the accumulation of osmolytes, thus improving the resistance of host plants to cold stress.

Chlorophyll plays a crucial role in capturing light energy during photosynthesis. While, cold stress significantly hinders the synthesis of chlorophyll or even causes its degradation, adversely affecting the photosynthesis of plants (Hajihashemi et al., 2018; Li et al., 2024). In this study, we found that inoculation with *P. indica* can effectively alleviate the decrease in chlorophyll and carotenoid content in tobacco under cold stress. This suggests that *P. indica* stimulates the synthesis of photosynthetic pigments, thereby reducing the negative impact of cold stress on plants. Furthermore, the results of this study indicate that photosynthesis in tobacco is severely inhibited under cold stress, which is particularly evident in the notable decreases observed in Pn, Gs, and Tr values. The reduction in Pn is likely associated with decreased Gs and the hindrance of CO_2 entry into the leaves. However, colonization with *P. indica* enhances the Pn, Tr, and Gs of tobacco leaves, and simultaneously reduces the Ci. The decreased Ci value in tobacco inoculated with *P. indica* may be attributed to the fact that CO_2 is an essential substrate for photosynthesis. The reduction in intercellular CO_2 actually reflects a higher consumption of CO_2 during the photosynthetic process, thereby promoting an increase in the Pn. In addition, cold stress can damage the enzymatic system involved in photosynthesis. Rubisco, a key rate-limiting enzyme in carbon assimilation during photosynthesis, experiences a significant decrease in activity under cold stress, thereby negatively affecting the process of photosynthesis (Salesse-Smith et al., 2020). However, our study found that inoculation with *P. indica* can increase the activity of Rubisco in tobacco, which is conducive to CO_2 assimilation under cold stress.

Cold stress can also cause damage to the PSII reaction center, resulting in reduced photosynthetic activity, decreased rate of electron transfer in leaves, and increased photoinhibition (Wei et al., 2022). The rapid accumulation of ROS can further inhibit the synthesis of

proteins necessary for PSII photodamage repair (such as D1 protein), exacerbating damage to the PSII reaction center (He and Chow, 2003). Fluorescence is an important indicator to measure PSII function and light capture efficiency. Under salt stress, inoculation with *P. indica* significantly enhances the PSII photochemical efficiency of tomatoes, manifested by notable increases in photosynthetic fluorescence parameters such as NPQ, qP, Fv/Fm, and ΦPSII (Ghorbani et al., 2018). Similarly, when plants are exposed to cadmium pollution, drought, or low temperatures, inoculation with *P. indica* can effectively enhance the PSII photochemical efficiency of the host plant, helping it better cope with various environmental pressures (Shahabivand et al., 2017; Li et al., 2021; Boorboori and Zhang, 2022). In this study, the tobacco colonized with *P. indica* exhibited less declines in Fv/Fm, ΦPSII, and ETR under cold stress compared to the uninoculated control group. The Fv/Fm ratio, which is sensitive to environmental stress, represents the maximum photochemical efficiency of PSII. Meanwhile, ΦPSII is closely related to the efficiency of light energy utilization in plants; when photosynthetically active radiation (PAR) is limited, a decrease in ΦPSII is indicative of a reduction in photosynthetic efficiency. ETR is indicative of the rate and efficiency of plants utilizing available light energy in the environment for photosynthesis. When illumination is insufficient, a decrease in ETR suggests a slowdown in the rate of photosynthesis. Additionally, qP, which represents the proportion of open PSII reaction centers, is positively correlated with plant photosynthetic activity. Low temperatures typically cause a decrease in qP (Banerjee and Roychoudhury, 2019). On the other hand, NPQ represents the photoprotection ability of plants to dissipate excess light energy through heat dissipation. The results show that after low-temperature treatment, the qP and NPQ values of tobacco leaves inoculated with *P. indica* were higher than those of the uninoculated control group. Considering *P. indica*'s ability to enhance the activity of antioxidant enzymes in tobacco, we hypothesize that it can alleviate the damage caused by excessive ROS generation to PSII reaction centers by activating the antioxidant enzyme defense system. Based on a comprehensive analysis of photosynthesis parameters, Rubisco activity, and fluorescence parameters, we believe that tobacco colonized by *P. indica* exhibits better photosynthetic capacity than uninoculated plants.

P. indica has been reported to enhance plant nitrogen use efficiency (Ansari et al., 2013). In this study, we observed that under cold stress, the colonization of tobacco plants by *P. indica* can significantly induce the expression of nitrate transporter genes, and enhance the activities of NR, NIR, GS, and GOGAT. The combined effect of these mechanisms promoted nitrogen uptake and assimilation in tobacco, resulting in increased nitrogen content in the roots. Nitrogen metabolism is a complex pathway that affects nearly all growth-determining processes in plants. Recent research has demonstrated that an increase in total nitrogen content and nitrogen assimilation plays a crucial role in enhancing plant tolerance to cold stress (Soualiou et al., 2023). This augmented nitrogen content promotes photosynthetic activity by enhancing nitrogen partitioning in the photosynthetic apparatus and inducing positive feedback effects on carbohydrate metabolism through nitrogen allocation to sink organs for growth (Yi et al., 2014; Bloom et al., 2010; Soualiou et al., 2023). In contrast, a reduction in nitrogen assimilation in plants directly impacts the nitrogen supply to the photosynthetic apparatus, thereby restricting the flow

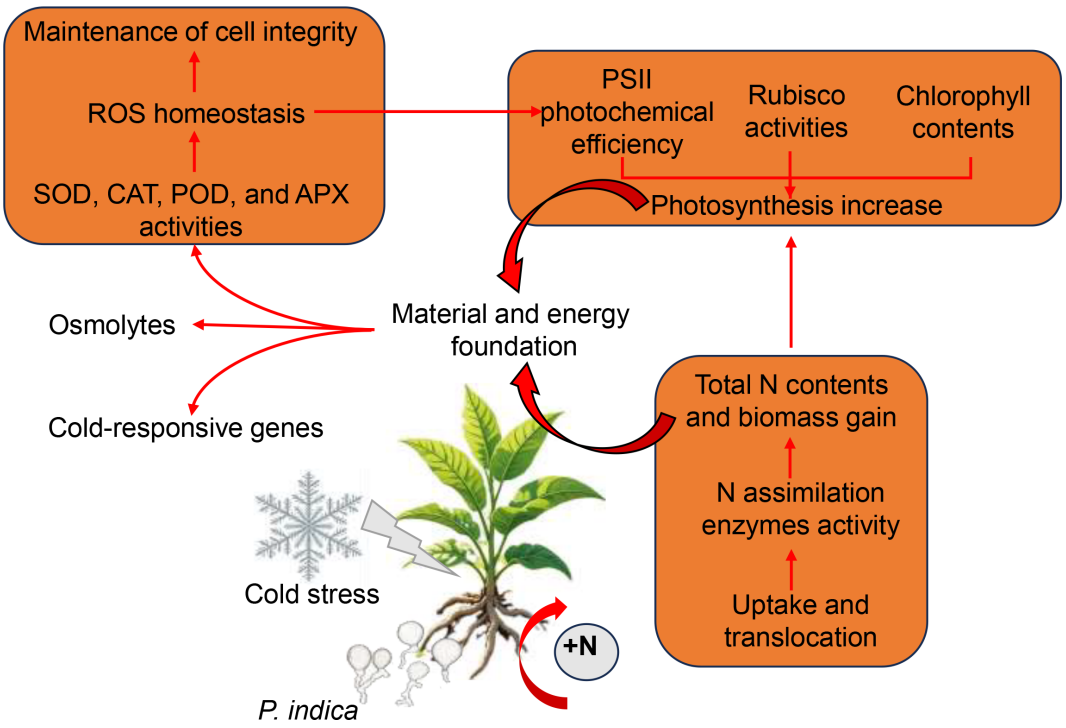

FIGURE 9
Proposed model of enhanced cold stress tolerance in tobacco through root colonization by *Piriformospora indica*. The orange boxes indicate strongly induced processes, and the red arrows represent positive regulatory processes.

of nitrogen to the photosynthetic system (Yi et al., 2014; Bloom et al., 2010; Soualiou et al., 2023). This limitation not only undermines photosynthetic capacity but also significantly lowers the electron transport rate at the PSII reaction centers, posing challenges to plant growth and adaptability (Soualiou et al., 2023). Therefore, the enhancing effect of *P. indica* on nitrogen utilization efficiency in tobacco may be beneficial to the photosynthesis, so as to improve the assimilation of nitrogen and carbon in tobacco under cold stress, thus providing a material and energy foundation for tobacco to resist cold stress.

The cascade of cold signaling composed of ICE-CBF-COR plays a crucial role in plant cold resistance (Zhao et al., 2016; Shi et al., 2018; Liu et al., 2019). The CBF transcription factors, as downstream regulatory genes of *ICE1* (INDUCER OF CBF EXPRESSION 1), can be transiently and rapidly induced by cold stress. The induced CBFs can bind to the cis-acting elements of the promoters of *COR* (cold-regulated) genes, thereby activating the expression of *COR*s to enhance the plant's ability to resist cold stress. This study demonstrates that, under cold stress, apart from *ICE1*, the expressions of *CBF1*, *CBF3*, and *DREB2B* (a homologous gene of *CBF1*) are significantly higher in tobacco colonized by *P. indica* than in the control group. The dehydration-responsive genes *NtERD10B* and *NtERD10C* are downstream regulatory factors of *CBF1* in tobacco, encoding LEA proteins (Shukla et al., 2006). Under cold stress, the expressions of *NtERD10B* and *NtERD10C* are also significantly higher in tobacco colonized by *P. indica* compared to the control group. Similarly, recent studies have shown that *P. indica* can enhance the tolerance of host plants to cold stress by activating the expression of *CBF*-dependent pathway genes (Jiang et al., 2020; Li et al., 2021). Therefore, we suggest that colonization by *P. indica* can enhance the expression of genes related to cold stress, thereby improving tobacco's adaptability to cold stress.

In summary, under low-temperature stress, *P. indica* enhances the activity of antioxidant enzymes in host plants, thereby facilitating the elimination of ROS and mitigating both peroxide-induced damage to plant cells and the detrimental effects of excessive ROS generation on the PSII reaction center (Figure 9). Additionally, *P. indica* stimulates the accumulation of osmolytes, contributing to the restoration of osmotic balance (Figure 9). When exposed to cold stress, *P. indica* not only promotes photosynthesis in tobacco by stimulating the synthesis of photosynthetic pigments, enhancing Rubisco activity, and improving PSII efficiency, but also strengthens tobacco's nitrogen assimilation by inducing the expression of nitrate transporter genes and activating enzymes related to nitrogen assimilation (Figure 9). The resultant improvement in nitrogen use efficiency further promotes photosynthetic activity by enhancing the allocation of nitrogen to photosynthetic enzymes, pigment content, and light absorption. This synergistic optimization of nitrogen and carbon assimilation provides a solid material and energetic foundation for tobacco plants to withstand cold stress (Figure 9). Furthermore, *P. indica* confers cold resistance to tobacco by stimulating the expression of cold-responsive genes (Figure 9). Our findings provide a valuable insight into the mechanisms by which *P. indica* enhances cold tolerance in tobacco and underscore the potential of using symbiotic fungi to improve crop resilience to abiotic stresses.

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

HL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. ZW: Investigation, Resources, Writing – review & editing. YY: Investigation, Writing – review & editing. WG: Methodology, Writing – review & editing. JZ: Formal analysis, Writing – review & editing. HZ: Investigation, Writing – review & editing. XL: Funding acquisition, Writing – review & editing. YL: Funding acquisition, Writing – review & editing.

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Conflict of interest

Author XL was employed by the company Guizhou Branch Company of China Tobacco Corporation.

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

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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.2024.1459882/full#supplementary-material

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Mechanisms of rhizosphere plant-microbe interactions: molecular insights into microbial colonization

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The rhizosphere, as the "frontline" of plant life, connects plant roots, rhizosphere microorganisms, and surrounding soil, plays a crucial role in plant growth and health, particularly in sustainable agriculture. Despite the well-established contribution of plant-microbe interactions to plant health, the specific molecular mechanisms remain insufficiently understood. This review aims to summarize the physiological adjustments and signal modulation that both plants and microorganisms undergo within this unique ecological niche to ensure successful colonization. By analyzing key processes such as chemotaxis, root attachment, immune evasion, and biofilm formation, we uncover how plants precisely modulate root exudates to either recruit or repel specific microorganisms, thereby shaping their colonization patterns. These findings provide new insights into the complexity of plant-microbe interactions and suggest potential directions for future research in sustainable agriculture.

KEYWORDS

rhizosphere, root colonization, molecular dialogue, root exudates, plant-microbe interactions

Introduction

The rhizosphere is the nutrient-rich zone of soil surrounding plant roots, which is characterized by intense microbial activity and diversity. It is regarded as one of the most intricate ecosystems on Earth, mediating subterranean interactions between plants and microorganisms (Goswami and Deka, 2022). These interactions, ranging from mutualism to parasitism, can be classified as forms of symbiosis (Plett and Martin, 2018; Du et al., 2021a). In mutualistic relationships, beneficial microorganisms such as rhizobia, mycorrhizae, endophytes (including plant growth-promoting microorganisms, PGPMs), and epiphytes establish positive interactions with plants, providing protection against stresses, enhancing growth, nutrient uptake, and improving soil conditions (Kiers et al., 2011; Zamioudis and

Pieterse, 2012; Jin et al., 2019; Ding et al., 2022; Koprivova and Kopriva, 2022). In contrast, parasitism causes diseases that negatively affect plant growth (Venturi and Fuqua, 2013; Zhang et al., 2023).

"Rhizosphere colonization" refers to the process through which microorganisms establish themselves in the rhizosphere soil or on the root surface, thereby forming stable communities (Liu et al., 2024). Among these microorganisms, bacteria are particularly significant due to their widespread presence and profound influence on plant health and soil ecosystems (Liu et al., 2024). The root surface serves as a critical interface for direct interactions between bacteria and plants, making it essential to understand the mechanisms of bacterial colonization in this area to elucidate plant-microbe interactions. Consequently, this review focuses primarily on the process of bacterial colonization on root surfaces. The colonization process typically follows four key steps (Figure 1): (i) chemotactic signal recognition; (ii) attachment to the root surface; (iii) evasion of plant immune defenses; and (iv) biofilm formation on the root surface. Upon completing these steps, endophytic bacteria may subsequently enter plant internal tissues, facilitating more direct interactions with the plant (Dudeja et al., 2021; Mushtaq et al., 2023). Throughout the colonization process, both plants and bacteria adjust their physiological states, guided by molecular signals, which include chemical molecules and effector proteins. These signals, whether specific or non-specific, are perceived through receptors and signaling pathways, influencing bacterial colonization behavior (Badri et al., 2009). This " molecular dialogue" is crucial for the successful colonization and the establishment of symbiotic relationships in the rhizosphere.

In this review, we comprehensively summarize the process of rhizosphere microbial colonization and its interactions with plants. By conducting an in-depth analysis of regulatory signals, such as chemical molecules and effector proteins, we delve into the "dialogue" mechanisms existing between plants and microorganisms. This review aims to provide a reference for future research and to advance the application of microorganisms in modern agriculture.

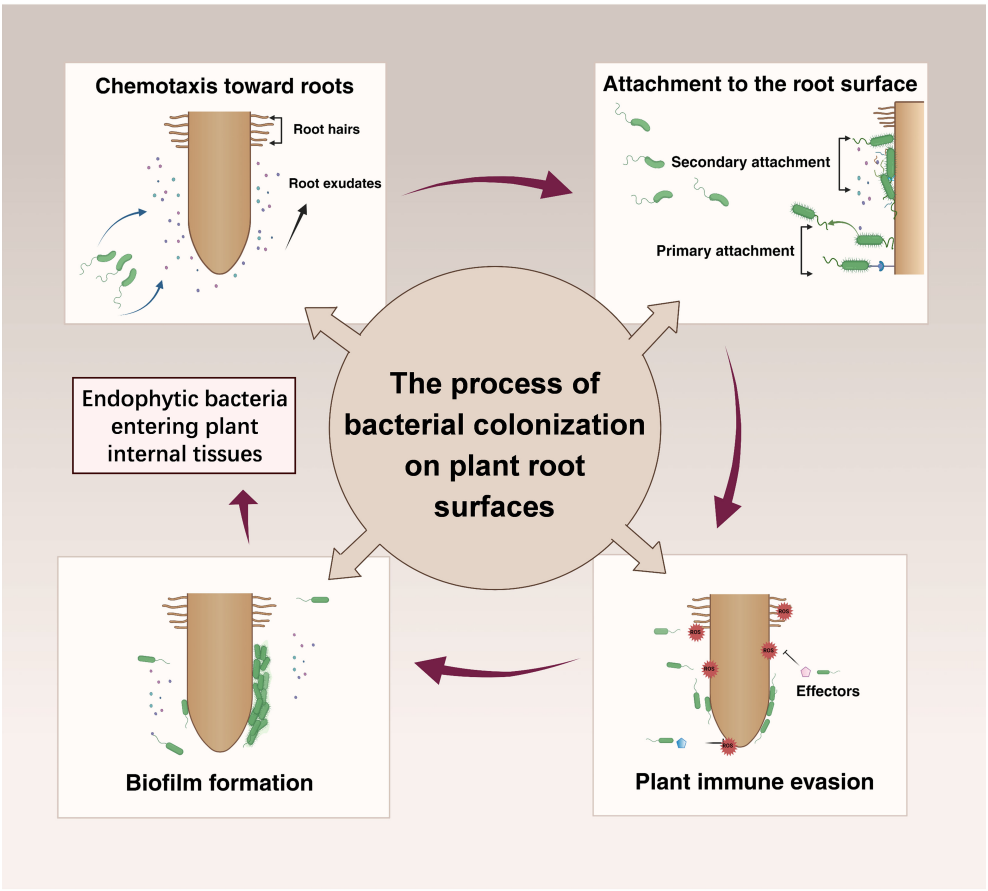

FIGURE 1
The colonization process of most bacteria can generally be divided into the following steps: chemotaxis, attachment, growth on the root surface, and biofilm formation. The chemotaxis and motility of microorganisms not only determine their movement toward the rhizosphere but also influence their initial positioning and migration of colonization sites. Following attachment to the root surface, microorganisms must overcome plant immune responses. Biofilm formation is essential for most bacteria colonizing the rhizosphere soil and root surfaces, and during this process, the bacteria utilize root exudates as a carbon source, which is a prerequisite for biofilm formation. Root exudates play a crucial role in influencing microbial colonization, and throughout the colonization process, a series of molecular dialogues occur between the interacting partners, indicating complex plant-microbe interactions.

Chemotactic signals emitted by plants roots

The chemotactic movement of soil microorganisms constitutes the fundamental basis of plant-microbe interactions, where root exudates frequently serve as communication molecules within this process (Chagas et al., 2018). Root exudates consist of significant amounts of carbon generated by plant photosynthesis, including low molecular weight compounds such as sugars, organic acids, and secondary metabolites (e.g., flavonoids, glucosinolates, and coumarins). These compounds make the rhizosphere a more active site for microbial colonization compared to bulk soil (Bais et al., 2006). Additionally, there are high molecular weight compounds like mucilage and proteins, despite their lower variability, they constitute the principal components of exudates (Chen and Liu, 2024). It is worth noting that due to the differential utilization and metabolic potential of various substrates among soil microorganisms, the chemical valence of rhizosphere exudates can drive positive or negative chemotactic movements in microbes (Feng et al., 2021; Knights et al., 2021).The following are detailed examples further illustrating the specific roles of root exudates in microbial chemotaxis:

1. Primary metabolites, such as sugars, amino acids, and organic acids, play essential roles in the rhizosphere by serving as carbon sources, chemoattractants, and chemical signals, thereby shaping microbial community structure and facilitating root colonization. Sugars are important carbon sources and colonization signals for rhizosphere microorganisms, influencing microbial community structure. In barley, key carbon compounds in root exudates help beneficial *Pseudomonas* adapt. A decrease in exudate diversity and higher glucose and fructose levels lead to more *Pseudomonas* (Pacheco-Moreno et al., 2024). Sugar molecules also positively influence the colonization of beneficial Gram-positive bacteria, particularly *Bacillus*. Additionally, sucrose, a widely present disaccharide, selectively shapes *Bacillus* in soil microbial communities. When *Bacillus subtilis* encounters sucrose exuded by Arabidopsis roots, it initiates the "levan detour" signaling cascade, activating solid surface motility (SSM), thereby enhancing its movement and colonization ability in the root environment (Tian et al., 2021).

In addition to sugars, amino acids in root exudates serve as key chemoattractants for various rhizosphere bacteria. For example, in *Sesbania rostrata*, the amino acids histidine, arginine, and aspartate act as chemoattractants for *Azorhizobium caulinodans*, not only facilitating bacterial chemotaxis but also upregulating the expression of genes involved in chemotaxis and motility. This dual role of amino acids underscores their importance in biofilm formation and root colonization (Liu et al., 2019). Moreover, in *Pseudomonas fluorescens* Pf0-1, the chemotaxis sensory proteins CtaA, CtaB, and CtaC have been identified as critical for amino acid-driven root colonization. Mutants lacking these proteins demonstrate reduced competitiveness, highlighting the pivotal role of amino acids as chemoattractants in the colonization process (Oku et al., 2012).

Organic acids further contribute to the rhizosphere's ecological dynamics by regulating soil pH and increasing mineral solubility, thereby promoting nutrient uptake by plants. They also act as chemical signals during the chemotactic colonization of rhizosphere bacteria. Studies have shown that the high release of citric, pyruvic, succinic, and fumaric acids may account for the enrichment of *Comamonadaceae* (Wen et al., 2020). When *Arabidopsis thaliana* leaves are infected by the tomato pathogen *Pseudomonas syringae* pv. tomato DC3000, roots secrete L-malic acid (MA) as a signal to recruit beneficial rhizosphere bacteria *Bacillus subtilis* FB17 (Rudrappa et al., 2008).

2. Secondary metabolites can serve as attractants for specific microbial strains, promoting beneficial interactions within the rhizosphere. For instance, the secretion of coumarins by *Arabidopsis thaliana* under iron deficiency attracts beneficial microbes, which enhance plant growth by improving iron uptake (Harbort et al., 2020). On the other hand, secondary metabolites often act as negative chemotactic agents, playing a crucial role in defending plants against pathogens. For instance, glucosinolates (GSLs), secondary metabolites unique to cruciferous plants, are hydrolyzed to produce glucosinolate aglycones, which rearrange into isothiocyanates with potent antimicrobial activity (Wittstock and Burow, 2010; Siebers et al., 2018). Moreover, studies on flavonoids provide important insights into another mechanism by which secondary metabolites resist pathogens. In high-density microbial populations, intercellular communication triggers the production of virulence factors through a process known as quorum sensing (QS) (Fuqua et al., 2001). While quorum sensing is a mechanism of communication that facilitates cooperative behavior in many microbial communities, it may not function as a virulence strategy in all microbes. Research has demonstrated that catechins significantly reduce the expression of key QS regulatory genes lasI, lasR, rhlI, and rhlR in *Pseudomonas aeruginosa*, leading to decreased production of QS factors and forming a line of defense against pathogen attacks (Vandeputte et al., 2010). This suggests that secondary metabolites can not only directly kill pathogens but also inhibit pathogen virulence by disrupting intercellular bacterial communication, thereby exerting negative chemotactic effects on pathogenic bacteria.

The regulatory role of secondary metabolites in the rhizosphere extends beyond direct interactions with pathogens. For instance, the decomposition product of benzoxazinoids, 6-methoxy-benzoxazolin-2-one (MBOA), indirectly determines the composition of the next generation of rhizosphere microorganisms by altering the associated microbial community around the roots, thereby extending its regulatory effects into the soil (Hu et al., 2018). Benzoxazinoids can also influence soil microbial communities by regulating the release of secondary metabolites from rhizosphere plants, particularly flavonoids (Cotton et al., 2019). These findings further demonstrate that secondary metabolites, as signaling molecules, play a crucial role not only in facilitating communication between plants and microorganisms, but also in influencing interactions among microorganisms themselves. Additionally, these compounds significantly impact the soil environment within the rhizosphere.

The regulatory effects of secondary metabolites are highly complex, depending on both the diversity of species that respond to these signals and the specific environmental conditions under which they function.

3. Volatile organic compounds (VOCs) primarily consist of terpenes, aromatic compounds, nitrogen-containing compounds, and fatty acid derivatives, as well as volatile plant hormones like methyl jasmonate and methyl salicylate as low molecular weight substances released by plant roots (Holopainen, 2004). VOCs, such as essential oils, have long been recognized for their broad antimicrobial activity, potentially playing a crucial role in mediating negative chemotaxis of pathogens. However, recent evidence indicates that VOCs can also serve as energy sources or signaling molecules, promoting the enrichment of beneficial bacteria by regulating bacterial growth, chemotaxis, and competitive ability, thereby exerting positive chemotactic effects (de la Porte et al., 2020; Sharifi et al., 2022). Due to their physicochemical properties, VOCs are more likely to diffuse throughout the soil layer, attracting bacteria at distances ranging from a few millimeters to as far as 12 centimeters from the roots, thereby regulating the structure of a broad microbial community (Schulz-Bohm et al., 2018). Schulz-Bohm et al. (2018) utilized an olfactometer system to verify that VOCs released by the roots of *Carex arenaria* could induce long-distance migration of soil bacteria toward the roots, explaining the crucial role of plant VOCs in facilitating long-distance plant-microbe interactions. Therefore, VOCs play a key role in long-distance interactions in the soil rhizosphere.

4. Complex polymers such as polysaccharides, proteins, and enzymes, known as high molecular weight (HMW) compounds, are also present in root exudates, although they are not easily utilized by soil microorganisms (Goswami and Deka, 2022). However, mucilage (polysaccharides) can form protective barriers, promote soil particle adhesion, and assist in the colonization of rhizosphere microorganisms (Read and Gregory, 1997). Additionally, proteins in root exudates, particularly those with enzymatic activity, enhance plant defense mechanisms through their constitutive release. For example, peroxidases can break down pathogen cell walls, induce defense signaling, and trigger plant immune responses, effectively curbing pathogen invasion. These enzymatic proteins can also recognize and recruit beneficial microorganisms, promoting the formation of symbiotic relationships and further enhancing plant health and stress resistance (Wen et al., 2007; De-la-Peña et al., 2010).

Chemotactic signal reception and response by microorganisms

Upon perceiving chemotactic signals from plants, soil microorganisms move along a chemical gradient towards (attraction) or away from (repulsion) the signal source, engaging in chemotactic movement (Kearns, 2010). The chemotaxis system in bacteria is one of the most complex signal transduction systems in prokaryotes, with signaling and regulatory mechanisms relatively conserved across all bacteria. Specifically, bacterial chemoreceptor sensor proteins (MCPs) serve as receptors for chemotactic agents, sensing rhizosphere chemical effectors (Allard-Massicotte et al., 2016; Compton et al., 2018). Typically, MCPs are transmembrane proteins that form a ternary complex with histidine kinase CheA and coupling protein CheW (Sampedro et al., 2015). MCPs are located in the ligand-binding domain (LBD) of the cell membrane, which exhibits high structural variability to sense a wide range of chemical signals. For instance, the PGPR strain *Bacillus velezensis* SQR9 can sense various substances, including organic acids, sugars, amino acids, and sugar alcohols, primarily due to the pivotal role of its eight MCPs in mediating host interactions (Corral-Lugo et al., 2016; Feng et al., 2019; Tohidifar et al., 2020). When MCPs selectively recognize and bind specific root exudates, they trigger subsequent signal transduction and execution modules (Sampedro et al., 2015). Specifically, signal transduction regulates the autophosphorylation rate of histidine kinase CheA in a CheW-dependent manner, and phosphorylated CheA further influences the transphosphorylation of the CheY response regulator (McEvoy et al., 1999; Lacal et al., 2010). Ultimately, phosphorylated CheY interacts with motility proteins, mediating bacterial movement (Sampedro et al., 2015). The motility of rhizosphere bacteria manifests in various phenotypic forms, including swarming motility, swimming motility, gliding motility, and twitching motility (Kearns, 2010). These motility forms are driven primarily by flagellar rotation of individual bacterial cells, coordinated flagellar complex movement within the population, an extension of polar type IV pili, or passive surface spreading (Mattick, 2002; Mignot, 2007; Kearns, 2010). This sophisticated chemotaxis system enables bacteria to keenly sense the concentration gradient of extracellular signaling molecules, allowing them to adapt to changes in their surrounding environment. However, current research primarily focuses on bacterial responses to single chemotactic signals. In reality, both root exudates and bacterial MCPs exhibit high diversity, indicating that responses to composite signals may differ significantly from responses to single signals (Figure 2). Therefore, future research should focus more on bacterial responses to composite signals to gain a more comprehensive understanding of their behavior in complex environments.

Bacterial attachment to root surfaces

Microorganisms move along a positive chemotactic signal gradient towards the host roots, selecting regions of the root with high concentrations of exudates as their colonization sites. Interestingly, microbial chemotaxis and motility play crucial roles in the selection of colonization sites on the roots. For instance, organic acids are strong attractants for *Azospirillum brasilense*, and *A. brasilense* mutants lacking chemotaxis fail to preferentially colonize the surface of the root maturation and elongation zones, which produce organic acids (O'Neal and Alexandre, 2020). The study also found that *A. brasilense* avoids the root tip region, which may produce toxic reactive oxygen species (ROS). This is because the root tip, being an area of active growth, generates hydrogen peroxide, which has a repellent effect on bacteria. Similarly, microorganisms must continuously migrate to avoid the

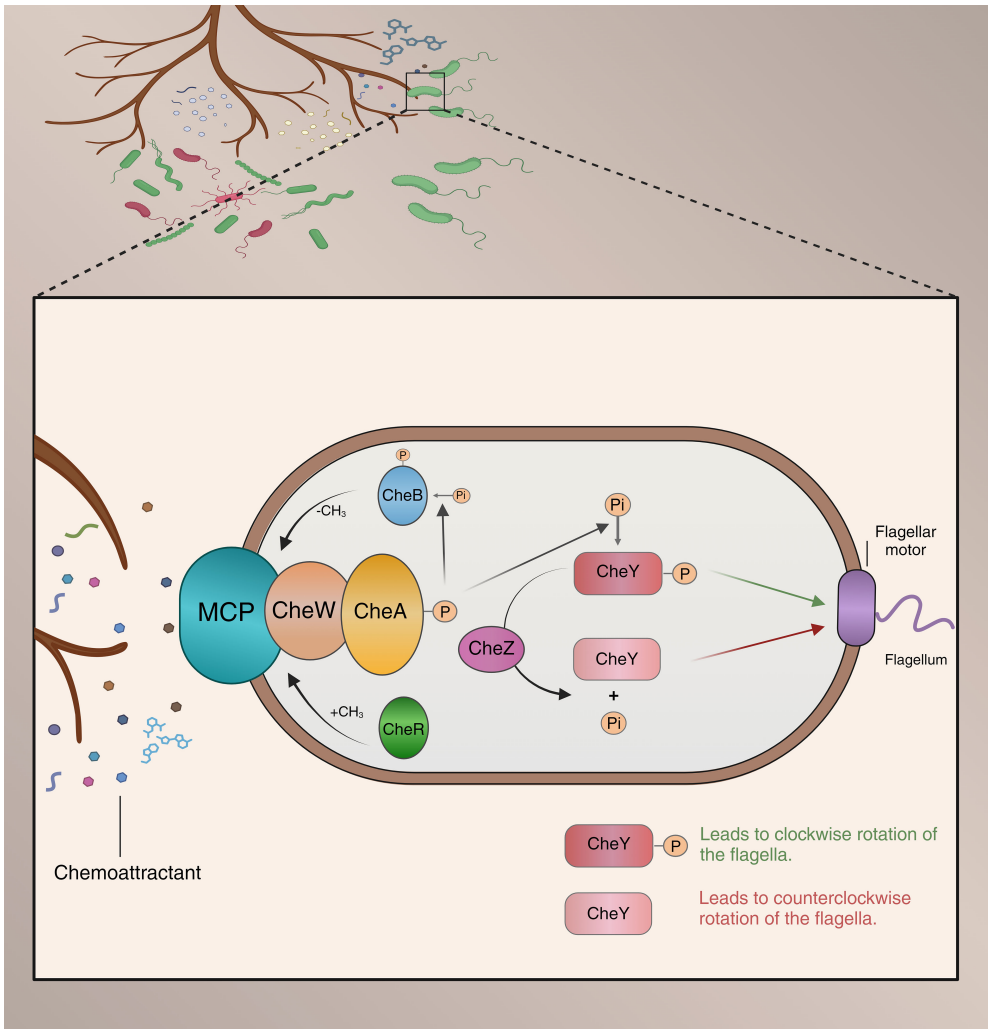

FIGURE 2
Model of microbial chemotaxis in the rhizosphere Chemical compounds secreted by plant roots attract beneficial bacteria while repelling harmful ones. These compounds are detected by transmembrane chemoreceptors on the bacteria, which regulate the autophosphorylation of CheA through the adaptor protein CheW. Once phosphorylated, CheA (CheA-P) phosphorylates the response regulator CheY. The phosphorylated CheY (CheY-P) binds to the flagellar motor, causing a switch in the direction of flagellar rotation from counterclockwise to clockwise, thereby altering the bacterial movement direction. The phosphatase CheZ dephosphorylates CheY-P, rapidly terminating the signal. CheB-P regulates methylation levels, working in conjunction with CheR to mediate the adaptive regulation of the chemoreceptors.

immunologically active sites that change as the root develops (Tsai et al., 2023). These findings suggest that the host plays a significant regulatory role in bacterial selection of attachment sites on the roots.

Subsequently, microorganisms cease movement and adhere to the root surface, a critical step in achieving root colonization. Most agricultural microorganisms follow a common biphasic model for root attachment, which includes an initial attachment phase and a secondary attachment phase. During the initial attachment phase, rhizosphere bacterial cells form weak and reversible bonds with the root surface; in the secondary attachment phase, the bacteria's attachment to the root surface becomes more secure, irreversible, and specific (Wheatley and Poole, 2018). Throughout the attachment process, microorganisms alter their physiological structures and secrete adhesive substances to successfully adhere. For example, bacteria may swing their flagella or pili to overcome electrostatic repulsion and secrete porins, outer membrane proteins, and lipopolysaccharides (LPS) as root surface adhesins (Berne et al., 2015; Knights et al., 2021). Additionally, bacteria secrete the signaling molecule di-AMP as an extracellular signal to regulate root attachment and biofilm formation, with *Bacillus subtilis* being a typical example (Townsley et al., 2018). This attachment is not merely a passive process; it involves active communication between the microbe and the plant. Plant surface molecules, such as lectins and arabinogalactan proteins, play a role in recognizing and binding microbial cells. This interaction is often species-specific, ensuring that only compatible microbes establish a symbiotic relationship (Imberty and Varrot, 2008). During this process, both the host and microorganisms change their lifestyles, achieving a balanced state through mutual regulation via molecular and physiological mechanisms.

After Pathogen-Associated Molecular Pattern (PAMP) recognition, PRRs located on the cell surface recruit the co-

receptor BRI1-Associated Kinase 1 (BAK1), forming a receptor complex that activates downstream Receptor-Like Cytoplasmic Kinase (RLCK)-VII family members, such as Botrytis-Induced Kinase 1 (BIK1). Subsequently, RLCKs phosphorylate downstream targets, including Respiratory Burst Oxidase Homolog D (RBOHD) and Mitogen-Activated Protein Kinase Kinase Kinases (MAPKKKs), triggering a series of defense responses, such as ROS burst, calcium influx, Mitogen-Activated Protein Kinase (MAPK) activation, transcriptional reprogramming, and the production of plant hormones.

The figure also highlights the role of Type III secreted bacterial effectors (T3Es) in modulating plant defense mechanisms. Since some effectors interfere with multiple plant targets, they are represented multiple times in the figure.

Bacterial immune recognition evasion

Once microorganisms attach to the root surface, overcoming the plant immune system becomes one of the crucial challenges for successful rhizosphere colonization. At this stage, microorganisms deploy a series of molecular strategies to adapt to and modify the plant environment, thereby alleviating the immune attacks they face during colonization (Jones and Dangl, 2006; Khavkin, 2021; Kong and Yang, 2023). The following chapter summarizes three strategies evolved by colonizing microorganisms (particularly pathogens and symbionts) in response to the evolutionary selection pressures exerted by plant pattern recognition receptors (PRRs) that activate host immunity (Figure 3).

Microorganisms possess widely conserved molecular patterns (MAMPs), such as flg22, elongation factor Tu (EF-Tu), cold shock proteins (CSP), lipopolysaccharides (LPS), chitin, phospholipids, and Nep1-like proteins, which can be recognized by different pattern recognition receptors (PRRs) in plants. The activation of immune signaling events, known as MAMP-triggered immunity (MTI), plays a crucial role in eliminating potential pathogenic infections and forms the first barrier to microbial colonization (Zipfel and Oldroyd, 2017). Microbial pathogens have evolved complex strategies to evade plant immunity, allowing them to effectively colonize roots. A key strategy involves avoiding detection by PRRs, which primarily includes altering the structure of MAMPs, degrading MAMP precursors, inhibiting their biosynthesis, or preventing MAMP release. For example, *Pseudomonas syringae* secretes the protease ArpA to degrade flagellin monomers, preventing the release of the immunogenic epitope flg22 and thus evading immune detection during root colonization (Pel et al., 2014).

Beneficial microorganisms also employ similar strategies to evade recognition by the plant immune system. However, unlike pathogenic microorganisms, which rapidly adjust the properties of

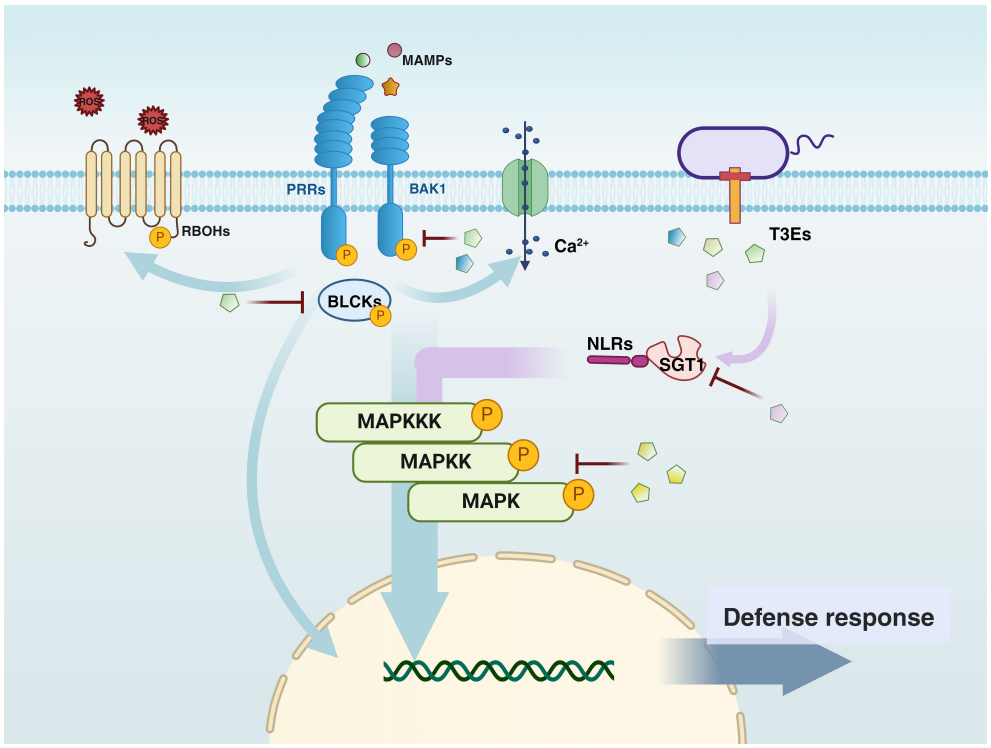

FIGURE 3
The figure illustrates the key components of PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) signaling pathways in plant defense against bacterial pathogens, as well as the interconnections between these pathways. Arrows indicate the flow of defense signals, with light blue representing Pattern Recognition Receptor (PRR)-dependent signals and purple representing Nucleotide-binding Leucine-rich Repeat (NLR)-dependent signals. The convergence of these two signaling pathways is depicted by arrows that blend purple and light blue. PRR and NLR signals jointly regulate the plant immune response and interact at multiple critical points.

MAMPs, the immune evasion of beneficial microorganisms is more often achieved through the positive selection of PRRs over long-term evolution. For instance, variations in the flagellin protein sequence of the nitrogen-fixing symbiont *Sinorhizobium meliloti* result in its inability to activate immune responses in Arabidopsis (Felix et al., 1999). Similarly, the FLS2 receptor in grapevines has a weaker recognition of the flg22 epitope derived from beneficial *Bacillus subtilis* compared to that from pathogenic *Pseudomonas syringae* and *Xanthomonas campestris*, leading to a significantly reduced immune response (Trdá et al., 2014). Beneficial symbiotic microorganisms can also avoid detection by creating environments unfavorable to immune recognition. For example, *Pseudomonas capeferrum* WCS358 lowers environmental pH by secreting organic acids, thereby inhibiting plant recognition of flg22 (Yu et al., 2019). However, current research on immune evasion primarily focuses on flg22, and future studies should extend to other MAMPs to uncover the mechanisms by which rhizosphere microorganisms evade immune recognition during colonization. In addition to MAMPs, mutualistic symbiotic microorganisms also secrete symbiosis-related molecules, which are recognized by host symbiotic receptors and initiate signal transduction (Zipfel and Oldroyd, 2017). For example, Nod factors from rhizobia are lipochitooligosaccharides similar in structure to MAMPs like chitin and peptidoglycan, and they initiate the symbiotic process of rhizobia (Oldroyd, 2013). Interestingly, many symbiotic molecules from beneficial microorganisms seem to suppress the local immune responses triggered by MAMPs in roots (Gourion et al., 2015). For instance, Nod factors from rhizobia significantly suppress MAMP-induced immune responses in both leguminous and non-leguminous plants like Arabidopsis, leading to a significant reduction in the levels of homologous PRR proteins on the cell membrane (Liang et al., 2013; Gourion et al., 2015).

Although the mechanisms by which plant immunity regulates pathogenic and beneficial microorganisms have been elucidated (Yang et al., 2022), microorganisms in the rhizosphere often exist in community forms, which means plants need to sense the presence of entire communities. The FLS2 receptor in plants plays a crucial role in this process, acting as a community sensor to detect the relative proportions of danger signals (canonical peptides) from pathogens and signals (evading and modulating peptides) from safe microorganisms. This sensing mechanism influences the assembly of specific pathogenic and symbiotic microbial communities, enabling the plant to distinguish between friends and foes (Colaianni et al., 2021). This discovery challenges the traditional view that the flg22-FLS2 complex only drives immune signal transduction, showcasing the complexity and subtlety of plant regulation in immunity and symbiosis.

Bacterial effectors interfere with immune signaling

Bacteria can secrete effectors, primarily composed of effector proteins, into the apoplast or deliver them into plant cells through specialized secretion systems (Jones and Dangl, 2006). These effectors bypass plant MTI by targeting immune signal transduction components. Research into the mechanisms of pathogen effectors provides important insights into how bacterial effectors suppress plant immune responses by interfering with key immune signaling molecules. Specifically, BRI1-Associated Kinase 1 (BAK1) and Bacterial-Like Cytoplasmic Kinases (BLCKs) are major targets for various pathogen effectors (Wang et al., 2022). BAK1 acts as a co-receptor for multiple pattern recognition receptors (PRRs) and plays a critical role in immune signal transduction induced by various MAMPs (Yasuda et al., 2017). For example, the pathogen *Phytophthora sojae* effector PsAvh110 specifically binds to the soybean heterochromatin protein GmLHP1-2, disrupting its assembly with the transcriptional complex GmPHD6, thereby inhibiting the expression of a set of immunity-related genes, including the BRI1-associated receptor kinase GmBAK1-3 (Qiu et al., 2023). Botrytis-induced Kinase 1 (BIK1), a member of the BLCK-VII subfamily, is a core regulator of plant immunity, located downstream of PRR and BAK1 signaling pathways, and is responsible for transmitting signals to downstream MAPK cascades, initiating a series of cellular defense responses, and coordinating the plant's defensive actions (Ma et al., 2020). The type III secretion effector RipAC from the bacterium *Ralstonia solanacearum* targets the plant E3 ubiquitin ligase PUB4, suppressing pattern-triggered immunity (PTI) and leading to the degradation of the critical immune kinase BIK1 (Yu et al., 2022). Additionally, within plant cells, Nucleotide-binding Leucine-rich Repeat-like receptors (NLRs) recognize effectors either directly or indirectly, activating effector-triggered immunity (ETI), which triggers a more robust immune response (Gourion et al., 2015). Pathogen effectors also disrupt multiple stages of ETI signal transduction. As mentioned earlier, the *Ralstonia solanacearum* effector RipAC not only drives BIK1 degradation but also interferes with the phosphorylation of SGT1 (a regulator of NLR accumulation or activation) and its interaction with MAPKs (Yu et al., 2020). The multifaceted mechanisms by which RipAC regulates plant immune responses suggest that the targets of effectors are often not singular, reflecting the complex regulatory methods and further emphasizing the multi-layered strategies pathogens employ to evade plant immunity. It is noteworthy that pathogens can evade NLR receptor recognition by regulating effector gene expression, such as altering promoter regions or applying epigenetic modifications. However, as this involves more epigenetic aspects, it will not be detailed in this article, but relevant content can be found in other literature (Wang et al., 2022).

Activation of the MAPK cascade is an early signal transduction event shared by both PTI and ETI and plays a key role in regulating immune outputs such as callose deposition, hormone production, and transcriptional reprogramming (Tzipilevich et al., 2021). This critical node is also often targeted by pathogens to weaken host immunity. For instance, the *P. syringae* T3Es effectors HopA1 and HopF2 inactivate MAPKs and their upstream kinases through different mechanisms. HopA1 acts as a phosphothreonine lyase, physically inactivating MPK3 and/or MPK6, while HopF2 inactivates MAPK kinase 5 (MKK5) through ADP-ribosylation (Zhang et al., 2007; Wang et al., 2010). Additionally, the *P. syringae* effector AvrB interferes with plant hormone signaling by activating MAPK MPK4, thereby negatively regulating pathogen

defense (Cui et al., 2010). These findings demonstrate that the regulation by effector proteins is often not singular; they frequently activate different mechanisms to enhance their effects. The *Phytophthora infestans* RXLR effector PITG20303 further emphasizes this conclusion, targeting the stabilization of the potato MAPK cascade protein StMKK1, suppressing PTI, promoting pathogen colonization, and evading recognition by the resistance protein Rpi-blb2, thereby negatively regulating the plant's PTI response (Du et al., 2021b).

Similar to plant pathogens, symbiotic rhizobia in legumes use a type III protein secretion system (T3SS) to deliver toxin-like type III effectors (T3Es) into plant cells, utilizing multiple effectors to suppress plant immune activation. These T3Es not only inhibit the immune response in legumes but also stimulate the production of nodulation signals, which are crucial for establishing symbiosis (Ferguson et al., 2010; Oldroyd, 2013). The NopL effector secreted by *Sinorhizobium* sp. strain NGR234 is a typical example; its serine-proline motif is essential for its effector activity during symbiosis, and over-phosphorylated NopL mutants show significantly reduced activity during symbiosis (Ge et al., 2016). The mechanism by which NopL suppresses immune responses by interfering with the plant MAPK cascade is gradually being uncovered. For example, NopL forms a complex with the tobacco MAP kinase SIPK and is phosphorylated by SIPK *in vitro*, thereby suppressing MAP kinase signal-mediated defense responses. Additionally, NopL mimics MAPK substrates and interferes with their signal transduction, inhibiting host cell death and premature nodule senescence (Zhang et al., 2011). Thus, it can be inferred that NopL suppresses plant defense responses by interfering with the MAPK signaling process or its downstream events. Recent studies have shown that, unlike the strategy of pathogen and rhizobial symbionts that rely on T3SS to inject highly specific effector proteins, beneficial rhizosphere symbionts primarily interfere with MTI signal transduction through non-specific extracellular mechanisms. The synthetic communities (SynComs) constructed from diverse MTI-suppressing strains in Arabidopsis roots primarily inhibit MTI by modulating specific and conserved parts of the host immune system. Gene screening further revealed that although *Dyella japonica* MF79 carries T3SS genes, its potent MTI-suppressing function mainly relies on the type II secretion system (T2SS), while T3SS is not essential for its inhibitory activity (Ökmen et al., 2013).

Although the mechanisms of action for individual bacterial effectors have been extensively studied, the synergistic effects of multiple effectors within the same pathogen and the temporal and spatial regulation by different pathogens in secreting effectors at various infection stages to evade host defense systems remain unclear. These complex molecular dialogues and regulatory networks require further research to elucidate their synergistic mechanisms and interactions in plant immunity.

Microbial tolerance to immune responses

Activated immune signaling cascades ultimately trigger a series of powerful immune responses, including the secretion of proteases and inhibitors, the release of antimicrobial molecules, and bursts of ROS. Plants with activated defense responses synthesize and secrete various defense molecules and proteases, generating resistance to pathogenic microorganisms through different mechanisms, thereby altering the rhizosphere environment (Jones and Dangl, 2006; Wang et al., 2022). Meanwhile, symbiotic microorganisms cleverly evade these immune responses and even modify the environment to successfully promote their survival and proliferation. To successfully infect host plants and overcome their defense mechanisms, rhizosphere microorganisms have developed various strategies, which can be categorized into active neutralization and passive adaptation.

Active Neutralization Strategies include directly intervening in and regulating host plant immune responses by inhibiting the production of plant defense substances and secreting specific inhibitors. For example, the effectors AVRblb2 from *Phytophthora infestans* and Avh240 from *Phytophthora sojae* target plant PLCP C14 and aspartic protease AP1, preventing the secretion and activation of these defense proteins (Bozkurt et al., 2011; Guo et al., 2019). Similarly, the Kazal-like protease inhibitor EPI1 secreted by *P. infestans* can inhibit the plant resistance protease P69B, thereby hindering the maturation of immunogenic peptides (Paulus et al., 2020; Wang et al., 2021). In addition, pathogenic microorganisms suppress ROS production or interfere with their transport in host plants as a key strategy to actively neutralize host defense mechanisms. Host plants primarily produce ROS through peroxidases and membrane-bound NADPH oxidases. To suppress ROS, pathogenic microorganisms secrete various effectors. For example, *Ustilago maydis* effector Pep1 directly inhibits maize peroxidase POX12 (Hemetsberger et al., 2012), while *Phytophthora parasitica* effector PpE18 weakens the immunity of *Nicotiana benthamiana* by inhibiting the ROS-scavenging function of NbAPX3-1 and interfering with its interaction with NbANKr2 (Cao et al., 2024). Similarly, the *Phytophthora sojae* effectors Avr3b and CRN78 reduce ROS accumulation and transport by disrupting NADH availability and phosphorylating aquaporin PIP2;2 (Ai et al., 2021).

Passive Adaptation Strategies involve setting up biological barriers and altering physiological structures to adapt to plant defenses. Pathogenic microorganisms evade host immune responses through post-translational modifications of effector molecules. For instance, the virulence factor XEG1 produced by *Phytophthora sojae* undergoes glycosylation modifications to evade degradation by the host protease AP5 (Xia et al., 2020). Furthermore, pathogenic microorganisms can detoxify antimicrobial compounds synthesized by plants, such as the conversion of toxic α-tomatine into less toxic derivatives by the enzyme secreted by *Cladosporium fulvum* (Ökmen et al., 2013). Microbial pathogens have also developed various strategies to evade oxidative stress during infection. A typical example is that bacterial pathogens can produce extracellular polysaccharides to form protective layers, shielding themselves from oxidative stress induced by the host (Fones and Preston, 2012).

Similar to pathogenic microorganisms, beneficial microorganisms employ both active and passive adaptation strategies to cope with ROS bursts in plants, allowing them to successfully colonize the rhizosphere. Enhancing tolerance is a key strategy for responding to activated root immune responses, such as ROS bursts. For example, the colonization of beneficial bacterium *Bacillus velezensis* triggers plant immune

responses and ROS production, which in turn stimulate bacterial auxin production, reducing ROS toxicity and playing a crucial role in root colonization (Tzipilevich et al., 2021). *Bacillus subtilis* SQR9 induces oxidative bursts in cucumber and Arabidopsis through its flg22 homologs, demonstrating high H_2O_2 tolerance and the ability to suppress oxidative bursts, with the ResD-ResE signal transduction system in SQR9 playing a key role in tolerating plant oxidative stress and root colonization (Zhang et al., 2021). Additionally, recently revealed spatial adaptation mechanisms indicate that plant cortical, endodermal, and root hair cells exhibit different defense capabilities, and beneficial microorganism *Pseudomonas simiae* WCS417 selects colonization sites with lower stress, demonstrating a spatially host-immune-driven adaptation strategy (Verbon et al., 2023).

Active Regulation: For example, the NopM effector from *rhizobium* NGR234 significantly suppresses flg22-induced ROS bursts when expressed in *Nicotiana benthamiana*, blocking ROS-related defense responses (Xin et al., 2012). This demonstrates that beneficial microorganisms can suppress transient ROS bursts by secreting effectors, cleverly interfering with plant immune responses, and showcasing their strategies and potential in actively regulating plant immunity.

Through the detailed analysis of the strategies employed by beneficial and pathogenic microorganisms in coping with plant immune systems, we can see that both types of microorganisms use many similar strategies in immune recognition evasion, immune signal interference, and immune response suppression. However, their ultimate goals are entirely different: pathogenic microorganisms aim to overcome plant immune systems to cause infection, while beneficial microorganisms regulate plant immune systems to establish symbiotic relationships.

Biofilm formation and stable colonization

Biofilm formation is a key strategy employed by microbes to ensure stable colonization on the root surface. Biofilms are complex communities of microorganisms embedded in a self-produced extracellular matrix, which provides protection against environmental stresses and host immune responses (Flemming et al., 2023). This matrix mainly consists of polysaccharides, proteins, amyloids, lipids, extracellular DNA (eDNA), as well as membrane vesicles and humic-like microbially derived refractory substances (Flemming et al., 2023). The properties of these polymers confer resistance or antimicrobial tolerance to biofilms under certain adverse conditions. Beneficial rhizosphere bacteria first halt their motility and then form biofilms, a process generally controlled by one or more of their own transcriptional regulators (Arnaouteli et al., 2021; Nie et al., 2022; Ivanova et al., 2023). However, increasing evidence suggests that this process is also co-regulated by global transcriptional regulators mediated by environmental factors such as root exudates. For example, *Bacillus subtilis* colonizes Arabidopsis roots by forming biofilms dependent on extracellular matrix genes, a process triggered by plant polysaccharides and transmitted through the regulation of the phosphorylation state of the master regulator Spo0A (Beauregard et al., 2013). Interestingly, these polysaccharides not only serve as signals but also as sugar sources for synthesizing the extracellular polysaccharides (ESP) in the matrix, demonstrating the critical role of external regulation in bacterial root colonization (Beauregard et al., 2013). Similarly, phenolic compounds in root exudates attract and promote endophyte biofilm formation on the host root surface, facilitating colonization. After colonization, endophytes enhance the host's intrinsic defense mechanisms by increasing the levels of phenolic antimicrobial substances in the rhizosphere exudates, significantly boosting the plant's resistance to pathogen attacks (Ray et al., 2018). Root exudates also influence biofilm formation in pathogenic bacteria. *Pseudomonas aeruginosa* strains PAO1 and PA14 can infect the roots of Arabidopsis and sweet basil, forming biofilms and causing plant death. Inducing the secretion of rosmarinic acid (RA) from the host roots before infection or the exogenous addition of RA effectively counters *Pseudomonas aeruginosa* (Walker et al., 2004). Notably, although chemotaxis and biofilm formation are two distinct processes in rhizosphere colonization, the same molecule secreted by the host can regulate chemotaxis or biofilm formation in rhizosphere microorganisms depending on its concentration (Zhang et al., 2014; López-Farfán et al., 2019). This dose-dependent signaling is very common in the regulation of biofilm formation and chemotaxis in rhizosphere bacteria, highlighting the critical role of concentration gradients of environmental signals in microbial behavior.

Summary and future perspectives

Previous research on microbial colonization processes has predominantly focused on isolating beneficial strains from rhizosphere bacteria. However, future studies should prioritize the regulation of these colonization processes to effectively mitigate pathogen invasion and enhance the efficiency of beneficial microbes in field applications. Potential regulatory strategies may involve manipulating the composition of root exudates, modulating microbial signaling pathways, or employing gene-editing technologies to optimize the colonization potential of beneficial microorganisms. By elucidating the interaction mechanisms between various microbial functional groups in the rhizosphere, we can enhance microbial symbiosis and competitive dynamics under conditions that closely mimic natural environments, thereby promoting plant health and advancing agricultural sustainability.

Future research should focus on investigating microbial colonization mechanisms within complex ecological contexts, examining the roles of soil physicochemical properties, microbial community structure dynamics, and interspecies interactions in shaping plant health. A multi-layered, systems-based approach that integrates the soil microenvironment and the ecological dynamics of microbial communities will provide a more precise theoretical foundation for agricultural management practices. This, in turn,

will enhance crop resilience, optimize yield, and contribute robustly to sustainable agricultural development.

Author contributions

LY: Formal analysis, Investigation, Visualization, Writing – original draft. XQ: Investigation, Conceptualization, Writing – review & editing. ZZ: Investigation, Writing – original draft. YW: Investigation, Writing – original draft. GD: Methodology, Supervision, Writing – review & editing. XX: Methodology, Supervision, Writing – review & editing, Funding acquisition.

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Conflict of interest

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Halotolerant bacterial endophyte *Bacillus velezensis* CBE mediates abiotic stress tolerance with minimal transcriptional modifications in *Brachypodium distachyon*

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Nitrogen and water are the primary resources limiting agricultural production worldwide. We have demonstrated the ability of a novel halotolerant bacterial endophyte, *Bacillus velezensis* CBE, to induce osmotic stress tolerance in *Brachypodium distachyon* under nitrogen-deprived conditions. Additionally, we aimed to identify the molecular factors in plants that contribute to the beneficial effects induced by *B. velezensis* CBE in *B. distachyon*. To achieve this, we conducted transcriptomic profiling using RNA-seq on 18-day-old *B. distachyon* seedlings treated with *B. velezensis* CBE in the presence or absence of available nitrogen, with and without osmotic stress. These profiles were then compared to those obtained from *B. distachyon* treated with known plant growth-promoting bacterial strains, *Azospirillum brasilense* Cd and *Azoarcus olearius* DQS4, under the same growth conditions. We identified differentially expressed genes (DEGs) in response to the combinations of bacterial strains and stress treatments. Interestingly, only 73 transcripts showed significant differential expression in *B. velezensis* CBE-treated plants under stress conditions, compared to 1,078 DEGs in plants treated with *A. brasilense* Cd and 2,015 DEGs in *A. olearius* DQS4. Our findings suggest that the novel endophyte *B. velezensis* CBE mediates osmotic stress tolerance in *B. distachyon* through the fine-tuning of molecular mechanisms with minimal transcriptional modifications.

KEYWORDS

abiotic stress, beneficial microbes, endophytes, transcription, tolerance

1 Introduction

Stresses such as drought, salinity, and nutrient limitations are the main limiting factors for plant growth and productivity (Fahad et al., 2017). The situation has been exacerbated by the drastic and rapid changes in global climate. For instance, drought due to water scarcity is one of the most critical issues potentially compromising global food security (Raza et al., 2019). Environmental stress factors are known to provoke complex transcriptional changes in plants, and comprehensive transcriptome profiling has led to the identification of two major categories of genes (Debnath et al., 2011). The first group includes genes that encode proteins involved in cellular homeostasis and protection from stress, such as osmolytes, chaperones, antioxidative enzymes, metabolic enzymes, and lipid-transfer proteins. The second group mainly includes kinases and transcription factors that regulate the stress signal transduction and stress-responsive gene transcription (Yoshida et al., 2014; Sewelam et al., 2016; Haak et al., 2017; Tiwari et al., 2017).

Stress tolerance in plants can be enhanced through the treatment of plants or seeds with certain natural and synthetic compounds or microorganisms (Rai et al., 2021). Plant-beneficial bacteria, commonly referred to as plant growth-promoting bacteria (PGPB), are known to be associated with several plant species (Glick, 2012). They may have different mechanisms to support plant growth and health, including the ability to mediate abiotic stress tolerance (Liu et al., 2020; Yang et al., 2009). Thus, certain PGPB strains are extensively investigated for their potential to control plant stress (Vurukonda et al., 2016; Naing et al., 2021). For instance, it was reported that *A. brasilense* can mediate abiotic stress tolerance in several plant species including salt stress in barley (Omar et al., 2009) and drought stress in wheat (Kasim et al., 2013) and maize (Curá et al., 2017). Furthermore, Naveed et al. (2014) reported the ability of *Paraburkholderia phytofirmans* PsJN to mediate drought stress tolerance in maize. *P. phytofirmans* PsJN was also found to induce salt stress tolerance in *Arabidopsis thaliana* (Pinedo et al., 2015). PGPB utilize several mechanisms to induce abiotic stress tolerance in plants (Dimkpa et al., 2009; Yang et al., 2009). PGPB can enhance plant growth directly by providing plants with nutrients such as nitrogen via nitrogen fixation or by supplying phosphorus from soil-bound phosphate (Di Benedetto et al., 2017). PGPB are known for their ability to synthesize several plant growth hormones such as auxin and cytokinins and further modulate the plant stress hormone ethylene via 1-aminocyclopropane-1-carboxylate (ACC) deaminase production (Glick, 2014).

Plant responses to PGPB are not well characterized; however, they are thought to be dependent on numerous factors, including plant genotype and the associated bacterial strain (Vandana et al., 2020). Reports suggest that the plant PGBP crosstalk is complex and can manifest in significant metabolic and physiological responses (Abd El-Daim et al., 2019). The metabolic and physiological changes caused by PGPB are expected to be driven by complex molecular and signaling tuning through plant transcriptional regulation (Abd El-Daim et al., 2018; Backer et al., 2018; Abd El-Daim et al., 2019). PGPB appear to program relatively similar plant molecular factors to mediate abiotic stress tolerance in plants, e.g., classical defense response pathway genes such as the well-characterized plant transcription factor WRKY, ABA-responsive MYB, and ethylene-responsive factors ERFs are known to be modulated by PGPB treatment (Bruto et al., 2014; Abd El-Daim et al., 2018). However, due to the vast diversity of PGPB, it is also possible that certain PGPB strains will have different impacts on such broad transcriptional pathways. For instance, *Enterobacter* sp. strain C7 alleviated drought stress by reducing the expression of ethylene-responsive genes in tomatoes (Ibort et al., 2018). In contrast, *B. megaterium* inoculation mediated drought stress tolerance by inducing the expression of ethylene response genes in tomatoes (Ibort et al., 2018). Furthermore, certain PGPB strains may induce abiotic stress tolerance by molecular tuning of more specific genes. The PGPB *Dietzia natronolimnaea* strain STR1 confers salt tolerance to wheat via increased expression of the *SOS4* pyridoxal kinase, which controls N^+ and K^+ balance through adjusting ion transporter activity (Rosier et al., 2018). Moreover, Pinedo et al. (2015) reported that the ability of *P. phytofirmans* PsJN to mediate salt stress tolerance in *A. thaliana* is related to the accumulation of proline and transcription of *RD29A* and *RD29B* genes.

B. velezensis CBE is a novel endophyte isolated from the stem of the *Calluna vulgaris* plant growing on the coastal path near Aberystwyth, Wales, where it experienced regular salt spray. The strain is highly tolerant toward salt stress and further showed the potential of improving *B. distachyon* vegetative growth under salt stress conditions (Supplementary Figure S1). The present study aimed to elucidate the plant molecular factors involved in *B. velezensis* CBE's ability to mediate abiotic stress tolerance in *B. distachyon*. Differentially expressed genes were identified by whole-transcriptome shotgun sequencing (RNA-Seq) analysis on total RNA extracted from *B. distachyon* seedlings treated with novel hallo-tolerant endophyte *B. velezensis* CBE and grown under osmotic stress and nitrogen-free conditions. Furthermore, we aimed to test whether the plant molecular modulations induced by *B. velezensis* CBE are common transcriptional regulations involved in PGPB-mediated abiotic stress tolerance in Brachypodium. To achieve this, we compared the transcriptome profile of *B. distachyon* treated with *B. velezensis* CBE with the profiles obtained for *B. distachyon* treated with known PGPB strains *A. brasilense* Cd and *A. olearius* DQS4.

2 Materials and methods

2.1 *Bacillus velezensis* CBE isolation and characterization

The strain, along with others, was isolated from a surface-sterilized stem of *Calluna vulgaris*, collected along the coastal path between Clarach and Aberystwyth (52°25′53.9″ 4°04′42.2″W) (Supplementary Figure S1A). The sterilized stem was divided into three parts: one left whole, and two ground in 1 ml of sterile water, with one of the ground samples further diluted 1^{10} in sterile water. These three sample subsets (whole, ground, and diluted ground tissue) were used to inoculate nutrient agar plates supplemented

with either rock salt or sea salt (SAXA Rock Salt and Cornish Sea Salt) at final concentrations of 3.5% w/v and 1% w/v, respectively, to selectively isolate halotolerant endophytes. The plates were sealed with parafilm and incubated for 4 weeks at ambient lab temperature, allowing slower-growing, less-characterized endophytes to emerge under natural day/night cycles. Colonies of interest were picked from the agar plates and streaked onto fresh nutrient agar plates for further identification and characterization.

The bacterial isolate was identified by amplifying and sequencing the 16S rRNA gene using the primers 8F (AGAGTTTGATCCTGGCTCAG) and 1378R (CGGTGTGTA CAAGGCCCGGGAACG). The amplified PCR product was sequenced via Sanger sequencing using an ABI 3730 DNA analyzer at Aberystwyth University. The resulting sequence was analyzed in CHROMAS, and a BLAST search was performed using the NIH Bacterial 16S reference library. The strain used in this study was identified as *Bacillus velezensis*. The sequence has been deposited in GenBank under accession number PQ498196.

The strain's salt tolerance was characterized on nutrient agar plates supplemented with 1 to 2 M NaCl (methodology used is described as Supplementary Information). CBE demonstrated the ability to survive at the highest tested salt concentration for up to 186 h (Supplementary Table S1). The ability of *B. velezensis* CBE was found to improve the vegetative biomass of salt-stressed *B. distachyon* (Supplementary Figure S1B).

2.2 Plant growth, bacterial and abiotic stress treatment

Brachypodium distachyon Bd21 seeds were sourced from the National Plant Phenomics Centre (NPPC), Aberystwyth, UK. Seeds were dehusked in order to increase germination efficiency and uniformity. Dehusked seeds were submerged in 70% ethanol for 5 min, rinsed in sterile dH_2O, and submerged in 4% bleach solution for 5 min before being rinsed again in sterile dH_2O. The final wash was plated on nutrient agar plates to determine the sterility of the seed surfaces. Surface sterilized seeds were transferred to Petri dishes containing presterilized filter paper moistened with 5 ml of sterile dH_2O. The germination plates were sealed with micropore tape and placed into darkness for one day at room temperature, followed by 3 days on the windowsill in full day/night light cycle conditions until the seed coating had split and the root had begun to emerge.

A. brasilense Cd and *A. olearius* DQS4 were obtained from Dr. Euan James at the James Hutton Institute, Invergowrie, Dundee, UK. *A. brasilense* Cd, a rhizosphere strain originally isolated from mangrove roots by Holguin et al. (1992), is known for its ability to fix nitrogen and mediate salt stress tolerance in plants (Bacilio et al., 2004). *A. olearius* DQS4, a nitrogen-fixing bacterium isolated by Chen et al. (2013), is genetically and phenotypically similar to the model grass endophyte *A. olearius* BH72 (Faoro et al., 2017; Raittz et al., 2021). It is recognized for its ability to endophytically colonize and promote plant growth across various species (Faoro et al., 2017).

B. velezensis CBE, *A. brasilense* Cd, and *A. olearius* DQS4 were cultured separately on full-strength tryptic soya broth (TSB) media (Sigma Aldrich, Germany) overnight at 28°C with 180 RPM shaking. Four days old *B. distachyon* Bd21 seedlings were bacterially treated by soaking in TSB based bacterial solution consisting of 10^7 ml^{-1} of the desired bacterial strain for 20 min at room temperature. Seedlings reserved for both negative and positive controls were treated by sterile TSB media.

Four bacterial treated or untreated seedlings were transferred to sterile plastic containers filled with 50 ml of pure semisolid autoclaved water agar or supplemented with either 3 mM ammonium nitrate, 5% polyethylene glycol (PEG 8000), or both. The containers were transferred to a plant growth cabinet and maintained in a controlled environment at 22/16°C (day/night), with a 16/8-h photoperiod, light intensity of 450 µmol m^{-2} s^{-1}, and 80% humidity. The sealed containers supported plant growth for up to 14 days, after which the seedlings began to deteriorate due to nutrient depletion and the limitations of the closed system. Consequently, 18 days old seedlings (4 days old when transferred to the containers + 14 days inside the containers), were harvested, phenotyped, and imaged.

To assess bacterial colonization, samples were taken from both the water agar and roots and inoculated on TSA plates for bacterial counting using CFU methods. Seedlings were air-dried at 60°C and monitored regularly until a constant weight was achieved to determine the dry weight. The dry weight measurements represent the average dry weight of six biological replicates, with each replicate including the entire seedling (both shoots and roots). The dry weight is expressed as mg per seedling. Samples reserved for transcriptomic analysis were immediately frozen in liquid nitrogen and stored at −80°C until further processing.

2.3 RNA extraction and quantification

Six seedlings per replicate for each treatment were flash-frozen in liquid nitrogen, and ground into powder, and RNA was extracted using Trizol (Thermo Fisher Scientific, Hemel Hempstead, UK) following the manufacturer's guidelines. RNA was purified using the QIAGEN RNeasy Mini Elute Kit, and quality was assessed by agarose gel. RNA quantification was determined by analyzing 2 µl on a Qubit fluorometer (Thermo Fisher Scientific, Hemel Hempstead, UK). Samples were diluted with DEPC water and sent to the sequencing service provider (Earlham Institute, Norwich, UK) where Illumina RNA-seq libraries were prepared and sequenced using the HiSeq 2500 platform.

2.4 RNAseq processing, quality control, and mapping

Prior to mapping, raw reads were processed using Trimmomatic v.0.33 to remove adapters with the following parameters (optimized after several run tests): ILLUMINACLIP: TruSeq3-PE-2.fa LEADING:15 SLIDINGWINDOW:4:15 MINLEN:30 HEADCROP:12 (Bolger et al., 2014). The quality of the resulting trimmed and cleaned reads was assessed using FastQC v.0.11 (Andrews, 2014). Reads were then mapped to assembly

version 3.1 of the *Brachypodium distachyon* genome, downloaded from Phytozome (https://phytozome.jgi.doe.gov). Briefly, reads were mapped to the reference genome using the splice-aware mapper Hisat2 v.2.0.0 (Kim et al., 2015).

2.5 Preprocessing and quantification of transcripts

Prior to calling DEGs, preprocessing filtering was performed to remove potential artifacts and assess the quality of the replicates. Count matrices were derived from the bam files above using the Genomic Features and Genomic Alignments R libraries. Transcripts with a count lower than 10 in any samples were discarded. We applied the regularized logarithm transformation (rlog) as implemented in the DESeq2 package to decrease the variance among gene expression values, as proposed by Love et al. (2014). We then calculated a distance matrix between samples and performed a principle component analysis (PCA) to quantify experimental covariates and batch effects among samples and replicates (Van Belle et al., 2004).

2.6 Estimating the completeness of transcriptomes

The transcriptome in each sample was assessed for its completeness as a measure of the quality of the sequencing. Clean reads were mapped to the reference genome (above) and were assembled and merged using StringTie v1.1.0 using default parameters (Pertea et al., 2015). The completeness of each transcriptome was assessed using BUSCO on the early-release plantdb set, composed of 1,440 core genes (Simao et al., 2015).

2.7 Identification of DEGs using Salmon and DESeq2

Quantification of transcripts was done using Salmon using precomputed mapping files (bam files) computed as described above using the *–ValidateMappings –gcBias* and *–numBootstraps* set to 1,000 to improve the quantification (Patro et al., 2017). Derived counts were used as inputs to call DEG using DESeq2 comparing the different treatments to control treatment (sterile water agar). The overlap of DEGs between different treatments was computed using the UpSetR library(doi: https://doi.org/10.1093/bioinformatics/btx364).

2.8 Functional annotation of DEGs and GO term enrichment

The reference genome was functionally re-annotated using Blast2GO 5.25 (Pro) as a prior step before computing GO term enrichments (Camacho et al., 2009). The functional annotation was done as follows: BLAST searches were performed on the nr database (release March 2019) using the BLASTx command from ncbi-blast-2.2.28+ release at an *e*-value cut-off of 0.000001 and selecting the top 20 hits (Jones et al., 2014). InterPro searches were performed using InterProScan v.5.18-57 on TIGRFAM, PFAM, SMART, PANTHER, Gene3d, and PIRSF databases (Wu et al., 2004; Letunic et al., 2006; Lees et al., 2010; Haft et al., 2013; Mi et al., 2013; Finn et al., 2016). Finally, enriched GO terms in all three categories: molecular function (MF), biological process (BP), and cellular component (CC) among identified DEGs were identified using the Fisher's exact test as implemented in Blast2GO 5.25 (Pro) at a *p*-value cut-off of 0.01 (Conesa and Gotz, 2008).

2.9 Statistical analysis and visualization

Data based on replicates (at least three biological replicates) were subjected to different statistical analysis methods using several packages. Analysis of variance (ANOVA) test to determine the significance between the different treatments was carried out using Costas (CoHort software, Pacific Grove, CA, USA). The heat map was generated based on using Pearson and Ward for distance measure and clustering algorithm using the XLSTAT package. Analysis using the MapMan package was used to visualize differences in biological functions among different treatments (Usadel et al., 2009).

3 Results

3.1 *B. velezensis* CBE-mediated osmotic stress tolerance in *B. distachyon*

The effect of osmotic stress (5% PEG8000) on *B. distachyon* growth was observed after 18 days (postgermination). As illustrated in Figure 1, the stressed seedlings exhibited a clear deleterious impact. The stress treatment significantly reduced the seedlings' dry weight and resulted in a characteristic stressed root phenotype (Figures 1A, B).

Seedlings treated with *B. velezensis* CBE showed far greater tolerance to the osmotic stress. Bacterial treatment had a significant effect on osmotic stressed *B. distachyon*. Hence, the inhibitory effects of PEG treatment on the plant phenotypes were halted after CBE treatment, where a significant dry weight increase was evident in stressed CBE-treated seedlings (Figure 1A). CBE also resulted in different root phenotypes than unstressed and stressed *B. distachyon* seedlings (Figure 1B).

The ability of *B. velezensis* CBE to mediate osmotic stress tolerance in *B. distachyon* was compared with the relatively similar stimulation effect of the well-known PGPB strains *A. brasilense* Cd and *A. olearius* DQS4 under limited and normal nitrogen regimes. We did not find a clear difference in *B. distachyon* growth after treatment with any of the strains under stress conditions, as all strains appeared to effectively improve *B. distachyon's* ability to withstand osmotic stress imposed by PEG treatment (Figure 1). However, *B. velezensis* CBE was superior under normal nitrogen conditions, as a significantly higher dry

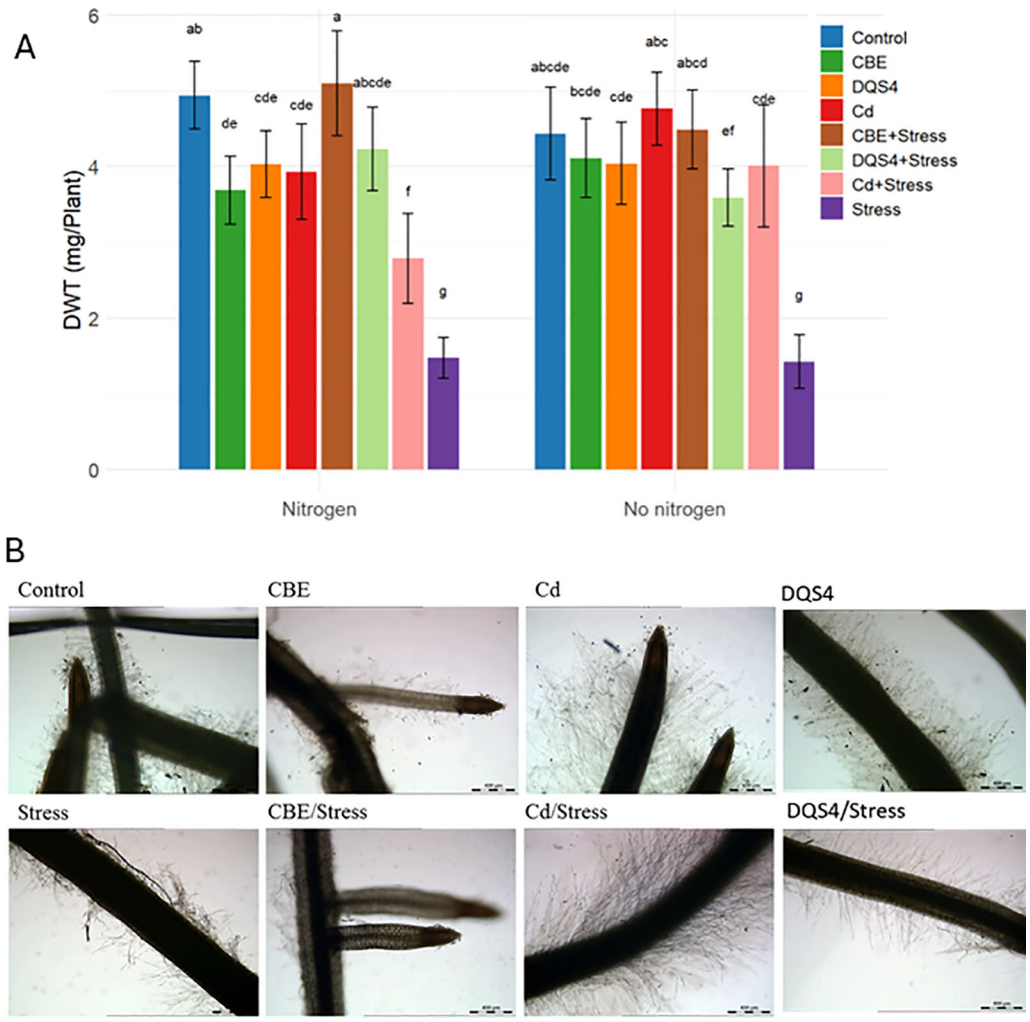

FIGURE 1
Response of *B. distachyon* bd21 seedlings to the inoculation with novel halotolerant endophytic bacterial strain *B. velezensis* CBE, *A. olearius* DQS4, and *A. brasilense* Cd (reference control strains). Four-day-old seedlings were grown for 14 days either in sterile water agar (2.5 g/ml) or in 5% PEG8000 (osmotic stress) under normal nitrogen supply (3 mM NH_4NO_3) or no nitrogen supply. **(A)** Seedling dry weight (mg/plant). Bars indicate standard deviation (n = 6). Treatments labeled with identical small letters are not significant at $p \leq 0.01$. **(B)** Photomicrographs showing root phenotypes.

weight was recorded in stressed *B. velezensis* CBE-treated seedlings compared with stressed *A. brasilense* Cd and *A. olearius* DQS4-treated seedlings grown in nitrogen-supplemented water agar. In the case of seedlings grown without nitrogen, both strains significantly increased *B. distachyon* dry weight under stress conditions (Figure 1A). *A. brasilense* Cd treatment-induced lateral roots and root hair growth in both stressed and stressed seedlings, which was not the case in roots of *B. velezensis* CBE-treated seedlings (Figure 1B).

Bacterial colonization was assessed in the same sterile system by quantifying bacterial presence using the CFU method on TSA plates. A significant increase in the total bacterial count for *B. velezensis* CBE, *A. brasilense* Cd, and *A. olearius* DQS4 was observed in the medium, roots, and surface-disinfected roots under all tested conditions, compared to the uninoculated treatment. Both *B. velezensis* CBE and *A. olearius* DQS4 demonstrated an equal ability to colonize plant roots endophytically, as indicated by the CFU counts. However, in plants treated with the *A. brasilense* Cd strain, no significant increase in bacterial count was observed in the disinfected roots, suggesting the nonendophytic nature of this strain (Supplementary Figure S2).

3.2 Profiling of the *B. distachyon* seedling transcriptome

Transcriptome profiling was conducted on 18-day-old *B. distachyon* seedlings grown under osmotic stress (5% PEG8000) and/or nitrogen supply (3 mM ammonium nitrate) to identify differentially expressed genes (DEGs) in response to treatment with three different PGPB strains (*B. velezensis* CBE, *A. brasilense* Cd and *A. olearius* DQS4) using shotgun RNA-Seq analysis. The transcriptomes of all treatments were compared to the transcriptome obtained for the control treatment (14-day-old seedlings grown in liquid water agar). Data obtained for all

treatments were further analyzed for hierarchical clustering, and a heat map was generated to illustrate global transcriptional changes (Supplementary Figure S3). Data presented in Table 1 shows the numbers of significant identified DEGs (p-values < 0.01 and $\log_2$ fold-change > 2). Nitrogen treatment had the most pronounced transcriptional effects on *B. distachyon* seedlings, where 8,584 DEGs were counted (4,144 upregulated and 4,440 downregulated DEGs). Osmotic stress (5% PEG8000) treatment, on the other hand, resulted in much lower transcriptional changes in *B. distachyon* seedlings (160 DEGs, 78 upregulated and 82 downregulated).

All tested PGPB strains were able to modulate gene expression in *B. distachyon* (Table 1). Transcriptional changes in response to each tested PGPB strain were diverse and depended on the bacterial strain and the stress or nitrogen conditions. Compared to the other tested PGPB strains (*A. brasilense* Cd and *A. olearius* DQS4), *B. velezensis* CBE treatment-induced minimal transcriptional modulations in *B. distachyon* seedlings under all tested conditions. The maximum DEGs recorded in CBE-treated seedlings were found in unstressed *B. distachyon* grown under nitrogen-free conditions (331 DEGs, 50 upregulated and 281 downregulated) (Supplementary Table S2). Only 73 DEGs were identified in *B. velezensis* CBE-treated seedlings under stress conditions. DGEs number further fell to 18 in seedlings grown under normal nitrogen conditions.

TABLE 1 Numbers of significant (p < 0.01, n = 3) differentially expressed genes (DEGs) detected in *B. distachyon* Bd2.

Treatments	Differentially expressed genes (DEGs)		
	Upregulated	Downregulated	Total
Stress	78	82	160
Nitrogen	4,144	4,440	8,584
Stress + nitrogen	2,750	2,909	5,659
B. velezensis CBE	50	281	331
A. brasilense Cd	322	105	427
A. olearius DQS4	127	221	348
Stress + CBE	52	21	73
Stress + Cd	601	477	1,078
Stress + DQS4	1,045	970	2,015
Nitrogen + CBE	1	17	18
Nitrogen + Cd	192	250	442
Nitrogen + DQS4	81	119	200
Stress + nitrogen + CBE	24	38	62
Stress + nitrogen + Cd	653	1,181	1,839
Stress + nitrogen + DQS4	572	883	1,455

Treatments represent seedlings inoculated with novel halotolerant endophytes (*B. velezensis* CBE), and control strains (*A. brasilense* Cd or *A. olearius* DQS4). Four-day-old seedlings were grown for 14 days either in sterile water agar (2.5 g/ml) or in 5% PEG8000.

3.3 Overlapping DEGs in PGPB-treated *B. distachyon*

More overlapping DEGs between all tested PGPB strains were detected in unstressed *B. distachyon*. Data illustrated in Figure 2A show 180 overlapping DEGs between *B. velezensis* CBE and *A. olearius* DQS4 and 58 overlapping DEGEs between *B. velezensis* CBE and *A. brasilense* Cd. In stressed seedlings, it was evident that Cd and DQS4 have resulted in several transcriptional modifications. Furthermore, 590 DEGs were overlapped between *A. brasilense* Cd and *A. olearius* DQS4 (Figure 2B). Very few overlapping DEGs were found in *B. velezensis* CBE-treated stressed seedlings; hence, only 37 overlapping DEGs were identified between CBE and *A. olearius* DQS4, while 24 overlapping DEGs were detected between *A. brasilense* Cd and *B. velezensis* CBE (Figure 2B). It was noticeable that, in contrast to the 113 overlapping DEGs between *A. brasilense*

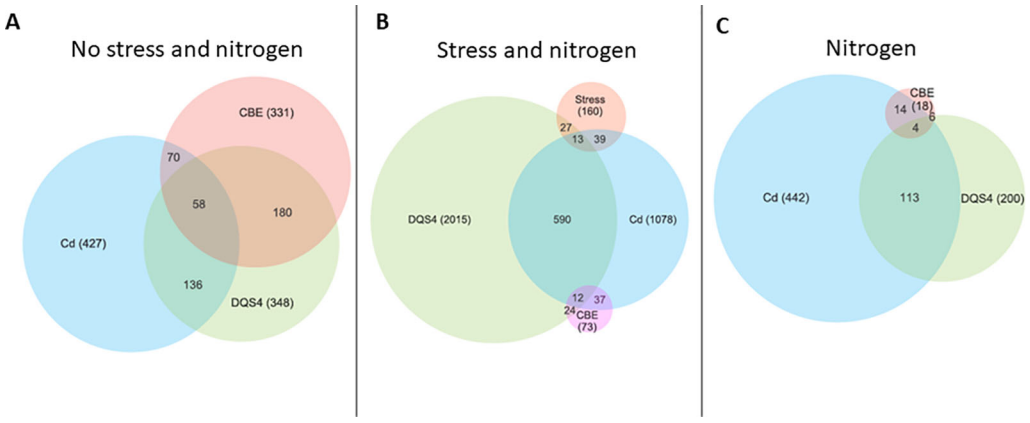

FIGURE 2
Venn diagrams showing the numbers of unique and overlapping differentially expressed genes (DEGs; significant difference at $p \leq 0.01$, n = 3) in 14-day-old *B. distachyon* Bd2 seedlings treated with a novel halotolerant endophyte (*B. velezensis* CBE) and control strains (*A. brasilense* Cd or *A. olearius* DQS4) compared to control (not treated with bacteria). **(A)** Unstressed seedlings. **(B)** Osmotic stressed (5% PEG8000) seedlings. **(C)** Normal nitrogen supply (3mM NH_4NO_3).

Cd- and *A. olearius* DQS4-treated seedlings, there were only six overlapping DEGs between *B. velezensis* CBE and *A. olearius* DQS4, and 14 overlapping DEGs between *B. velezensis* CBE and *A. brasilense* Cd (Figure 2C).

3.4 GO enrichment analysis showed potential biological processes associated with different PGPB treatments in unstressed *B. distachyon* seedlings

To identify potential biological functions of the DEGs (up- and downregulated) by each PGPB strain treatment, we performed a GO terms enrichment analysis of those genes in three sub-trees of GOs (biological process, molecular function, and cellular component). The enrichment analysis showed variable GO representation in unstressed *B. distachyon* seedlings in response to treatment with each PGPB strain. The heatmap illustrated in Figure 3A shows that upregulated genes due to treatment with *B. velezensis* CBE and *A. olearius* DQS4 strains enriched relatively similar GOs that involved several biological functions, including responses to stresses and stimulus and cellular metabolic processes. There was a clear contrast in the GOs enhanced for the downregulated genes in response to *B. velezensis* CBE and *A. olearius* DQS4, as they were clearly separated in the heatmap (Figure 3A). Molecular functions related to cellular membranes and macromolecule metabolic processes represented GOs related to the downregulated genes in *B. velezensis* CBE-treated seedlings (Figure 3A). Unlike *B. velezensis* CBE and *A. olearius* DQS4, up- and downregulated genes in *A. brasilense* Cd-treated seedlings resulted in related GO enrichment, as illustrated in the clustering analysis. However, it was clear that some GOs were enriched for the upregulated genes, such as cytoplasmic and transcriptional regulation functions, while others were overrepresented for the downregulated genes, such as organelle-related functions (Figure 3A).

GO term enrichment analysis for DEGs unique to *B. velezensis* CBE treatment showed that only four functions were overrepresented (Figure 3B). Cell wall, external encapsulating structure, and extracellular region functions were all found to be overrepresented in response to all tested bacterial strains (Figure 3B). GO term enrichment analysis for overlapping DEGs in response to the three tested bacterial strains showed enriched six biological functions including response to stresses and stimulus and cell periphery (Figure 3B).

3.5 GO enrichment analysis of DEGs showed potential biological processes associated with different DQS4 and Cd treatments, but not CBE treatment, in *B. distachyon* seedlings under osmotic stress and normal nitrogen supply conditions

Up- and downregulated DEGs identified in *B. distachyon* Bd21 seedlings treated with sterile TSB media (control), *B. velezensis* CBE, DQS4, or Cd bacterial strains in response to either osmotic stress (5% PEG8000) or nitrogen supply (3 mM NH_4NO_3) were subjected to GO term enrichment analysis across the three sub-trees of GOs (biological process, molecular function, and cellular component). Osmotic stress treatment led to the enrichment of GOs often associated with stress response, such as primary and organic substance metabolic process (Figure 4A). Nitrogen treatment induced substantial transcriptomics changes; therefore, several functions were overrepresented in *B. distachyon* Bd21 seedlings grown under nitrogen supply (Figure 4B). Functional enrichment for DEGs in response to bacterial treatments under osmotic stress or nitrogen supply conditions was highly variable and strain-dependent. However, the most noticed finding was that the GOs enrichment for *B. velezensis* CBE-related DEGs did not indicate any potential biological functions in seedlings grown under either osmotic stress or nitrogen supply conditions. On the other hand, contrasting functional enrichments were found for DEGs identified in DQS4- or *A. brasilense* Cd-treated seedlings grown under either osmotic stress or nitrogen supply conditions (Figures 4A, B).

Functional enrichment analysis of unique DEGs resulting from *A. olearius* DQS4 treatment revealed that several GO terms were overrepresented in *B. distachyon* Bd21 seedlings grown under osmotic stress conditions (Figure 5). Furthermore, most of these functions were enriched in DEGs that overlapped between *A. olearius* DQS4 and *A. brasilense* Cd treatments (Figure 5).

3.6 Minor transcriptome modifications in *B. distachyon* Bd21 during *B. velezensis* CBE-mediated osmotic stress tolerance

The effect of *B. velezensis* CBE treatment on the overall *B. distachyon* Bd21 transcriptome was relatively minor. Hence, only 73 genes were found to show significant differential expressions (the data illustrated in Figure 6A shows fold changes in the expression of genes representing up- and downregulated genes). Functional classification of the identified genes showed that up to 27% did not correspond to any known genes (Figure 6B). Most of the functionally known genes (25%) were involved in plant metabolism (Figure 6B). The remaining genes were assigned to different functions, including signaling, cellular transport, and transcriptional regulation (Figure 6B).

The transcriptomes of *B. velezensis* CBE, *A. olearius* DQS4, and *A. brasilense* Cd-treated stressed (5% PEG8000) *B. distachyon* Bd21 seedlings were mapped to different functional categories using the MAPMan tool to reveal molecular processes associated with each strain's ability to mediate stress tolerance. The cellular response overview confirmed that all tested strains impacted genes involved in various cellular responses, including redox, biotic, and abiotic stress responses. However, it was clear that *B. velezensis* CBE had a lower influence on the overall cellular response than DQS4 and *A. brasilense* Cd (Figure 7). Developmental and biotic stress-responsive genes were the most represented genes in the transcriptome of *B. velezensis* CBE-treated seedlings under osmotic stress (Figure 7A). That was also the case in *A. olearius* DQS4 and *A. brasilense* Cd-treated seedlings, yet significantly more genes were involved in mounting the required developmental and

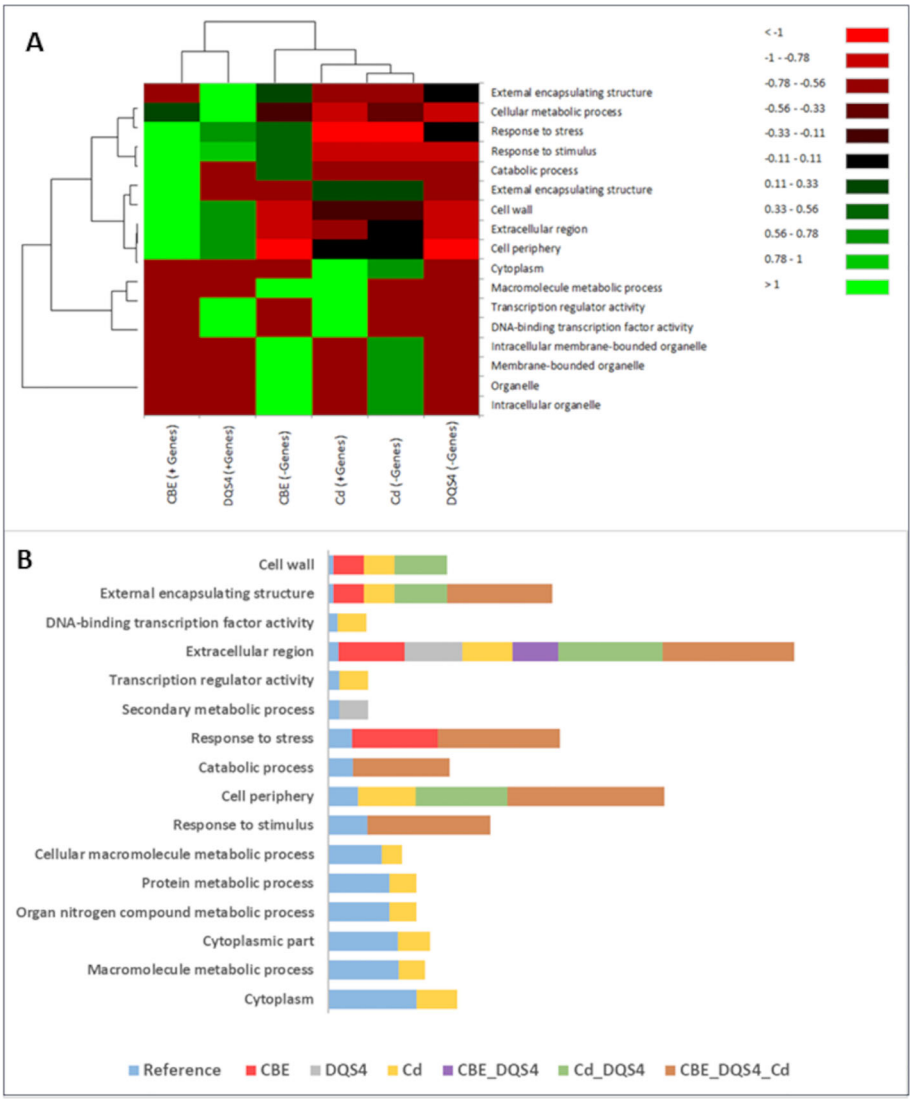

FIGURE 3
Gene Ontology annotations (GO) for significant DEGs found in 18-day-old *B. distachyon* Bd2 seedlings treated with a novel halotolerant endophyte (*B. velezensis* CBE) and control strains (*A. brasilense* Cd or *A. olearius* DQS4) compared to control (not treated with bacteria). Annotations were performed for the three sub-trees of GOs: biological process, molecular function, and cellular component, using Blast2GO tools. **(A)** Heat map showing variation in enriched GOs between up- and downregulated DEGs in *B. distachyon* Bd2 seedlings following bacterial treatments. The map was generated based on Pearson and Ward methods for distance measurement and clustering with the XLSTAT package. **(B)** Enrichment bar chart showing over- and underrepresented enriched functions along with the corresponding sequence percentages for unique and overlapping DEGs in *B. distachyon* Bd2 seedlings after bacterial treatments. Sequence % compare treated plants with the reference *B. distachyon* Bd2 genome. A higher sequence % for a given function (compared to the reference) indicates that the function is overrepresented in the treated plant.

biotic stress responses (Figures 7B, C). Furthermore, several abiotic stress-related genes were found in *A. olearius* DQS4 and *A. brasilense* Cd-treated seedlings under osmotic stress conditions but not in the *B. velezensis* CBE-treated seedlings, where only a few genes were associated with abiotic stress response (Figure 7).

MAPMan was also used to map transcription factors contributing to the strain's potential to mediate stress tolerance. It was evident that *B. velezensis* CBE treatment has resulted in minor changes in the expression of different transcription factors compared to the other two tested strains (Figure 8). For instance, upregulation of one AP2-EREBP transcription factor was detected in seedlings treated with *B. velezensis* CBE under stress conditions (Figure 8A). However, more than five AP2-EREBP transcription factors were upregulated in *A. olearius* DQS4-treated seedlings (Figure 8B), and more than nine were upregulated in *A. brasilense* Cd-treated seedlings (Figure 8C). A similar trend was observed with other transcription factors, such as BHL-Hs, WARYs, and MYBs (Figure 8).

4 Discussion

The use of microorganisms to enhance plant tolerance to abiotic stress is considered an environmentally sustainable strategy for mitigating stress-related effects. Consequently, extensive research is underway to identify and characterize novel microorganisms with

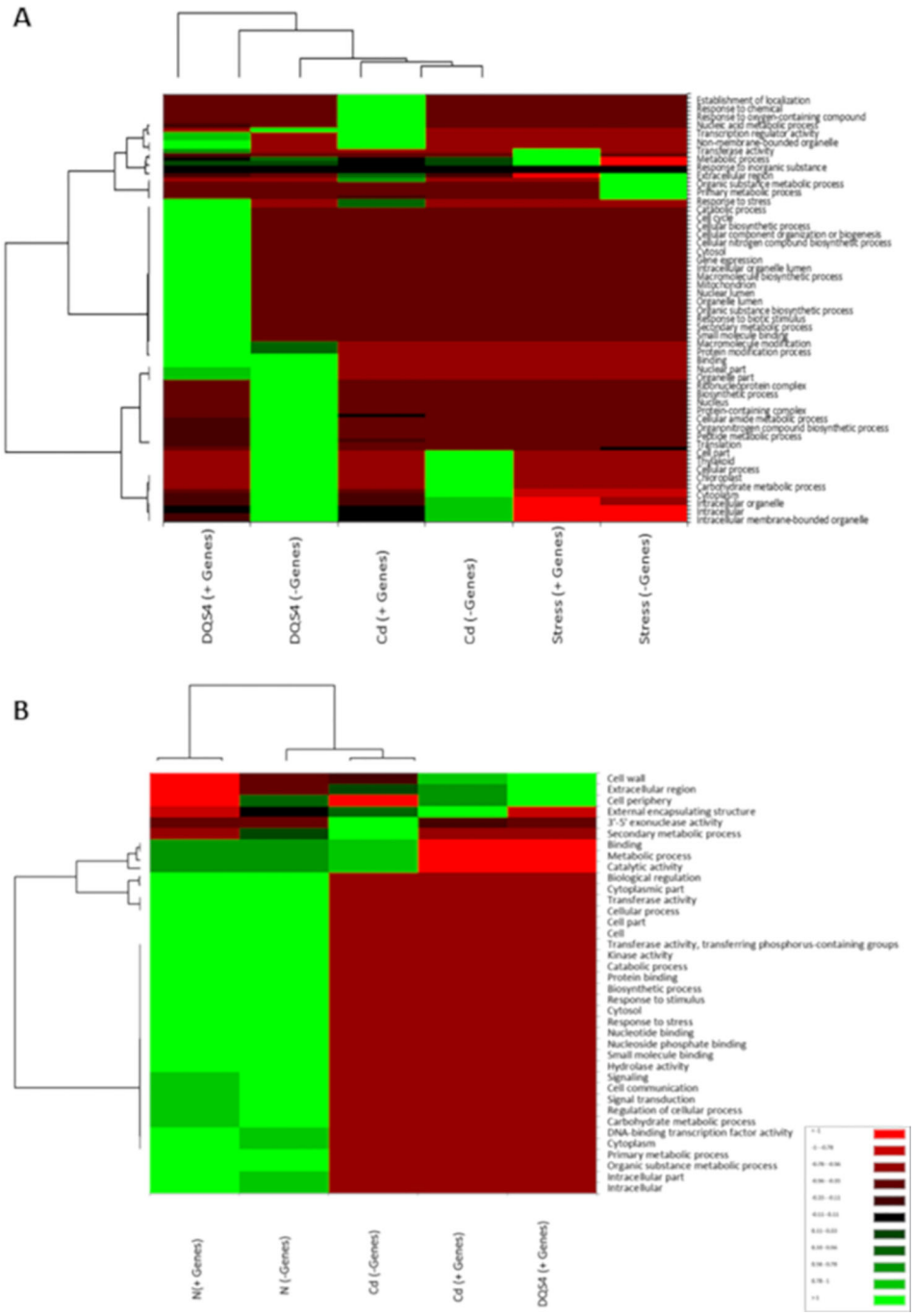

FIGURE 4
Gene Ontology annotations (GO) for significant DEGs found in 18-day-old stressed *B. distachyon* Bd2 seedlings treated with bacterial strains (*A. brasilense* Cd or *A. olearius* DQS4) compared to control (not treated with bacteria). The annotations were performed for the three sub-trees of GOs: biological process, molecular function, and cellular component, using Blast2GO tools. **(A)** Heat map depicting variations in enriched GOs between up- and- downregulated DEGs in osmotically stressed (5%PEG8000) *B. distachyon* Bd2 seedlings after bacterial treatments. **(B)** Heat map showing variation in the enriched GOs between up- and downregulated DEGs in *B. distachyon* Bd2 seedlings grown under normal nitrogen supply (3 mM NH_4NO_3). The maps were generated using Pearson and Ward methods for distance measurement and clustering, implemented in the XLSTAT package.

potential to augment abiotic stress tolerance (Singh et al., 2023). In this study, we demonstrate the ability of the newly discovered halotolerant strain, *B. velezensis* CBE, to enhance osmotic stress tolerance in *B. distachyon* Bd2 seedlings under both normal and nitrogen-depleted conditions. Notably, the effectiveness of *B. velezensis* CBE in improving osmotic stress tolerance was comparable to or even superior (especially under normal nitrogen levels) to the well-established PGPB species (*A. brasilense* Cd).

Noticeably, while the application of *B. velezensis* CBE did not stimulate the formation of lateral roots and root hairs in treated plants, it is crucial to note that Cd treatment clearly promoted these features. The influence of various beneficial microbes, including

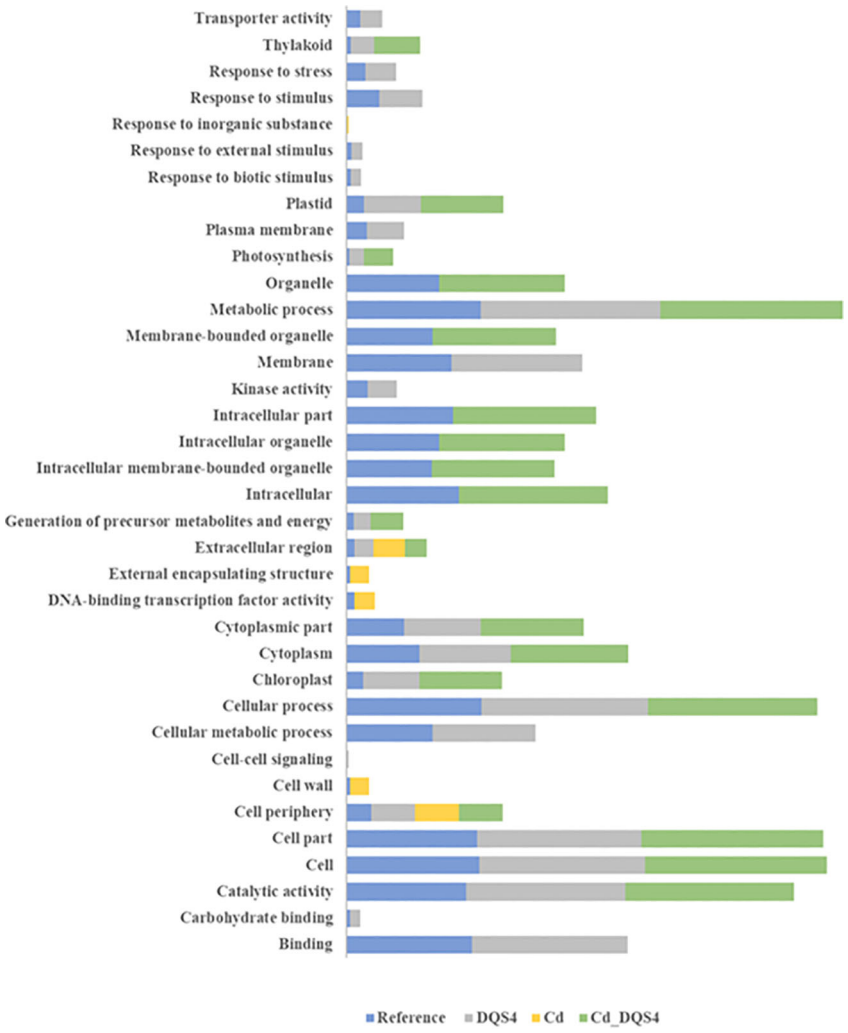

FIGURE 5
Enrichment bar chart showing over- and underrepresented enriched functions and the corresponding sequence percentages for unique and overlapping DEGs in osmotically stressed (5%PEG8000) *B. distachyon* Bd2 seedlings after bacterial treatments. The sequence % represent comparisons between treated plants are compared with sequence % in reference *B. distachyon* Bd2 genome. Higher sequence % for a given function (compared to the reference) indicate that the function is overrepresented in the treated plant.

Azospirillum, on plant root systems is well-documented in scientific literature, as reviewed by Grover et al. (2021). This effect is often attributed to the microbes' ability to enhance plant nutrient and water uptake (Grover et al., 2021). Additionally, changes in the root system response to beneficial microbes may be attributed to changes in signaling within key hormonal pathways that regulate plant root development, such as auxin and cytokinin (Verbon and Liberman, 2016). However, the limited impact on the root systems of plants treated with *B. velezensis* CBE suggests that each bacterial species may employ a unique mechanism to confer advantageous traits to plants under stressful conditions. The differences in observed root phenotypes among the tested strains may be attributable to the significant phylogenetic divergence between species. Furthermore, the distinct ecological niches occupied by each respective strain—*B. velezensis* CBE as a plant endophyte and *A. brasilense* Cd as a plant rhizosphere colonizer—could exert varying effects on the host plant. Consequently, these disparities in microbial characteristics are expected to lead to diverse responses. These variations could lead to the production of different microbial bioactive metabolites, including phytohormones, osmolytes, and antioxidants, which may directly influence the plant host in distinct ways.

Considering the distinct phenotypic responses observed in *B. velezensis* CBE-treated plants, we hypothesized that the plant transcriptional regulation involved in mediating osmotic stress tolerance in *B. distachyon* Bd2 differs from that induced by other beneficial microbes, such as *A. brasilense* Cd. To investigate this hypothesis, we conducted comprehensive transcriptomic profiling on *B. distachyon* Bd2 seedlings treated with *B. velezensis* CBE, aiming to identify the molecular factors contributing to the induced osmotic stress tolerance. Additionally, we performed transcriptomic analysis on plants treated with other beneficial microbes, specifically *A. brasilense* Cd and *A. olearius* DQS4, to elucidate similarities and differences in plant transcriptional responses to each bacterial strain.

Beneficial microbes possess the capacity to reprogram the plant's transcriptomes, frequently leading to shifts in gene expression profiles (Mukherjee, 2022). These modifications can

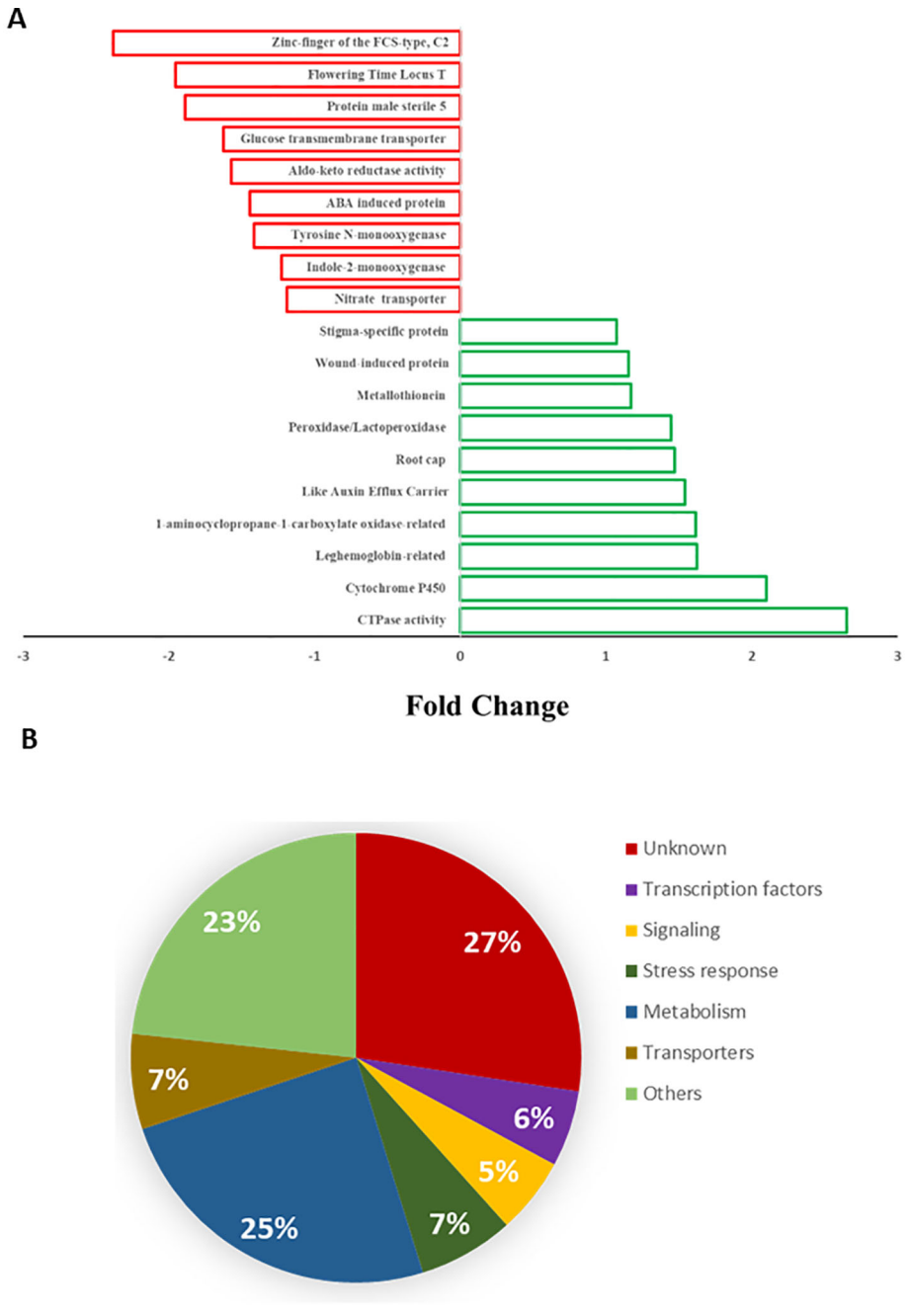

FIGURE 6
Representing DEGs detected in *B. distachyon* Bd2 during *B. velezensis* CBE-mediated osmotic stress tolerance. **(A)** Fold change (relative to control unstressed treatment). **(B)** Putative comparative classification according to the biological function of genes corresponding to the DEGs based on database queries using BLAST.

subsequently govern various molecular and metabolic processes (Mukherjee, 2022). In the present study, we observed transcriptional changes in *B. distachyon* Bd21 seedlings treated with the novel *B. velezensis* CBE strain, both under normal and osmotic stress conditions. As anticipated, substantial alterations in the transcriptome were also noted in seedlings treated with *A. brasilense* Cd and *A. olearius* DQS4. However, the extent of the plant transcriptional modulations varied significantly among the different strains, as indicated by the substantially higher numbers of DEGs identified in the *A. brasilense* Cd and *A. olearius* DQS4-treated plants under all tested conditions. Furthermore, it appeared that *A. brasilense* Cd and *A. olearius* DQS4 induced more pronounced changes in the plant transcriptome profiles under both normal and osmotic stress conditions compared to *B. velezensis* CBE, indicated by the higher DEGs in seedlings treated with *A. brasilense* Cd and *A. olearius* DQS4.

Numerous genes have been implicated in modulating the interactions between plants and beneficial microbes (Liu et al.,

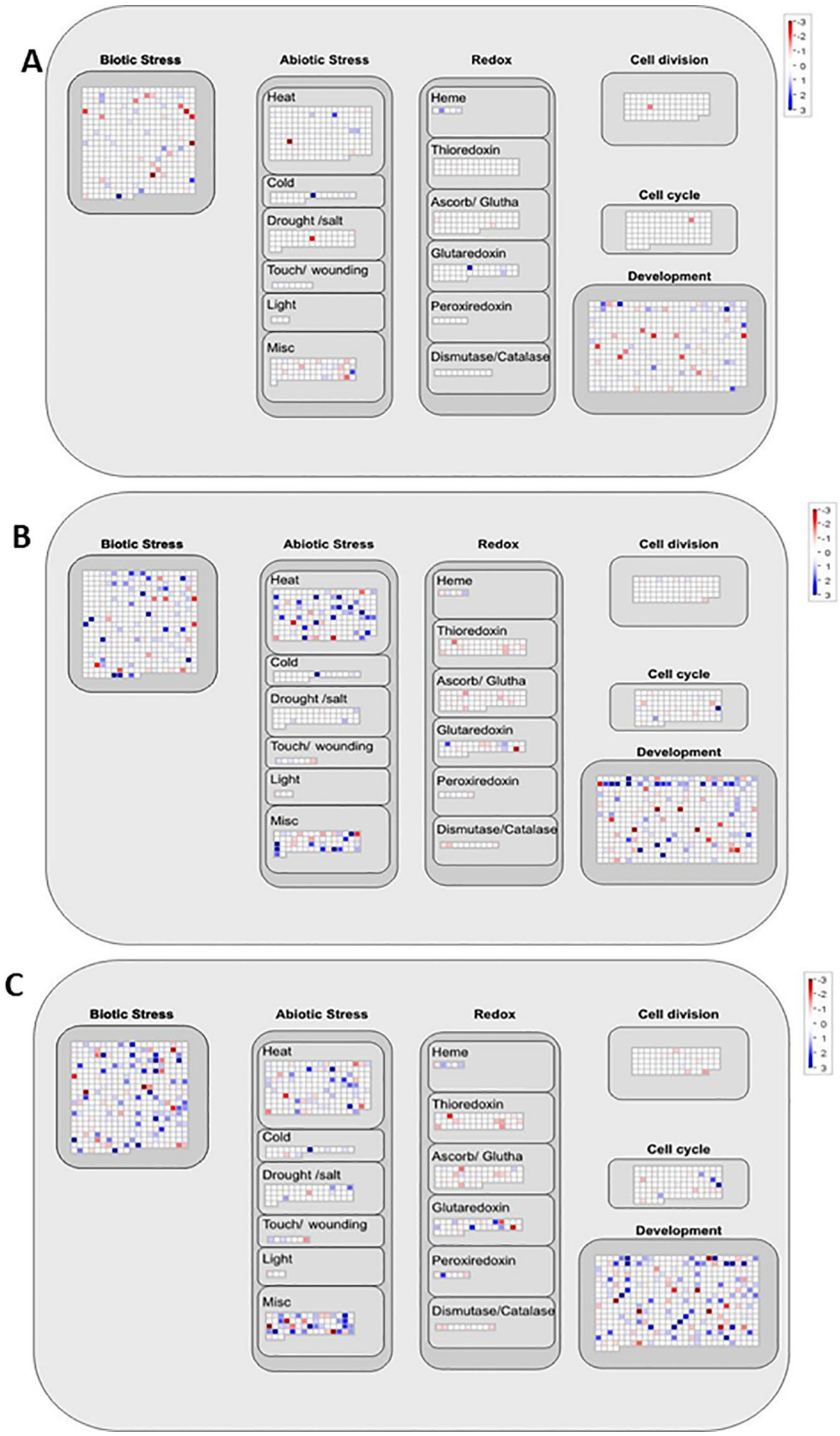

FIGURE 7
MapMan overview of transcriptional changes involved in overall cellular responses in *B. distachyon* Bd21 stressed seedlings treated with CBE **(A)**, DQS4 **(B)**, and Cd **(C)**. Each square represents a separate DEG; red indicates that gene expression was induced, and blue indicates that gene expression was repressed compared with the control.

2023). Our results show that treatment with strains *B. velezensis* CBE, *A. olearius* DQS4, and *A. brasilense* Cd-induced notable changes in plant gene expression profiles, observable under both standard growth conditions and in response to osmotic stress. The genes affected are associated with a diverse array of plant molecular and cellular functions, including but not limited to heat shock proteins (HSPs), cellular transport mechanisms, metal-ion binding proteins, components of signal transduction pathways, and transcription factors. Such molecular processes are posited to be essential for the facilitation of abiotic stress resilience by the beneficial microbes (Conde et al., 2011; Scieglinska et al., 2019; Mukherjee, 2022; Liu et al., 2023). Consequently, it was evident that

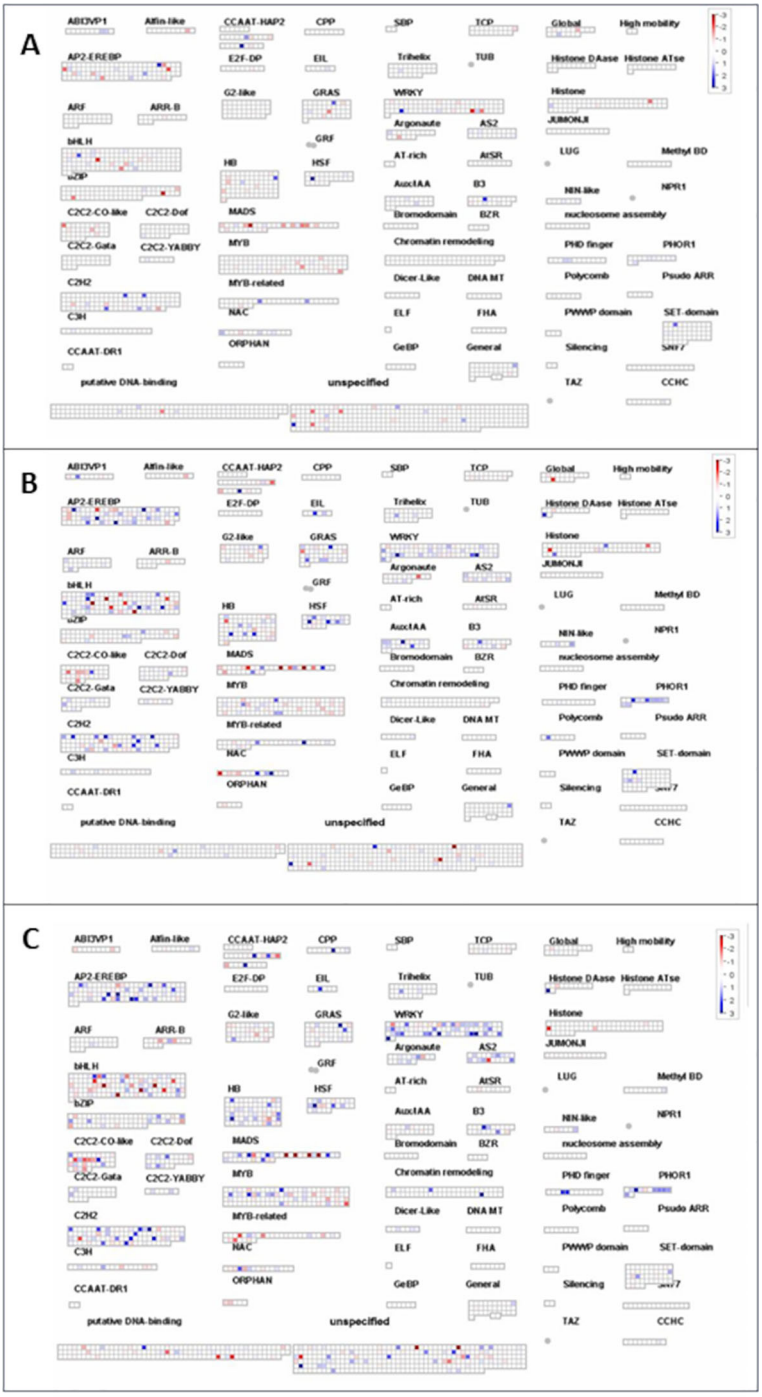

FIGURE 8
MapMan overview of transcription factors expression changes in *B. distachyon* Bd21 stressed seedling treated with CBE **(A)**, DQS4 **(B)**, and Cd **(C)**. Each square represents a separate DEG; red indicates that gene expression was induced, and blue indicates that gene expression was repressed compared with the control.

all microbial treatments were capable of mounting the requisite molecular alterations. Notably, transcriptional analysis revealed that each strain modulated a distinct set of genes to influence relatively comparable biological processes. However, strain *B. velezensis* CBE was observed to effect similar functional outcomes by regulating a smaller subset of genes, thereby suggesting a more targeted or efficient molecular regulation, in contrast to the broader gene expression changes induced by strains *A. olearius* DQS4 and *A. brasilense* Cd.

Transcription factors (TFs) play a key role in regulating various cellular processes, including plant responses to stresses and interactions with microbes (Strader et al., 2022). Among these, the MYB family of proteins, present in all eukaryotes, is known for its diversity and functionality. These proteins typically function as

transcription factors, possessing varying numbers of MYB domain repeats that enable them to bind to DNA (Yang et al., 2021). We observed differential expression of several *MYB* genes in plants treated with all tested beneficial microbes. Previous studies have highlighted the involvement of *MYBs* in plant responses to diverse abiotic and biotic stimuli, including stress factors, plant pathogens, and beneficial microbes (Sarosh et al., 2009; Ambawat et al., 2013; Yang et al., 2021; Biswas et al., 2023). For example, *MYB102* and *MYB41* have been linked to resistance against insects, as well as wounding and osmotic stress tolerance in Arabidopsis (Zhu et al., 2018). Additionally, *MYB41* was found to regulate ABA-mediated stomatal closure in response to abiotic stresses (Wei et al., 2020).

WRKY transcription factors play a crucial role in regulating the complex network of signaling pathways in plants in response to biotic and abiotic stresses, as well as interactions with beneficial microbes. For instance, several WRKYs were found to be associated with improved drought stress tolerance in several plants (Li et al., 2024). Upregulation of certain WRKY transcription factors appears to be crucial for microbes to mediate abiotic stress tolerance in plants, as suggested by Abd El-Daim et al. (2018), who reported that the *B. velezensis* 5113 lost its ability to mediate drought and heat stress tolerance after knocking down ABA-responsive WRKYs, indicating the involvement of ABA signaling in microbially mediated abiotic stress tolerance. Our results show both up- and downregulation of several *WRKYs* in plants treated with the beneficial microbes *B. velezensis* CBE, *A. olearius* DQS4, and *A. brasilense* Cd. It is also evident that the number of *WRKYs* exhibiting differential expression varies depending on the strain. For instance, plants treated with *B. velezensis* CBE showed differential expression in only three *WRKY* genes.

5 Conclusions

Microbially mediated abiotic stress tolerance in plants is an important process, long regarded as an eco-friendly approach to sustainable agriculture. This study demonstrates the potential of the novel halotolerant endophyte *B. velezensis* CBE in enhancing osmotic stress tolerance in *B. distachyon* Bd21 seedlings. We further explored the molecular modulations in plants associated with *B. velezensis* CBE activity through transcriptome profiling, which revealed that *B. velezensis* CBE regulates several molecular functions known to be involved in microbially induced stress tolerance. By comparing *B. velezensis* CBE with other microbes capable of mediating stress tolerance in plants, we highlighted that molecular regulation was strain-dependent. The critical difference among the microbes was the cellular and molecular reprogramming required by *B. velezensis* CBE to confer osmotic stress tolerance in *B. distachyon* Bd21 seedlings. This was achieved through subtle changes in gene expression that regulate these functions. For instance, in plants treated with *A. brasilense* Cd and *A. olearius* DQS4, we observed both up- and downregulation of numerous transcription factors, such as *MYBs* and *WRKYs*. In contrast, *B. velezensis* CBE-inoculated plants exhibited differential expression in only a few of these transcription factors, suggesting that *B. velezensis* CBE's impact on plant transcription is more specific compared to the broader effects observed with the other tested microbes.

This work is significant because it highlights how different PGPB strains can mediate stress tolerance through relatively similar molecular processes, yet each strain induces these processes through distinct transcriptomic changes. Understanding these differences is crucial and may underpin the design of microbial synthetic communities comprising multiple strains. It will be particularly interesting to investigate plant responses to combinations of PGPB and to determine to what extent these responses are predicted by the response to individual strains. It can be expected that designing a consortium of strains with complementary gene expression changes may enhance stress resilience compared to single strains.

Data availability statement

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB36975. It includes the fastq files with the filtered reads and bam files with the mapped reads.

Author contributions

IA-D: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. GR: Investigation, Methodology, Writing – review & editing. NF: Data curation, Formal analysis, Methodology, Software, Writing – review & editing. SH: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. AC: Investigation, Methodology, Writing – review & editing. KF: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, 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.2024.1485391/full#supplementary-material

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GmWRKY33a is a hub gene responsive to brassinosteroid signaling that suppresses nodulation in soybean (*Glycine max*)

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Brassinosteroids (BRs) are key phytohormones influencing soybean development, yet their role in symbiosis remains unclear. Here, the RNA-Seq was used to identify important gene associated with BRs and symbiotic nitrogen fixation, and the function of candidate gene was verified by transgenic hairy roots. The result shows that the RNA-Seq analysis was conducted in which BR signaling was found to suppress nodule formation and many DEGs enriched in immunity-related pathways. WGCNA analyses led to the identification of *GmWRKY33a* as being responsive to BR signaling in the context of symbiosis establishment. Transgenic hairy roots analyses indicated that GmWRKY33a served as a negative regulator of the establishment of symbiosis. The qRT-PCR analysis confirmed that BR signaling upregulates *GmWRKY33a*, leading to nodulation suppression and activation of soybean immune responses. In summary, our research revealed that BR suppresses root nodule formation by modulating the immune signaling pathway in soybean roots. We further identified that GmWRKY33a, a crucial transcription factor in BR signaling, plays a negative role in the symbiotic establishment.

KEYWORDS

soybean, symbiosis, brassinosteroids (BRs), RNA-Seq, WGCNA, GmWRKY33a

1 Introduction

Soybean (*Glycine max* L. Merr.) serves as a major source of vegetable oil and protein for human consumption, in addition to being a cornerstone crop for the development of sustainable agriculture (Ma et al., 2023; Wang et al., 2023a). Current soybean production efforts, however, require large amounts of industrial nitrogen-based fertilizers, thus posing

a substantial threat to soil microbes and the overall soil environment (Wang et al., 2023b). Soybean plants can establish a symbiotic relationship with rhizobia that results in the efficient conversion of atmospheric nitrogen into a form that can be used to support growth and development in a mutually beneficial manner (Ma et al., 2024a). As soybean production is forecast to grow by 55% as of 2050 and symbiotic nitrogen fixation can provide large volumes of nitrogen to fuel soybean growth, the key importance of this symbiotic nitrogen fixation capacity will be increasingly relevant to agricultural productivity and sustainability (Ciampitti et al., 2021).

Nodule development necessitates signaling interactions between leguminous plants and the associated rhizobia, together with the orchestration of complex programs of gene regulation within these legumes (Roy et al., 2020). In response to the detection of host-derived flavonoids, rhizobia can secrete a variety of nodulation factors (NFs) (Denarie and Cullimore, 1993; Zhang et al., 2009). Symbiotic receptors including Nod factor receptor 1 (NRF1) and Nod factor receptor 5 (NRF5), in turn, allow the legume hosts to respond to these NFs (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Smit et al., 2007), which induce a spike in nuclear calcium signaling that is decoded by calmodulin-dependent protein kinase (CCaMK) (Mitra et al., 2004; Gleason et al., 2006), leading to the phosphorylation and activation of CYCLOPS (Yano et al., 2008; Singh et al., 2014; Rudaya et al., 2022). The resultant signals drive the upregulation of a series of symbiotic downstream genes involved in the common symbiosis signaling pathway (CSSP), including *Ethylene Responsive Factor Required for Nodulation 1* (*ERN1)* and *Nodule Inception* (*NIN*) (Cerri et al., 2017; Roy et al., 2020). The transcription factor Nodulation Signaling Pathway 1 (NSP1) is capable of binding the promoter regions upstream of both *ERN1* and *NIN* to activate their expression, with its transcriptional activity being subject to Nodulation Signaling Pathway 2 (NSP2)-mediated regulation in the context of symbiosis (Hirsch et al., 2009; He et al., 2021). To date, the molecular and genetic mechanisms that underlie the establishment of this symbiotic relationship remain poorly understood.

As with all other plant developmental processes, nodulation is regulated by phytohormones (Yang et al., 2011; Vriet et al., 2013; Planas-Riverola et al., 2019). Brassinosteroids (BRs) are polyhydroxylated steroid phytohormones that are detected by the BR Insensitive 1 (BRI1) membrane receptor (Wang et al., 2001; Kinoshita et al., 2005) and BRI1-associated kinase (BAK1) as a co-receptor (Li and Chory, 1997; Vicentini et al., 2009; Sun et al., 2013), allowing for the regulation of a diverse array of plant developmental processes. BR signaling is transmitted from the membrane to the nucleus through complex and dynamic interactions between BRI1 Suppressor 1 (BSU1) (Tang et al., 2008; Wang et al., 2011), BR-insensitive 2 (BIN2) (Kim et al., 2009, 2011; Wang et al., 2012), and Brassinazole-Resistant 1/BRI1-EMS-Suppressor 1 (BES1/BZR1) (Wang et al., 2002; Yin et al., 2002). When BR signaling is initiated, BIN2 is degraded, leading BES1 and its homologs to be dephosphorylated whereupon they accumulate in the nucleus and control the expression of genes related to BR responses (Zhu et al., 2017). In addition to shaping plant growth and development, BR signaling processes are central to the orchestration of microbial stress responses (De Bruyne et al., 2014; Wei and Li, 2016). BRs can enhance the ability of plants to resist a wide range of pathogens. While BR-mediated disease resistance (BDR) is evident following BR treatment (Nakashita et al., 2003), its precise mechanistic basis remains poorly understood.

In different legume species, the precise roles that BRs play in the establishment of symbiotic relationships vary (Chen et al., 2023). In *M. truncatula* and *Pisum sativum*, *Mtbri1* mutants lacking the BR receptor present with fewer nodules (Ferguson et al., 2005; Cheng et al., 2017). The *lk* (5α reductase-deficient) and *lkb* (sterol C-24 reductase-deficient) mutant pea lines exhibiting impaired BR biosynthesis similarly exhibit reductions in nodule numbers (Ferguson et al., 2005). The application of BRs to leaves following rhizobial inoculation in *Pisum sativum* is associated with significantly higher root nodule numbers (Shahid et al., 2011). While these results emphasize the positive roles that BRs can play in nodulation for certain leguminous species, the leaf application of BRs to *Phaseolus vulgaris* or the BR inoculation treatment of *Lens culinaris* were associated with impaired nodule formation (Upreti and Murti, 2004). BR signaling can negatively regulate NF signaling activity in soybean plants, adversely affecting the establishment of symbiosis (Chen et al., 2023). When brassinaz, an inhibitor of BR biosynthesis, was exogenously applied to the Enrei cultivar, this led to better nodule formation, whereas the application of BRs to the roots of these plants effectively suppressed nodulation (Hunter, 2001). Overexpressing *BES1/BZR1* and homologs thereof has similarly been shown to decrease the formation of root nodules in soybean (Yan et al., 2018). The key BR signaling pathway component GmBES1-1 has been shown to inhibit nodulation through its interactions with GmNSP1 and GmNSP2 and the inhibition of the ability of GmNSP1s to bind DNA (Chen et al., 2023). BRs are central regulators of plant immune function, and the effective establishment of rhizobial symbiosis necessitates a homeostatic balance between signals conducive to antimicrobial immunity and symbiosis (Wei and Li, 2016). Beyond their ability to directly affect NF signaling activity during the establishment of symbiosis, BRs may also be capable of regulating the immune response to shape nodulation in this context, although the underlying mechanisms through which BR signaling can modulate immunity in this setting remain unknown.

WRKY zinc-finger motif-containing proteins frequently function in the coordination of defense responses in *Arabidopsis* (Eulgem and Somssich, 2007; Liu et al., 2018), with defense signaling or pathogen infection frequently provoking the upregulation of large numbers of WRKY genes that activate an array of downstream genes linked to disease resistance including *NPR1* and *PRs* (Li et al., 2020). WRKY33 is among the best-studied members of this family, with several studies having documented its mechanistic importance in response to biotic stressors (Zheng et al., 2006; Liu et al., 2017). WRKY33 can also mediate pathogen-associated responses in plants such that, when overexpressed, it can engender greater host resistance against many microbes (Liu et al., 2015). *WRKY33* is capable of responding to pathogen infection through its ability to modulate calcium ion, calmodulin, and hormone signaling (Zhou et al., 2020), in addition to shaping

redox homeostasis (Zheng et al., 2006) and autophagy (Lai et al., 2011). MAPK and CDPK-dependent WRKY33 phosphorylation upstream of pathogen infection also shapes its functional effects (Mao et al., 2011; Zhou et al., 2020). Beyond these pathogen-induced responses, WRKY33 also shapes the ability of plants to tolerate abiotic stressors including salinity (Jiang and Deyholos, 2009), hypoxia (Tang et al., 2021), heat (Li et al., 2011), and cold stress (Guo et al., 2022). While there have been several studies analyzing soybean *GmWRKY33* functions in the context of pathogen resistance, its impact on symbiotic signaling has yet to be established.

Here, treatment with eBL (Epibrassinolide) was found to significantly reduce root nodule numbers and to promote the expression of defense-associated genes including PRs. Through subsequent RNA-seq analyses, differentially expressed genes following eBL treatment were found to be enriched in nodulation and plant-pathogen interaction-related pathways. WGCNA and qPCR analyses led to the identification of *Glyma.09G280200*, which encodes a GmWRKY33 protein, as a hub gene involved in coordinating the BR signal transduction processes related to symbiosis establishment. This gene was designated *GmWRKY33a*. Through transgenic analyses, *GmWRKY33a* was found to negatively regulate symbiosis, with BRs suppressing nodulation through the promotion of GmWRKY33a expression. Together, these findings offer new insight into BR signaling processes and their role in symbiosis establishment and efficient nitrogen fixation in soybeans.

2 Materials and methods

2.1 Materials and cultivation conditions

Sinorhizobium fredii HH103 (hereafter HH103) and GUS-tagged HH103 (HH103-GUS) (Wang et al., 2023a) were utilized to conduct all nodulation experiments described herein. Both HH103 and HH103-GUS were cultured in TY medium with appropriate antibiotics (50 μg/mL) at 28°C. Soybean plants were cultivated in a greenhouse (16 h light/8 h dark, 25°C).

2.2 Inoculation and BR sensitivity assays

After using Cl_2 (generated from 96 mL sodium hypochlorite and 4 mL concentrated hydrochloric acid in drying bottle) to sterilize the surfaces of Dongnong 50 (DN50) seeds, they were sown in vermiculite in plastic jars that had been autoclaved. Irrigation was performed with a nitrogen-deficient nutrient solution. HH103 or HH103-GUS were cultured in TY medium to an OD_{600} of 0.8, at which time the medium was washed away with 10 mM $MgSO_4$ and the OD_{600} was adjusted to 0.5 for rhizobial inoculation.

BR sensitivity assays were conducted by growing soybeans to the VC stage. To determine how eBL affects soybean nodule development, a nitrogen-deficient nutrient solution containing eBL was added in place of the initial nutrient solution, changing this medium once per week, whereas control plants instead received an equivalent volume of nitrogen-deficient nutrient solution with ethanol. On day 30 post-inoculation, nodule number and nodule dry weight were assessed.

2.3 Infection event analyses

Acetone was used to fix select lateral roots from eBL-treated or control soybean plants on day 24 post-inoculation with HH103. As the β-glucuronidase reporter gene was encoded by the HH103-GUS strain, GUS staining was conducted as in prior reports after using 75% alcohol for decolorization. The utilized GUS staining solution consisted of 1 mM potassium ferricyanide, 1 mg/L X-gluc, and 100 mM sodium phosphate (pH=7.5). These analyses were performed using lateral roots from near the root-stem interface, with infection events being visualized and counted via light microscopy (Leica LM2500, Germany). These analyses were conducted using three biological replicates.

2.4 qRT-PCR

Rhizobia-inoculated soybean roots were collected, crushed in liquid nitrogen, and the FreeZol Reagent (Vazyme, China) was used for RNA extraction, followed by the use of a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme) to produce cDNA. cDNA then used for qPCR analyses performed with a Roche 480 instrument (Stratagene, CA, USA) and the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The $2^{\wedge(-\Delta CT)}$ algorithm was used to assess relative gene expression, using *GmUNK1* (*Glyma.12g020500*) for normalization (Ma et al., 2024b).

2.5 RNA-Seq

Genes responsive to BRs at the time symbiosis is established were identified by isolating total RNA from the roots of eBL-treated DN50 seedlings inoculated with HH103 on day 24 post-inoculation. The TruePrep RNA Library Prep Kit for Illumina (Vazyme) was used for library preparation, followed by RNA sequencing. The resultant data for each sample were assembled with the Cufflinks or StringTie software. Gene and transcript levels were separately quantified with RSEM, and expression levels were normalized with DEseq2 to conduct differential expression analyses according to a negative binomial distribution (Love et al., 2014). Differentially expressed genes (DEGs) were identified as those with a normalized fold change > 2, a p-value < 0.01, and a false discovery rate (FDR) < 0.01.

2.6 Hairy roots transformation

A. rhizogenes strain K599 containing the pSoy10 *GmUbi3:GmWRKY33a-GFP* or B7gWWIWG2(II)-*GmWRKY33a* vectors were used for soybean hairy root transformation, using empty

vector (EV1 or EV2)-containing strains as a control. A portable fluorescent protein excitation light source (LUYOR) was used to identify transgenic roots, removing any roots that were not transgene-positive (Ma et al., 2024a). The confirmation of GmWRKY33a silencing or overexpression in these roots was achieved by qPCR. Positive hairy roots were then inoculated with HH103 under conditions of eBL treatment, using an equal volume of ethanol-containing nutrient solution as a control. On day 30 post-inoculation, nodulation testing was performed. Three independent experiments with 25 biological replicates each were used when assessing nodulation phenotypes.

3 Results

3.1 BRs suppress the formation of infection threads during the establishment of symbiosis

In the Williams 82 cultivar, BRs have previously been shown to inhibit soybean nodule formation and root development (Hunter, 2001). Consistently, significant reductions in nodule number and nodule dry weight were observed for the root systems of epibrassinolide (eBL)-treated soybean plants as compared to controls (Figures 1A–C). Nodule cross-section (NCS) staining with toluidine blue did not reveal any apparent differences in the numbers of cells infected within these established nodules (Figure 1). This suggests that while BRs suppress root nodule formation, their effects on the later stages of nodule development are less pronounced. Quantification of the infection threads (ITs) revealed that their numbers were significantly reduced following eBL treatment (Figures 1D, E). This supports the ability of BRs to primarily impact nodule formation through their effects on IT formation.

The role that signaling pathways downstream of BRs play in symbiosis establishment was assessed by evaluating relative symbiosis-related (*NIN1a*, *NSP1a*, and *ERN1*) and defense-related (*PR1*, *PR2*, and *PR5*) gene expression 24 h after infection. This approach revealed that treatment with BRs led to the downregulation of symbiosis-related genes together with defense-related gene upregulation (Figures 1F–H; Supplementary Figure S1). This suggests that the ability of BRs to regulate symbiosis establishment is related to their regulatory effects on both symbiosis-associated genes and defensive responses.

3.2 Identification of eBL treatment-related DEGs associated with the establishment of symbiosis

To gain additional insight into how BR signaling shapes symbiosis establishment, RNA-seq analyses of roots from DN50 seedlings inoculated with HH103 or $MgSO_4$ (mock control) under eBL or control treatment conditions were next conducted. In the absence of eBL, HH103 inoculation led to the upregulation of 727 DEGs and the downregulation of 1,234 DEGs (Supplementary Figure S2A, Supplementary Table S1). 'KEGG (Kyoto Encyclopedia of Genes and Genomes)' enrichment analyses of these DEGs revealed that the downregulated genes were primarily enriched in the fatty acid biosynthesis pathway (Figure 2A). 'GO (Gene Ontology)' enrichment analyses further indicated that downregulated DEGs were enriched in the flavonoid metabolic process, monocarboxylic acid biosynthetic process, cutin biosynthetic process, and glutathione biosynthetic process terms, together with the significant downregulation of the cutin biosynthetic process and glutathione metabolic process pathways (Figure 2B). HH103 inoculation under conditions of eBL treatment was associated with 1,206 and 1,456 upregulated and downregulated DEGs, respectively (Supplementary Figure S2A, Supplementary Table S2). KEGG enrichment analyses of these genes revealed that they were primarily enriched in the phenylpropanoid biosynthesis and plant-pathogen interaction pathways (Figure 2C), while also being enriched in the protein phosphorylation and developmental process GO terms (Figure 2D). Chitin response-related genes were significantly upregulated, while nodulation-related genes were significantly downregulated. These DEG expression patterns and enriched pathways may play important roles in the effects of BRs on IT formation.

3.3 BRs promote immune-related gene upregulation during symbiosis establishment

In order to gain a deeper insight into BR-related signaling mechanisms involved in the initiation of symbiosis and the associated genetic components, RNA-sequencing data were meticulously examined. This analysis revealed 1,074 genes that were upregulated and 1,362 genes that were downregulated in response to BRs during the symbiosis establishment process (Supplementary Figure S2B, Supplementary Tables S3, 4). These genes were primarily found to be involved in the plant-pathogen interaction and phenylpropanoid biosynthesis pathways (Figure 3A). These BR-responsive DEGs were also involved in plant cell wall organization or biology, morphogenesis-related anatomical structure formation, and nodulation process GO terms (Figure 3B).

As the establishment of a symbiotic relationship between soybean plants and rhizobia hinges on symbiotic gene activation and the inhibition of immune-related genes, and most BR-induced DEGs identified above were enriched in immunity and symbiosis-related processes, the effects of BRs on these signaling pathways were further explored by screening for genes associated with both symbiosis establishment and plant immunity under conditions of BR exposure. This approach revealed that BR treatment led to the downregulation of symbiosis-associated genes including *NIN1b*, *ENOD93*, and *ENOD40* (Figure 3C), together with the upregulation of immune genes that included *WRKY33s*, *PR1, PBS1s*, and *EDS1s* following HH103 inoculation (Figure 3D). These data offer evidence in support of the ability of BRs to inhibit symbiotic nodulation through the suppression of most symbiotic gene expression and by disrupting the ability of soybean plants to hinder immune-related gene expression in the context of symbiosis establishment.

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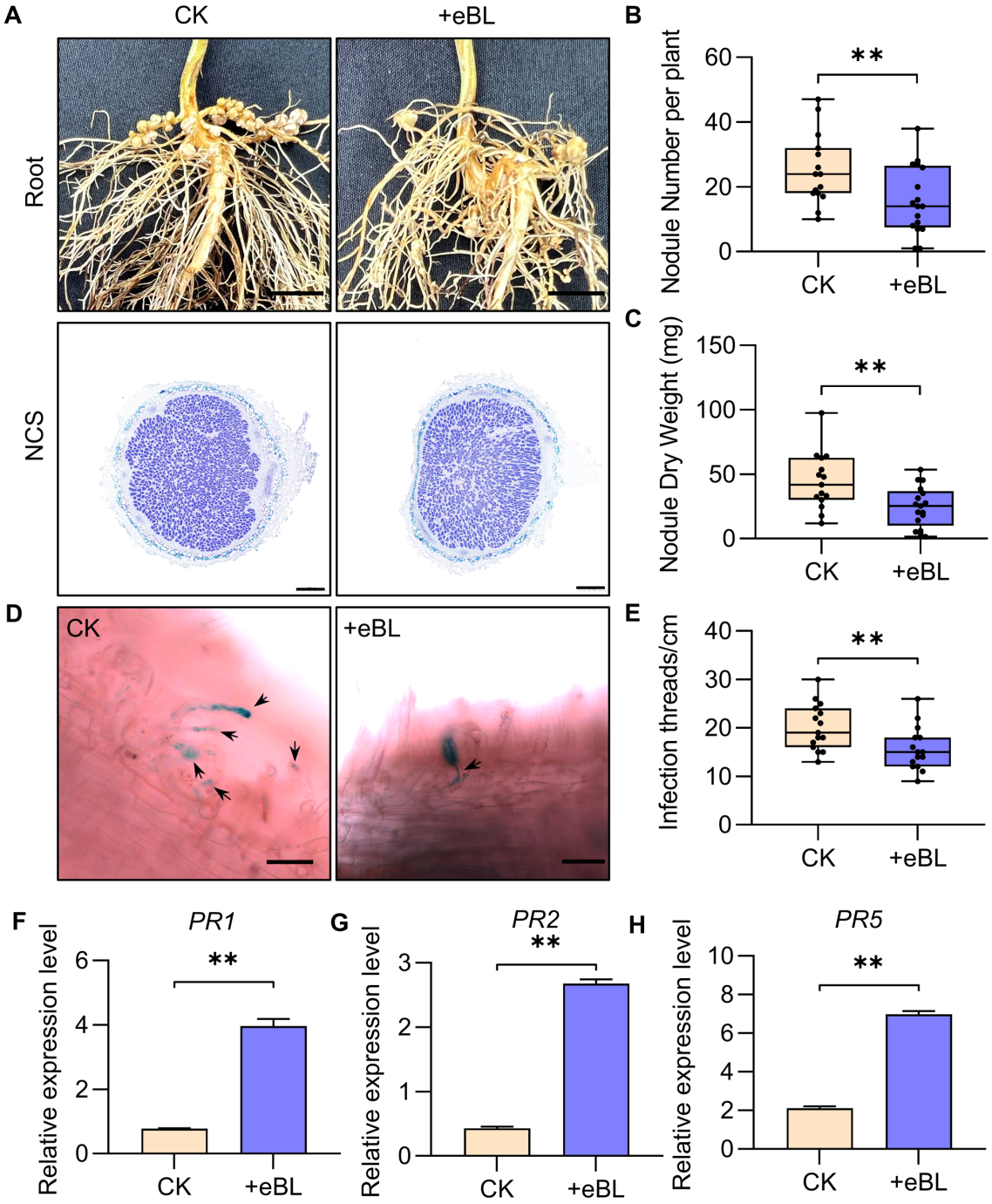

FIGURE 1
BR suppresses the formation of root nodules in DN50 soybean plants. **(A)** The root and nodular phenotypes of soybean plants subjected to eBL or CK treatment. Scale bars: 1 cm for roots, 500 μm for nodule cross-sections (NCS). **(B, C)** Nodule number **(B)** and nodule dry weight **(C)** values for the plants shown in (A). $^{**}P < 0.01$, Student's *t*-test (n=15). **(D)** Infection thread (IT) penotypes in DN50 plants inoculated with HH103-GUS on 1 dpi under eBL treatment or CK conditions. Scale bar: 50 μm. **(E)** IT numbers per cm for DN50 plants from (D). $^{**}P < 0.01$, Student's t-test (n=15). **(F–H)** Relative defense-related gene (*GmPR1*, *GmPR2*, *GmPR5*) expression under conditions of eBL or CK treatment was assessed with the $2^{-\Delta Ct}$ method, with *GmUNK1* (*Glyma.12g020500*) for normalization. Data were compared with Student's *t*-tests (n=3), $^{**}P < 0.01$.

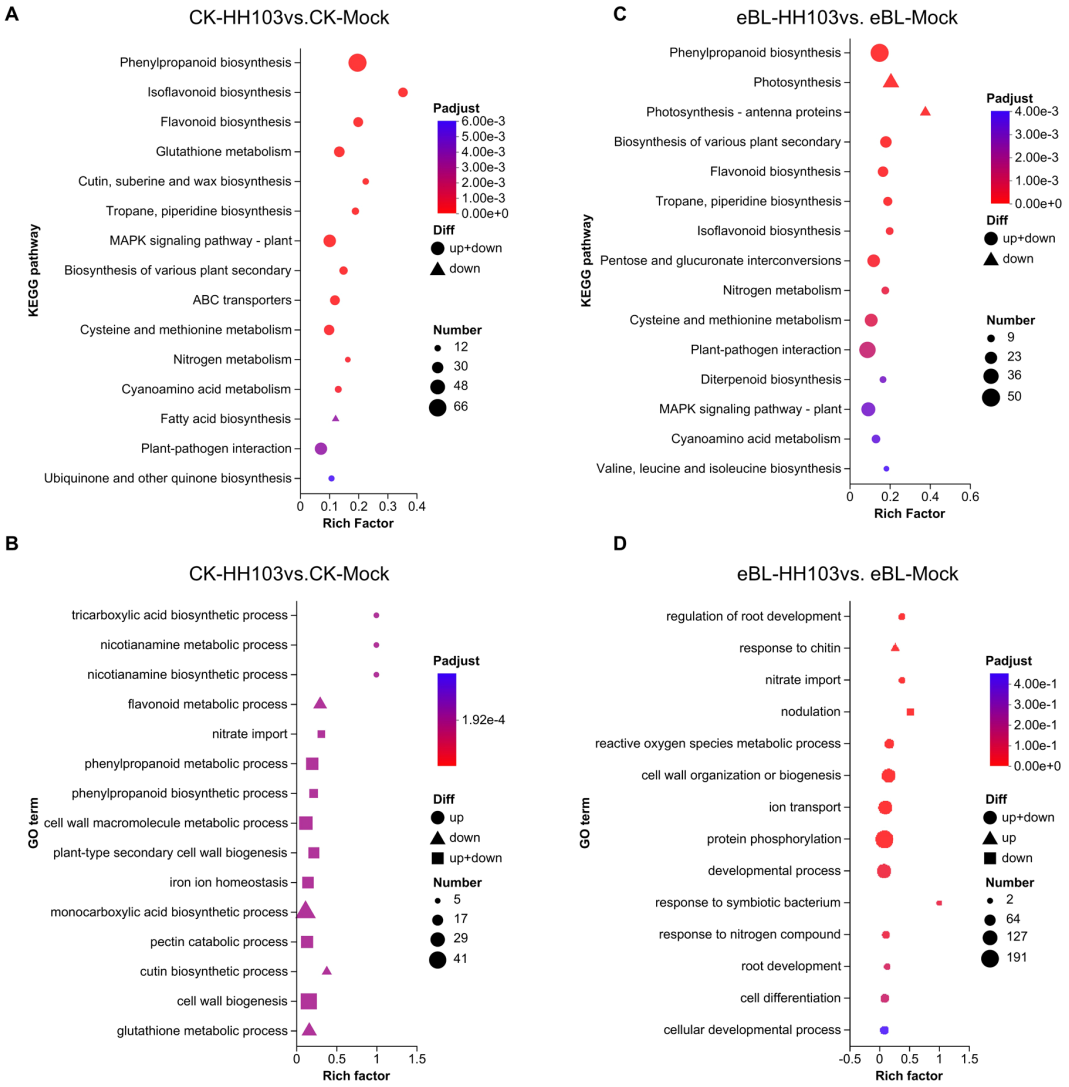

FIGURE 2
Functional enrichment analyses of DEGs identified under conditions of eBL treatment. **(A, B)** KEGG **(A)** and GO **(B)** enrichment analyses of DEGs identified when comparing the CK-HH103 and CK-Mock groups. **(C, D)** KEGG **(C)** and GO **(D)** enrichment analyses of DEGs identified when comparing the eBL-HH103 with eBL-Mock groups.

3.4 WGCNA-based identification of symbiosis-related gene expression following BR exposure

To gain further insight into the genes involved in the responses of soybean plants to BR treatment during the establishment of symbiosis, a weighted gene co-expression network analysis (WGCNA) was next conducted after screening those genes with low expression levels (FPKM < 0.01) from the RNA-seq dataset. This led to the classification of 9,671 genes into 13 co-expression clusters according to their correlations, with different colors being assigned to each module (Figure 4A). Trait-specific modules were selected at a *P*-value < 0.05, including four that were associated with HH103 responses. Of these, two modules were respectively enriched for BR signaling and HH103 responses (MEblack and MEred, respectively), with the MEblack and MEred modules respectively containing 780 and 862 genes (Supplementary Figure S3, Supplementary Table S5). Gene significance analyses of these modules revealed a significant correlation between the MEblack module and HH103 inoculation under BR treatment conditions (Supplementary Figures S4A, B), whereas it did not exhibit a significant correlation with HH103 inoculation in the absence of BR treatment. This suggests that the genes contained within the MEblack module may play a significant role in BR signaling responses during the establishment of symbiosis such that they were selected as the focus for further study. GO enrichment analyses of this gene module revealed that these genes were primarily involved in the adenyl ribonucleotide binding, adenyl nucleate binding, and protein kinase activity processes (Figure 4B). KEGG enrichment analyses also supported the enrichment of these genes in the plant-pathogen interaction and plant MAPK signaling pathways (Figure 4C; Supplementary Figures S5A, B).

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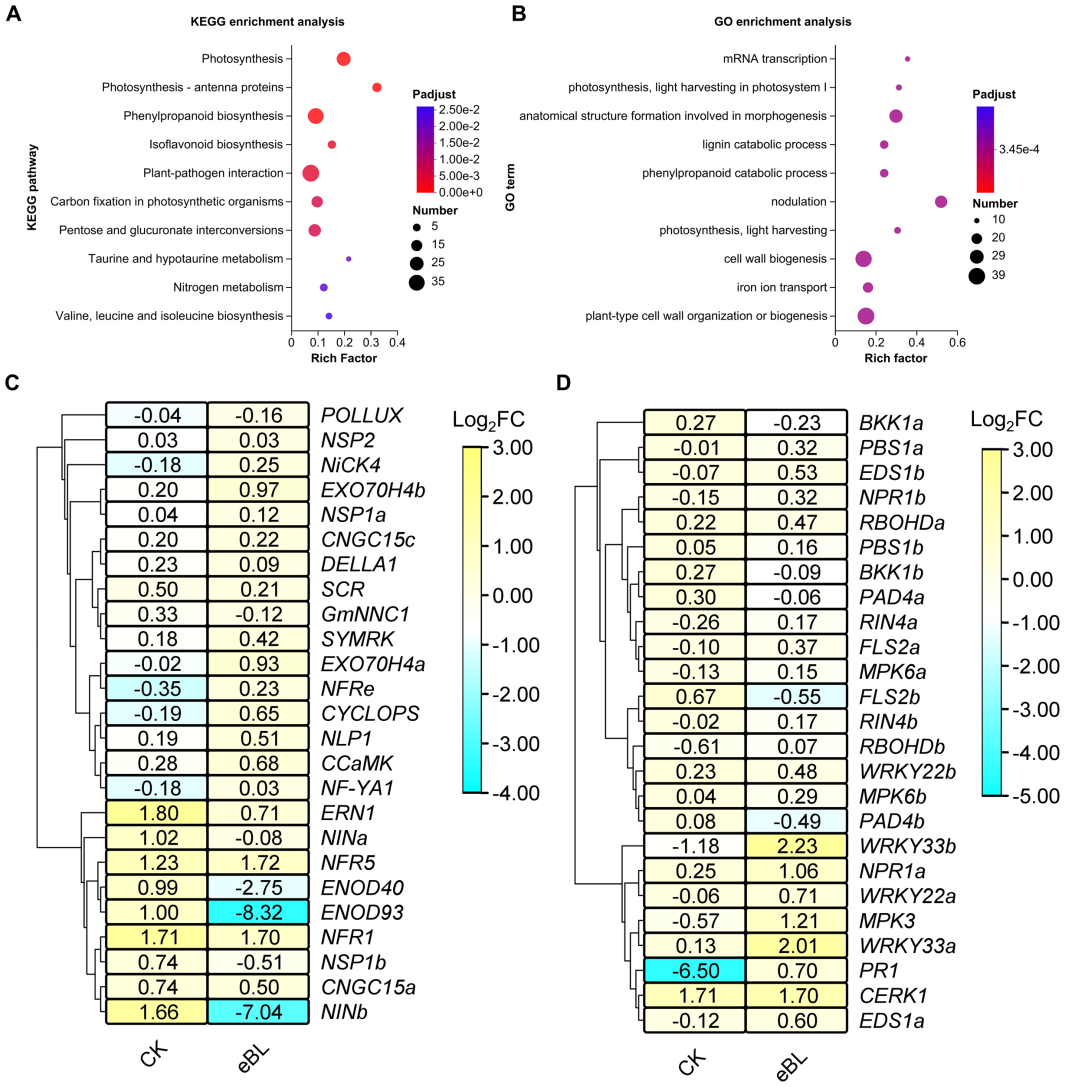

FIGURE 3
DEGs associated with responses to BR signaling in the context of symbiosis establishment. **(A, B)** KEGG **(A)** and GO **(B)** enrichment analyses of DEGs responsive to BR signaling. **(C)** A hierarchically clustered heat map of genes involved in shaping symbiosis establishment in roots under conditions of eBL treatment. Data are given as Log_2FC (Fold change) values. **(D)** A hierarchically clustered heat map of genes involved in regulating the immune status of plant roots under conditions of eBL treatment. Data are given as Log_2FC values.

3.5 *GmWRKY33a* is a hub gene responsive to BR signaling

Next, a module membership threshold of eigengene based connectivity (kME > 0.95), determined based on gene connectivity, was used to screen for the top 30 hub genes within the MEblack module, after which they were subjected to functional annotation (Supplementary Figure S6A, Supplementary Table S6). These analyses revealed that *Glyma.09G280200*, which encodes a GmWRKY33 protein (designated GmWRKY33a) exhibited high levels of connectivity and significant enrichment in the plant-pathogen interaction and plant MAPK signaling pathways (Supplementary Figure S6B). Given the importance of immune suppression in the context of symbiosis establishment, the ability of BRs to induce immune responses in leguminous plants during this process, and the status of WRKY33 as a key transcription factor associated with immune-related signaling, it was selected as the target for further study. In subsequent qPCR analyses, HH103 inoculation was found to significantly increase *GmWRKY33a* expression under conditions of eBL treatment relative to untreated plants (Figure 4D). These data are support that *GmWRKY33a* serving as a hub gene responsive to BR signaling in the establishment of symbiosis.

3.6 BR signaling suppresses nodulation via *GmWRKY33a*-mediated immune signaling activity

To determine the impact of *GmWRKY33a* on soybean symbiotic nodulation and BR signaling activity, *Agrobacterium rhizogenes* K599 carrying the antisense pB7gWWIWG2(II)-

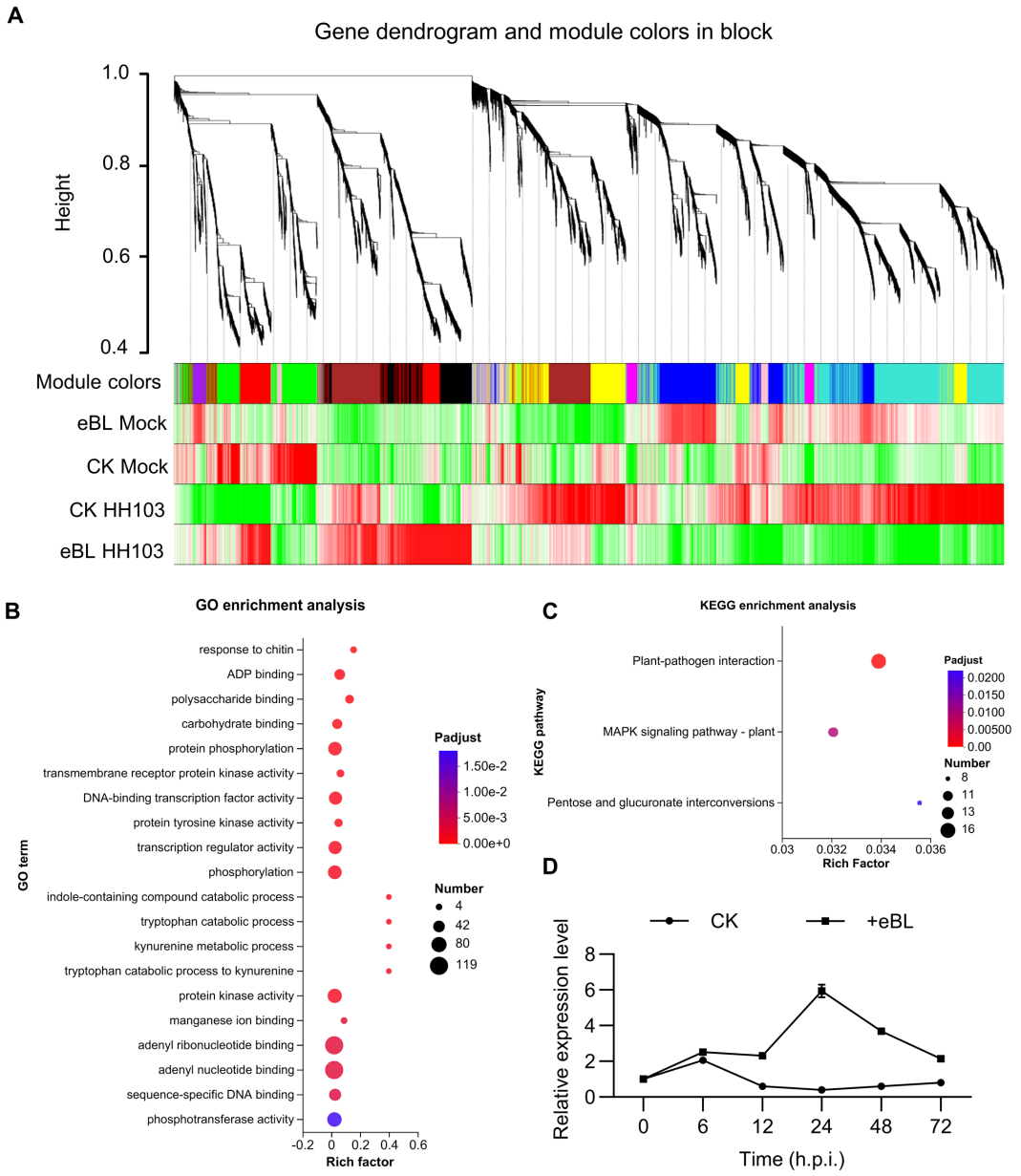

FIGURE 4
Identification of genes responding to BR after inoculation with HH103 by weighted gene coexpression network analysis. **(A)** A clustering dendrogram for genes showing the original and assigned colors of established modules. Each leaf in the dendrogram represents a gene. **(B, C)** GO **(B)** and KEGG **(C)** enrichment analyses of the genes in the MEblack module. **(D)** Relative *GmWRKY33a* expression under eBL treatment conditions during the establishment of symbiosis was assessed with the $2^{-\Delta Ct}$ method, using *GmUNK1* for normalization.

GmWRKY33a construct was used to silence this gene. Alternatively, *GmWRKY33a* overexpression (OE) was achieved with a K599 strain harboring the *GmUbi3: GmWRKY33a-GFP* construct. Soybean hairy roots were transformed with these strains or empty vector controls (EV1 or EV2), confirming successful *GmWRKY33a* OE or knockdown in these hairy roots via qPCR (Supplementary Figure S7). Nodule phenotype analyses revealed that *GmWRKY33a* silencing led to significant increases in root nodule numbers and dry weight, whereas its OE reduced the number of nodules (Figure 5), consistent with a role for GmWRKY33a as a negative regulator of the establishment of symbiosis. Following treatment with eBL, *GmWRKY33a* silencing led to less numbers and dry weight as compared to EV1 transformation, but the decrease in the nodule number and dry weight of these hairy roots relative to that for EV1, and there was no significant difference in nodule numbers for hairy roots overexpressing *GmWRKY33a* following eBL treatment relative to the corresponding control (Figure 5).

Relative symbiosis and immune-related gene expression was also analyzed at 24 h post-infection in transgenic hairy roots, revealing that silencing *GmWRKY33a* promoted *NIN1a* and *NSP1a* upregulation without affecting *ENOD40* relative to EV1, while overexpressing *GmWRKY33a* led to the downregulation of *NIN1a*, *NSP1a*, and *ENOD40* (Figures 6A–C). Transgenic hairy roots in which *GmWRKY33a* was silenced exhibited *NIN1a*, *NSP1a*,

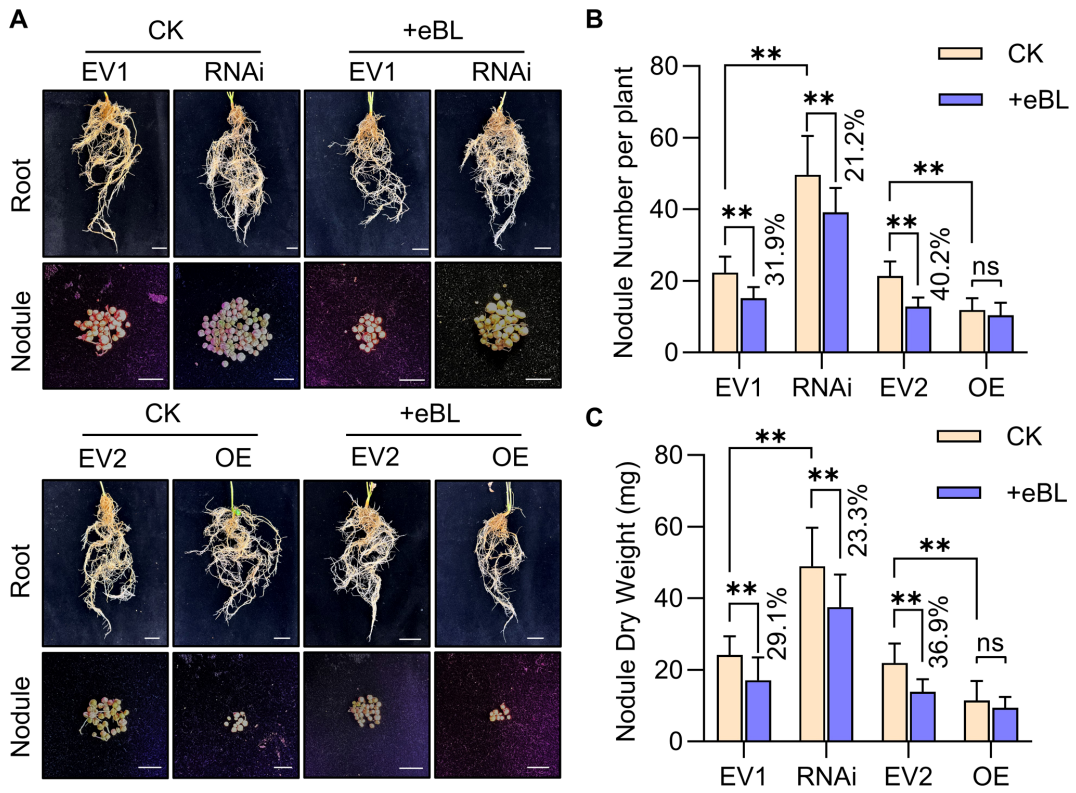

FIGURE 5
The effect of *GmWRKY33a* knockdown or overexpression on nodule phenotypes. **(A)** Nodule phenotypes were assessed for hairy roots following EV1, RNAi, EV2, or OE construct transformation and eBL or CK treatment. **(B, C)** Nodule number **(B)** and nodule dry weight **(C)** were analyzed for roots established as in **(A)**. Results were compared with Student's *t*-tests (n=25), $^{**}P < 0.01$; ns, not significant. Percentages denote decreases or increases as compared to CK.

and *ENOD40* downregulation following eBL treatment, with the downregulation of *NIN1a* and *NSP1a* being less than that for EV1 hairy roots under eBL treatment conditions (Figures 6A–C). The immune-related *PR1*, *PR2*, and *PR5* genes exhibited expression patterns opposite those of *NIN1a* and *NSP1a*, with *GmWRKY33a* silencing reducing their expression (Figures 6D–F). Treatment with eBL in soybean hairy roots in which *GmWRKY33a* was overexpressed led to increases in the expression of these target genes relative to CK (Control Check) treatment, while this increase was smaller relative to EV2 transfection (Figures 6D–F). Given the importance of WRKY33a as an immune-related transcription factor, these data support the ability of BR signaling to inhibit the establishment of symbiosis through the upregulation of *GmWRKY33a* and the modulation of *NIN1a*, *NSP1a*, and immune-related gene expression.

4 Discussion

BRs are a key class of phytohormones responsible for controlling the growth and development of plants (Santner and Estelle, 2009). The present data highlight the ability of BRs to suppress symbiotic nodule formation, although the effects of BRs on nodulation have been reported to vary across leguminous species such that they are positive and negative in particular contexts. These effects may be attributable to differences in BR concentration, BR composition, differences in growth states, or rhizobia genotypes, and the specific genotypes that regulate these effects are poorly understood. Previous studies have demonstrated that BR signaling via GmBES1 suppresses GmNSP1 activity and adjusts NF signaling to affect symbiosis (Chen et al., 2023), while this study suggests that BR signaling can activate immune-related gene expression to suppress the establishment of symbiosis. As establishing symbiosis necessitates activating plant symbiotic signaling while inhibiting certain immune-related genes, this may partially account for the dual roles that BRs exert in pea plants during different stages of nodulation. The GmBS1-1 homolog GmBEHL1 is also capable of interacting with GmNNC1, thereby inhibiting the establishment of symbiosis and affecting nodule numbers and IT numbers (Yan et al., 2018). These data indicate that BRs may thus regulate nodulation in legumes through a range of signaling processes and associated mechanisms.

Here, preliminary analyses revealed that BRs were capable of promoting *GmPR1*, *GmPR2*, and *GmPR5* upregulation. Additional transcriptomic analyses indicated that these BRs were capable of inducing the expression of many immune-related genes in DN50 plants in the context of symbiosis establishment. A growing body of evidence suggests that the failure to repress the expression of these genes can compromise the establishment of symbiosis between soybean plants and rhizobia. BAK1 is an important regulator of pathogenesis in plants, serving as a co-receptor for many microbial

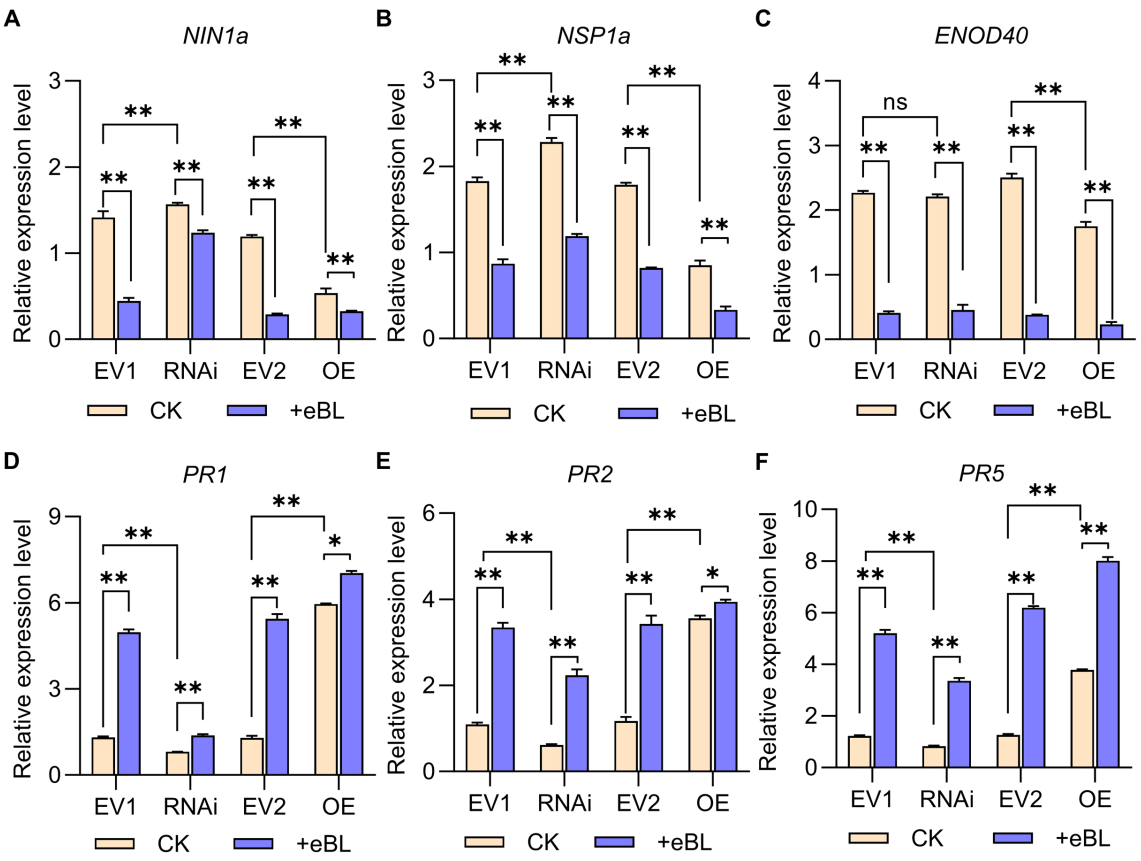

FIGURE 6
Relative symbiosis and immune-related gene expression. **(A–F)** the $2^{-\Delta Ct}$ method was employed to assess relative expression (*NINa*, *NSP1a*, *ENOD40*, *PR1*, *PR2* and *PR5*), with *GmUNK1* (*Glyma.12g020500*) for normalization. Data were compared with Student's *t*-tests (n=3), $^{*}P < 0.05$; $^{**}P < 0.01$; ns, not significant.

pattern recognition receptors that coordinates pathogen responses and serves as a key BR signaling-related receptor (Halter et al., 2014; Shang et al., 2016; Imkampe et al., 2017). Once symbiosis has been established, SymRK interacts with and inhibits BAK1 to strike a balance between symbiosis and pathogenesis (Feng et al., 2021). The upregulation of the *GmMPK3* and *GmMPK6a/b* kinases downstream of BAK1 is indicative of BAK1-mediated immune response activation. The dysregulation of BAK1 in the roots of soybean plants during the establishment of symbiosis may account for the lower numbers of nodules and ITs observed in this study. BR treatment can induce the expression of the salicylic acid (SA) signaling pathway marker *GmPR1* during symbiosis establishment, and BR signaling in *Arabidopsis* can coordinate SA responses and shape host immunity (Liu et al., 2022). BR-induced SA receptor *GmNPR1* expression during symbiosis establishment also suggests the ability of BR signaling to shape SA signaling and to synergistically modulate interactions between soybean roots and rhizobia. This BR signaling can influence SA signaling-related immune functionality in these plants, highlighting an important topic for further study. PBS1 can serve as another important receptor related to plant immune activity (Swiderski and Innes, 2001), interacting with NopT, a rhizobial type III effector, to control soybean immunity during rhizobial infection. *GmPBS1* is another hub gene that impacts the responses of soybean plants to the rhizobial type III effectors NopT and NopP (Khan et al., 2022; Li et al., 2023), with its significant upregulation in response to BR treatment supporting the ability of these phytohormones to modify the susceptibility of legume plants to pathogen infection, altering downstream immune signaling activity and thus explaining the ability of BR signaling to inhibit nodulation. Whether rhizobial type III effectors, which are important signals for the establishment of symbiosis, and involved in or impact BR signaling and the degree to which they are involved in immune activation mediated by BR during this process will need to be studied at length in the future. Notably, BR treatment suppressed many key symbiosis-related genes including *NIN1s*, *ERN1*, *ENOD40*, and *ENOD93*, although it did upregulate some symbiotic receptors, such as *SymRK* and *NFR5*, suggesting a need for additional mechanistic research aimed at better understanding these findings.

Through transcriptomic WGCNA analyses, genes included in the MEblack module were found to be primarily associated with responses to BR signaling and the control of soybean immune signaling against rhizobium during symbiosis establishment. Of the targets within this module, GmWRKY33a was previously identified as an important regulator of pathogen resistance that serves as a substrate for CDPK5/6 and MPK3/6 (Zhou et al., 2020). While some reports have found *GmWRKY33* to be upregulated during symbiosis establishment, the mechanisms through which it impacts symbiotic signaling have not

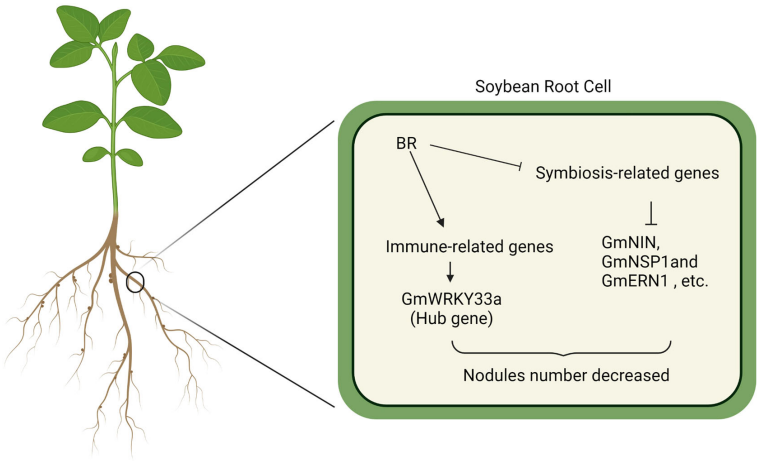

FIGURE 7
GmWRKY33 acts as a key BR signaling-related transcription factor, negatively regulating the establishment of symbiosis.

been clarified. Here, GmWRKY33 silencing was found to attenuate the impact of BR on nodule numbers, suggesting that knocking down *GmWRKY33a* may disrupt BR-driven immune signaling during symbiosis establishment, thereby limiting the impact of this phytohormone on symbiotic nodulation. GmWRKY33a is a transcription factor that controls *PR* gene expression (Liu et al., 2018), and treatment with BR induced *GmWRKY33a* expression, at least partially explaining the extensive *PR*-related gene upregulation observed in the RNA-seq dataset. In contrast, *GmWRKY33a* overexpression was associated with the significant downregulation of *NIN1a*, *NSP1a*, and *ENOD40a* in soybean hairy roots relative to EV2 control roots, potentially owing to the ability of GmWRKY33a to promote the upregulation of many immune-related genes and the accumulation of immune-related metabolites, ultimately leading to the loss of homeostatic balance between immunological and commensal signaling regulation in this context. *GmWRKY33a* thus appears to serve as a central hub mediator of BR signaling when symbiosis is being established. *GmWRKY33a* is also a SNAP1/2, SNAP1/2/4, and SNAP1/2/3/4 target (Wang et al., 2023b), although its potential involvement in SNAP-mediated root nodule responses to nitrogen and how BR signaling affects this involvement will need to be studied at length in the future.

In addition to *GmWRKY33a*, the MEblack hub genes *Glyma.01G224800* and *Glyma.05G215900* encode two additional WRKY transcription factors. As WRKY family transcription factors are essential for the regulation of the ability of plants to respond to microbial infections. The patterns of *Glyma.01G224800* and *Glyma.05G215900* expression in response to BR signaling may be similar to those of *GmWRKY33a*. Moreover, *Glyma.02G023800*, *Glyma.05G082500*, and *Glyma.16G136200* were identified as soybean hub genes that were responsive to BR signaling in the setting of symbiosis establishment, encoding leucine-rich repeat-containing disease resistance proteins (CC-NBS-LRR-like). R proteins are directly involved in plant immune responses, and the upregulation of certain R proteins following BR treatment may thus represent one additional process through which BR influences symbiosis. These include the downstream target of GmBZL3, *Glyma.05G082500*, which encodes a BZR1-like protein that serves as a key BR signaling regulator potentially involved in BR responses through the recognition of rhizobial PAMPs (Song et al., 2019), thus leading to the activation of various immune responses following the establishment of symbiosis.

5 Conclusion

The present analysis indicates that BR treatment is sufficient to inhibit the establishment of symbiosis, leading to reduced numbers of nodules and infection threads (IT) in soybean roots. BR signaling induces host immunity, with many differentially expressed genes (DEGs) enriched in immunity-related pathways. Further WGCNA identified *Glyma.09G280200*, which encodes the GmWRKY33 protein, as a central gene involved in BR signaling responses during symbiosis establishment. GmWRKY33 acts as a key BR signaling-related transcription factor, negatively regulating the establishment of symbiosis (Figure 7). In summary, these data highlight a novel mechanism through which BR signaling activity governs symbiosis, providing a foundation for efforts to select soybean varieties with more efficient nitrogen fixation.

Data availability statement

All raw sequencing data have been deposited at the NCBI Sequence Read Archive Archive https://www.ncbi.nlm.nih.gov/sra, PRJNA1124407.

Author contributions

MY: Conceptualization, Methodology, Writing – original draft. CLe: Data curation, Investigation, Visualization, Writing – original draft. CM: Data curation, Investigation, Visualization, Writing – original draft. XH: Data curation, Investigation, Visualization,

Writing – original draft. MY: Data curation, Investigation, Visualization, Writing – original draft. LM: Data curation, Investigation, Visualization, Writing – original draft. EL: Data curation, Investigation, Visualization, Writing – original draft. LX: Data curation, Investigation, Visualization, Writing – original draft. SW: Data curation, Investigation, Visualization, Writing – original draft. CLi: Conceptualization, Methodology, Supervision, Writing – review & editing. QC: Conceptualization, Methodology, Supervision, Writing – review & editing. DX: Conceptualization, Methodology, Supervision, Writing – review & editing. CX: Conceptualization, Methodology, Supervision, Writing – review & editing. JW: Conceptualization, Funding acquisition, 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.

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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.2024.1507307/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Relative NIN1a, NSP1a, and ERN1 expression. The $2^{-\Delta Ct}$ method was employed to assess relative expression, with *GmUNK1* (*Glyma.12g020500*) for normalization. Data were compared with Student's *t*-tests ($n=3$), $^{*}P < 0.05$; $^{**}P < 0.01$; ns, not significant.

SUPPLEMENTARY FIGURE 2
Identification of DEGs in DB50 roots under conditions of eBL treatment or control conditions. **(A)** Numbers of DEGs at 1dpi with HH103-GUS under eBL treatment or CK conditions. **(B)** Venn diagrams highlighting the numbers of DEGs detected in DN50 samples under conditions of eBL or CK treatment.

SUPPLEMENTARY FIGURE 3
Module-trait linkage WGCNA analyses. Rows correspond to module-trait genes. Columns correspond to treatments (HH103 inoculation status, eBL treatment status). Cells contain the correlation coefficient and P-values, with color coding as indicated.

SUPPLEMENTARY FIGURE 5
KEGG pathway maps for MEblack module genes. Genes from the MEblack module exhibited enrichment in the plant-pathogen interaction **(A)** and plant MAPK signaling **(B)** pathways.

SUPPLEMENTARY FIGURE 6
MEblack module hub genes. **(A)** 30 hub genes from the MEblack module. **(B)** Phylogenetic tree analysis of GmWRKY33a.

SUPPLEMENTARY FIGURE 7
Relative gene expression in transgenic hairy roots following *GmWRKY33a* silencing or overexpression. $^{**}P<0.01$; ns, not significant; Student's *t*-test. Error bars indicate the standard deviation.

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Interaction between arbuscular mycorrhizal fungi and dark septate endophytes in the root systems of *Populus euphratica* and *Haloxylon ammodendron* under different drought conditions in Xinjiang, China

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Background and Aims: Arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) are known to enhance the tolerance of host plants to biotic and abiotic stresses, but the mechanism of their interaction under natural conditions has not been extensively studied.

Methods: We analyzed the endophytic fungal diversity and colonization characteristics in the typical desert plants *Populus euphratica* and *Haloxylon ammodendron* and the relationship between them and environmental factors.

Results: Except for DSE in the roots of *H. ammodendron*, the colonization rates of AMF and DSE were significantly positively correlated with drought severity. The abundance of AMF and DSE under medium and mild drought conditions was greater than that under severe drought conditions. The root colonization rate and abundance of AMF were lower than those of DSE under the same drought conditions. The species diversity and abundance of AMF and DSE in *P. euphratica* were greater than those in *H. ammodendron*. AMF were more susceptible to soil factors such as soil water content, soil nitrogen and phosphorus content, and urease, whereas DSE were more affected by pH.

Conclusion: Drought stress has different effects on AMF and DSE in the roots of *P. euphratica* and *H. ammodendron*. DSE have a greater advantage in extremely arid environments. This study demonstrates the interaction between AMF and DSE with the host plants *P. euphratica* and *H. ammodendron* as well as their effects on the adaptation of host plants to the desert environment, which can provide a basis for strengthening desert vegetation management.

KEYWORDS

arbuscular mycorrhizal fungi, dark septate endophytes, colonization strategy, colonization status, rhizosphere effect

1 Introduction

In natural ecosystems, most plants coexist with endophytic fungi, which can effectively promote plant growth and adaptation to stress (Rodriguez et al., 2009). During the long-term succession and evolution of arid desert plants, a series of special strategies for survival in arid environments have developed (Huo et al., 2022). The association between plants and endophytic fungi has also been recognized as a key strategy for desert plants to adapt to stressful environments (Liu et al., 2023), that is, endophytic fungi play an important role in host resistance to drought and other stresses.

Arbuscular mycorrhizal fungi (AMF) are widely distributed soil microorganisms that can form symbioses with a variety of plants (Gai et al., 2006), promote the growth of other soil microorganisms (Sun and Shahrajabian, 2023), transport mineral elements and water to the host plant, and improve the overall stress resistance of plants (Tariq et al., 2023), enabling plants to better survive in desert environments with scarce water and nutrient deficiency (Madouh and Quoreshi, 2023). Therefore, AMF play an important role in desert vegetation and ecological restoration (Alrajhi et al., 2024). As symbionts, dark septate endophytes (DSE) can enhance the absorption of nutrients—such as nitrate, phosphorus, and micronutrients—by plants (Farias et al., 2020; Wang et al., 2023), so that more nutrients are available to the host plants (Mandyam and Jumpponen, 2008). Moreover, the DSE are able to form the "microsclerotia" structure (Madouh and Quoreshi, 2023). Under adverse conditions, DSE increase host plants tolerance to biotic and abiotic stresses by activating physiological and biochemical responses (Ban et al., 2017). Their wide host range, especially its distribution in extreme environments, makes DSE no less ecologically significant than AMF and has potential applications value in ecological conservation and vegetation restoration and more (Malicka et al., 2022). Under natural conditions, AMF and DSE often co-infect plant roots, and their biological characteristics and ecological functions can play a positive role in ecological reconstruction and vegetation restoration after damage (Huo et al., 2021; Xie et al., 2024).

In arid and semiarid zones, changes in the colonization of plant roots by endophytes are mainly caused by changes in soil moisture (Ayala et al., 2023). *P. euphratica* and *H. ammodendron* are, respectively, typical arboreal and shrubby species in oases of arid regions (Tan et al., 2024). *P. euphratica* can regulate climate, prevent sandstorms and desert expansion, and protect oases (Yao et al., 2024). *H. ammodendron* can reduce wind speed, improve forest microclimate, and promote the settlement and growth of other desert plants (Yang and Lv, 2023). Therefore, they play an important role in maintaining the structure and function of the ecosystem. *P. euphratica* and *H. ammodendron* have formed stable colonization structures with AMF and DSE (Dang et al., 2022; Li et al., 2022). Moreover, the two plants have been extensively studied in previous studies, but there is a lack of comprehensive understanding of the colonization patterns of AMF and DSE fungi that colonize simultaneously in the roots of *P. euphratica* and *H. ammodendron*. Based on this, this study considers *P. euphratica* and *H. ammodendron* in the Ebinur Lake Basin as research objects to study the diversity of endophytic fungi, colonization status of the plant root system, and the relationship between colonization status and environmental factors under different moisture conditions. The following questions were raised: (1) What are the differences in the colonization rates and morphological structures of AMF and DSE under different drought conditions? Is there an interaction between these two mycorrhizal fungi, such as competition or mutualistic symbiosis? (2) What are the differences in AMF and DSE species compositions in rhizosphere and roots under different drought conditions? (3) What are the main influencing soil physical and chemical factors causing differences in AMF and DSE colonization across drought gradients?

2 Materials and methods

2.1 Overview of the study area

Located in Xinjiang Uygur Autonomous Region in northwestern China, Ebinur Lake Wetland National Nature Reserve (EWNR) is the lowest depression and water-salt aggregation center of Ebinur Lake Wetland on the western edge of the Gurbantunggut Desert (44°30′-45°09′N, 82°36′-83°50′E), with a typical desert ecosystem. The annual maximum temperature is 44°C and the minimum temperature is -34°C. The average annual precipitation is less than 100 mm, whereas the annual evaporation is 16 times the average annual precipitation (Li et al., 2023). Ebinur Lake is the largest saltwater lake in Xinjiang. The soils in this basin are mostly grey desert soil, grey brown desert soil, and aeolian sandy soil with severe salinization (Chen et al., 2023). The dominant plant communities are the salt and drought stress tolerant plants such as *P. euphratica*, *H. ammodendron*, and *Tamarix chinensis* (Wang et al., 2022).

2.2 Collection of samples

2.2.1 Collection of plant root samples

In May 2023, a sample transect was established north of the Dongdaqiao Management Station within the typical arid desert reserve in the Ebinur Lake Basin, perpendicular to the bank of the Aqikesu River for approximately 500 m, towards the Kumtag Desert. Based on different natural drought conditions, we selected high, medium, and low moisture gradients from near to far at intervals of 1,200 m from the riverbank (representing mild, medium, and severe droughts, respectively), labelled W1, W2, and W3 from high to low moisture. All three sampling points are located in areas with gentle terrain. The main vegetation includes *P. euphratica*, *H. ammodendron* and other herbaceous plants such as *Ceratocarpus arenarius* and *Nitraria tangutorum*. From a high to a low moisture gradient, the height, crown width, and abundance of plants decrease. Three repeated plots were taken for each moisture gradient, and three *P. euphratica* and three *H. ammodendron* plants with similar growth conditions were selected as replicates from east to west in each plot, totaling nine plots, and 54 samples were collected.

Root samples were collected from the concentrated distribution area of fine roots within the 0-20 cm soil layer around the trunk of the tree (Xia et al., 2015), divided, labelled, collected in sterile bags,

and immediately sealed and refrigerated. Among the 27 trees, two root samples were taken from each plant for a total of 54 fine root samples, of which 27 root samples were stored at -20°C for the determination of mycelial colonization rate, and the remaining 27 samples were stored at -80°C for DNA extraction. Due to scarce precipitation, local soil water and groundwater are mainly recharged by rivers, and the depth of groundwater decreases with distance from the Aqikesu River, resulting in significant changes in the soil water content gradient (Yang et al., 2022). We measured the water content of the 0-20 cm soil layer in the subsurface. The results showed that in May, the water content of W1, W2, and W3 was 16.54% ± 2.27%, 8.43% ± 1.61%, and 4.12% ± 1.54%, respectively.

2.2.2 Collection of soil samples

The samples were taken to the laboratory, and the surface soil adhering to the roots was shaken off. The root samples were transferred to a sterile 50 ml centrifuge tubes, 20 ml of sterilized 10 m*M* PBS solution was added, the shaker speed was set to 120 rpm, and the centrifuge tubes were placed at room temperature for 20 min. Sterile tweezers were used to remove the root system, the centrifuge speed was set to 6,000 × g, and the remaining suspension was centrifuged at 4°C for 20 minutes. The precipitate obtained after centrifugation was the required rhizosphere soil (He et al., 2021). There were eighteen samples per moisture gradient, for a total of fifty-four soil samples. The shaken soil was then placed in numbered ziplock bags at room temperature.

2.3 Determination of soil physical and chemical properties

The soil was collected in an empty aluminum box that had been weighed beforehand, weighed in the field, brought back to the laboratory, dried. The dry soil was weighed to calculate the soil moisture content (SWC). The shaken soil was dried naturally and then sieved for analysis of soil physical and chemical properties, including soil organic carbon (SOC), electrical conductivity (EC), soil pH (pH), total phosphorus (TP), total nitrogen (TN), available phosphorus (AP), ammonium nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), urease (UA), and alkaline phosphatase (ALP). The specific methods were as follows (Bao, 2000): organic carbon content was determined by high temperature exothermic potassium dichromate oxidizing capacity method; soil conductivity was measured by precision conductivity meter; pH value was measured by a pH meter in the solution with a water soil ratio of 2.5:1; soil total phosphorus content was determined by $HClO_4$-H_2SO_4 Mo-Sb colorimetric method; the content of soil total nitrogen was determined by H_2SO_4 mixed accelerant digestion; and available nitrogen content was determined by $NaHCO_3$ leaching Mo-Sb colorimetric method; KCl leaching-indophenol blue colorimetric method for the determination of soil ammonium nitrogen content; colorimetry method with phenol disulfonic acid for the determination of soil nitrate nitrogen content; phenol-sodium hypochlorite colorimetric method for the determination of soil urease content; and alkaline phosphatase content in soil was determined by phenol-sodium hypochlorite colorimetric method.

2.4 Determination of AMF and DSE colonization

Sufficient fine roots were randomly selected from the root samples. The cells were stained using the Phillips and Hayman staining method (Phillips and Hayman, 1970). The structures of AMF and DSE were observed under a microscope (Nikon ECLIPSE Ti2, Japan), and photographs of the arbuscules, hyphae, vesicles of AMF and hyphae, and microsclerotia of DSE were obtained. The root sample observation method and mycorrhizal colonization grading criteria were used to calculate the root colonization rate (F%), colonization density (M%), and arbuscule abundance(A%) (Trouvelot et al., 1986). Microsclerotia colonization rate (MS%) was measured and calculated using the cross method under a 200x microscope (McGONIGLE et al., 1990). F% = number of infected root segments/total number of microscopic examination root segments* 100%. M% = (0.95 * N5 + 0.7 * N4 + 0.3 * N3 + 0.05 * N2 + 0.01 * N1)/total number of root segments examined by microscopy* 100%, where 0.95, 0.7… respectively represent the weight of each level. N5=sum of fifth-level root segments, N4, N3, N2, and N1 have the same meaning. A% = sum of the intersection points of arbuscule colonization/sum of cross colonization points* 100%. MS% = number of cross-colonization points/sum of cross-colonization points * 100%.

2.5 Determination of endophytic fungal diversity

Fungal DNA extraction and detection were completed, followed by PCR amplification using ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC) primers (Gardes and Bruns, 1993; Lekberg et al., 2018; Wang et al., 2024), and the PCR products were analysed using QuantiFluor ™ - ST blue fluorescence quantitative system (Promega Corporation), followed by the construction of an Illumina library and Illumina sequencing. DNA extraction and sequencing were performed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) to rapidly detect target strains. Then we used UPARSE platform version 7.1 to analyze the obtained gene sequences. To improve the accuracy and stability of the results, we clustered the data sequences and eliminated all chimeric sequences, ensuring that those with 97% similarity were grouped into the same operational taxonomic unit (OTU) to optimize the features of these datasets. An in-depth comparative analysis of the SILVA rRNA database (Release 138 https://www.arb-silva.de/) with the Unite eukaryotic ITS region database (Release 8.0 http://unite.ut.ee/index.php) was performed. The Majorbio cloud computing platform (http://cloud.Majorbio.com) was used to analyze the microbial communities in the soil. The initial data was stored in NCBI Sequence Read Archive database with the accession number PRJNA1046269. The data were initially organized and statistically analysed. By integrating the AMF and DSE taxonomic information and references, we created a species abundance table using the Majorbio platform to screen and download biodiversity information and facilitate subsequent data analysis.

2.6 Processing and analysis

SPSS 27 (IBM Corporation, Armonk, NY, USA) was used to perform normality and homogeneity of variance tests on the colonization rates of AMF and DSE, and the colonization rates under different water gradients were compared using multiple group comparisons (Kruskal-Wallis test). Bar charts were plotted using Excel to represent the status of the rhizosphere soil physical and chemical factors in *P. euphratica* and *H. ammodendron*. The ggcor package for R (version 4.3.1) was used to analyze the correlation between fungal colonization in rhizosphere and roots of *P. euphratica* and *H. ammodendron* and soil physical and chemical factors. Heat maps were drawn using the ggpubr, corrplot, and ggplot2 packages to visualize the correlation between the AMF and DSE colonization status. AMF and DSE with different gradients in rhizosphere and roots were classified and visualized by genus using circular and statnet packages for R. Redundancy analysis (RDA) between AMF and DSE colonization status and soil physical and chemical factors in the roots of the two plants was performed using CANOCO 5. Prior to RDA analysis, a species-sample analysis was done using CANOCO 5 to determine the choice of RDA analysis. The collinearity degree between soil physical and chemical factors was assessed by variance inflation factor (VIF) analysis using the R package (version 4.3.0) to ensure that the respective VIF values for all factors were less than 10.

3 Results

3.1 Soil physical and chemical factors and root AMF and DSE colonization structure characteristics

3.1.1 Analysis of physical and chemical factors in rhizosphere soil

A one-way ANOVA was performed to analyze the significant differences in the physical and chemical properties of the soil of *P. euphratica* (Figure 1). SWC decreased with the intensification of drought, and there was a significant difference among the three gradients. Both NH_4-N and UA decreased with increasing drought severity, and there were significant differences ($P<0.05$) between the contents of the high and low moisture gradients. There was no significant difference between the medium moisture gradient and the other two gradients. The NO_3-N, content in the high-moisture gradient was significantly higher than those in the medium- and low-moisture gradients ($P<0.05$). EC, pH, SOC, and ALP decreased as the moisture content decreased, but these changes were not significant. TN, TP, and AP decreased with increasing drought severity and the content in the high water gradient were significantly higher than those in the other two gradients ($P>0.05$).

The result of one-way ANOVA in the *H. ammodendron* soil (Figure 2) showed that the variation patterns among SWC, TN, TP, NH_4-N, and AP were the same with significantly greater content under high moisture gradient than in the remaining two gradients ($P<0.05$). There was a significant difference in the UA between the high and low moisture gradients ($P<0.05$), whereas there was no significant difference between the medium moisture gradient and the other two gradients. There were no significant differences in EC, pH, SOC, NO_3-N, or ALP among the three moisture gradients.

In summary, all physical and chemical factors in soils of the two plants were higher in the high-moisture gradient than in the other two gradients. All physical and chemical factors decreased with a reduction in soil water content, except for NO_3-N in the rhizosphere soils of *P. euphratica*. TP, TN, and AP were significantly higher ($P<0.05$) in the high moisture gradient than in the medium and low moisture gradients, whereas there were no significant differences between the medium and low moisture gradients.

3.1.2 Colonization characteristics of AMF and DSE fungi

AMF and DSE colonization structures were observed in *P. euphratica* and *H. ammodendron* (Figures 3, 4). AMF and DSE were widely present in the roots of *P. euphratica* and *H. ammodendron*. AMF formed typical hyphae, vesicles, and arbuscule. The size and shape of vesicles varied, with both circular and elliptical shapes appearing. DSE formed dark septate hyphae and microsclerotia. The mycelia were mostly brown, and obvious mycelial meshes formed along the longitudinal axis of the roots. Microsclerotia were formed by closely packed and enlarged cells with thickened cell walls, with varying degrees of shape, size, and density. The color of the same microsclerotial cluster was brown with varying shades.

3.2 Differences and correlation in colonization rates between AMF and DSE in roots

It can be seen that the AMF colonization rate increased significantly from 13.89% to 71.83% in the roots of *H. ammodendron* from high to low water gradients (Table 1). The arbuscule abundance of *P. euphratica* decreased with a decrease in soil moisture, whereas that of *H. ammodendron* increased with a decrease in soil moisture. From high to low water gradients, the DSE colonization rate increased significantly from 53.24% to 92.41% in the roots of *P. euphratica*, and decreased significantly from 67.10% to 45.06% in the roots of *H. ammodendron* (Table 2). Similar to *P. euphratica*, the DSE colonization rate and density of *H. ammodendron* in the medium water gradient were the lowest among the three gradients, and the number of microsclerotia was the highest among the three gradients. In most cases, both AMF and DSE had higher colonization rates under low water gradient conditions, indicating that a certain degree of drought promotes the formation of mycorrhizal structures between AMF, DSE and plant roots.

Under the same water gradient, the colonization status of AMF and DSE in *P. euphratica* and *H. ammodendron* differed. Under a high water gradient, the AMF colonization rate of *P. euphratica* was significantly higher than that of *H. ammodendron*. AMF were not detected in the roots of *P. euphratica* under a medium water gradient, but were detected in all water gradients of *H.*

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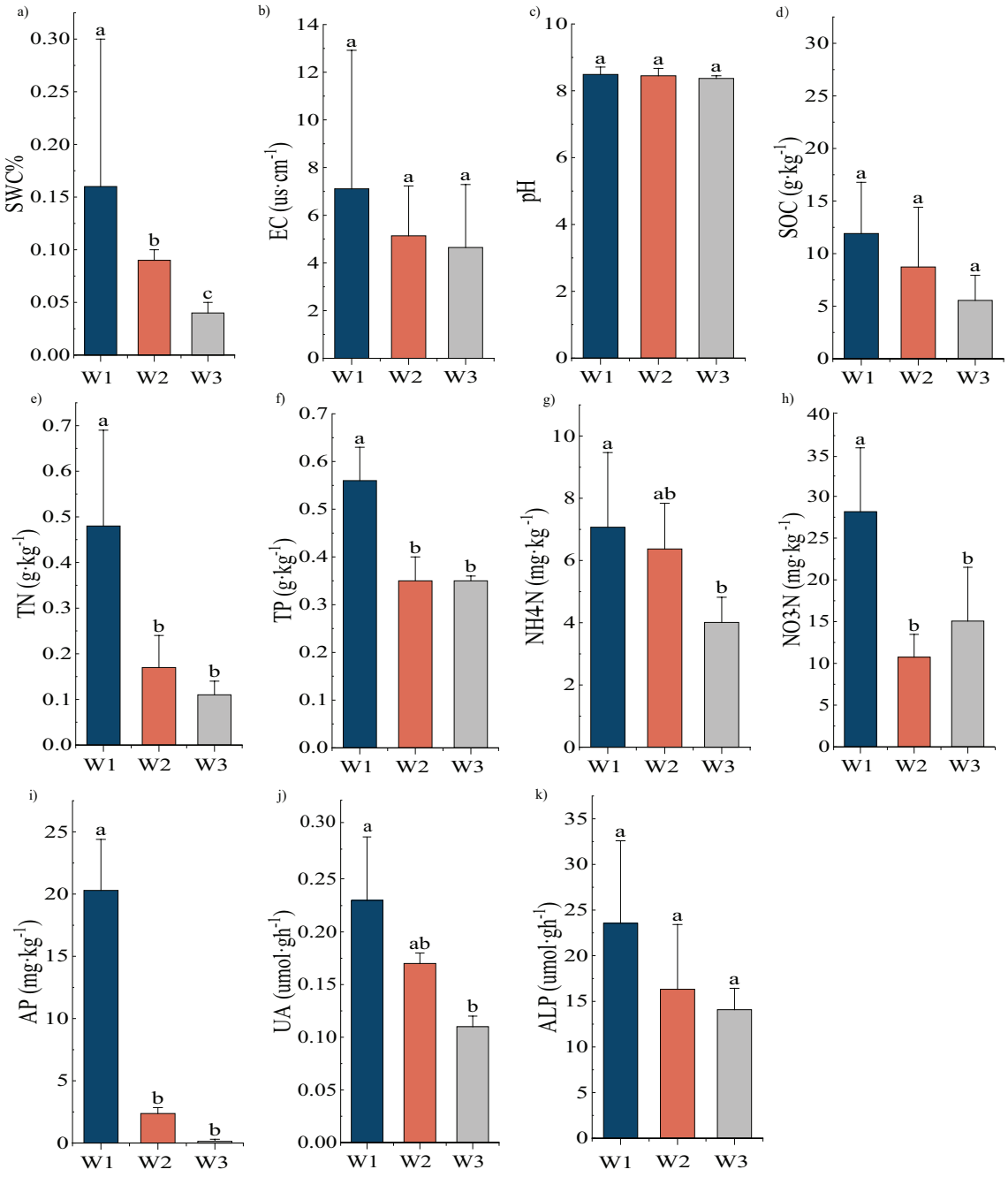

FIGURE 1
(A–K) Differences in physical and chemical properties of soil of *Populus euphratica* (mean ± SD, n=3). Different lowercase letters marked above the bars indicate significant differences in the physical and chemical properties of plant soil ($P<0.05$).

ammodendron. The DSE colonization rate of *P. euphratica* in the medium water gradient was significantly higher than that of *H. ammodendron*. Under a low water gradient, the DSE colonization rate, colonization density, and number of microsclerotia in the roots of *P. euphratica* were significantly higher than in *H. ammodendron*.

Different interactions were observed between DSE and AMF in the roots of *P. euphratica* and *H. ammodendron* (Figure 5). For example, in the roots of *P. euphratica*, AMF colonization (AM) was significantly positively correlated with the DSE colonization rate (DF) (R=0.74), and DF was significantly positively correlated with the colonization rate of AMF (AF) roots (R=0.72).

3.3 Diversity and abundance differences of AMF and DSE in the root and rhizosphere

Among all the 27 samples from the rhizosphere soil and roots of *P. euphratica*, 18 samples were detected in seven genera of AMF, with

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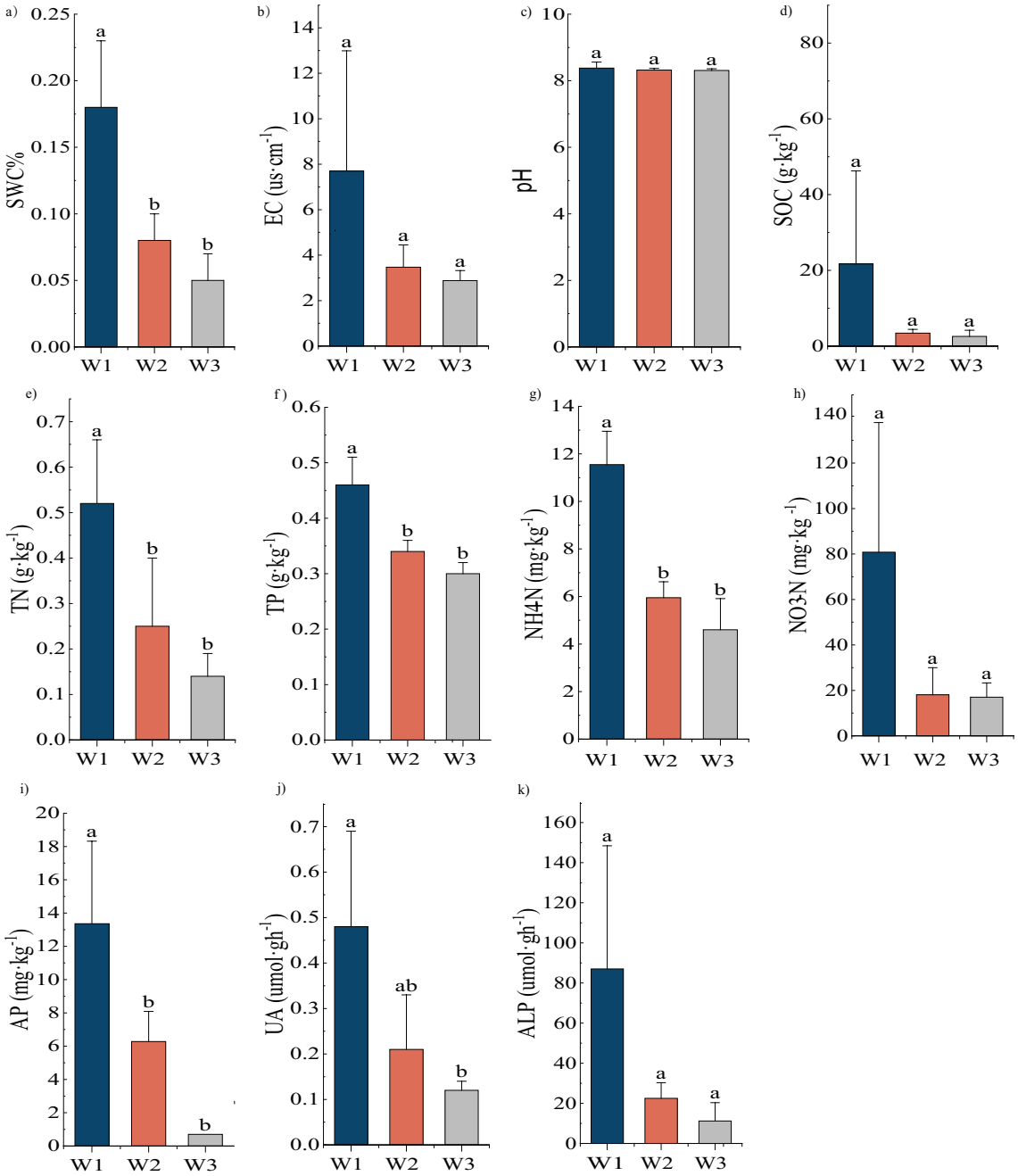

FIGURE 2
(A–K) Differences in physical and chemical properties of soil of *Haloxylon ammodendron* (mean ± SD, n=3). Same as Figure 1.

samples belonging to high and low water gradients in the rhizosphere and roots (Figure 6). More than one AMF genus coexisted in each of the 18 samples. At low water gradients, the dominant AMF genera from the rhizosphere to the root interior changed from *Kamienskia* to *unclassified_f__glomerraceae*. Under high water gradients, the dominant genus changed from *unclassified_p__Glomeromycota* in the rhizosphere to *unclassified_f__Glomerraceae* in the roots. Overall, *Kamienskia* had the highest abundance, followed by *unclassified_f__Glomerraceae*.

Among the 27 samples in the rhizosphere soil and within the roots of *H. ammodendron*, 18 samples were detected in three genera of AMF, belonging to high water gradients in the rhizosphere and high and low water gradients in the roots, all of which had AMF coexisting in one or more genera. Only two types of AMF were detected in the roots of *H. ammodendron* under a high water gradient, with *unclassified_p__Glomeromycota* being the dominant genus. Under a low water gradient, the dominant genus in the

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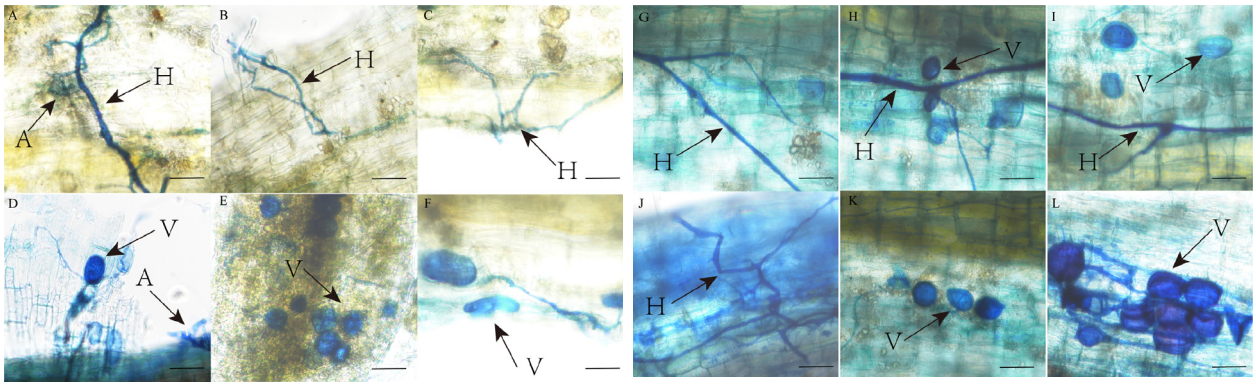

FIGURE 3
AMF colonization structure in roots of *Populus euphratica* **(A-F)** and *Haloxylon ammodendron* **(G-L)**. A, arbuscule; H, hypha; V, vesicle.

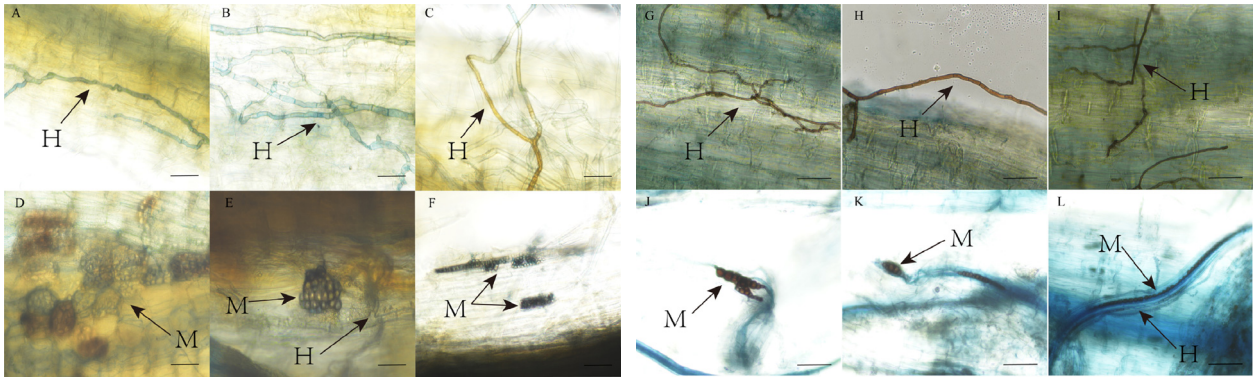

FIGURE 4
DSE colonization structure in roots of *Populus euphratica* **(A-F)** and *Haloxylon ammodendron* **(G-L)**. H, hypha; M, microsclerotia.

TABLE 1 Differences in AMF colonization rates in the roots of *Populus euphratica* and *Haloxylon ammodendron* under different water gradients.

Endophyte	Category	Species	W1	W2	W3
AMF	F%	*Populus euphratica*	60.15aA	0.00bA	85.42aA
		Haloxylon ammodendron	13.89bB	28.42bA	71.83aA
	M%	*Populus euphratica*	9.14abA	0.00bA	21.92aA
		Haloxylon ammodendron	0.14bA	0.55bA	22.77aA
	A%	*Populus euphratica*	6.14aA	0.00aA	5.59aA
		Haloxylon ammodendron	0.04aA	0.12aA	7.99aA

Different uppercase letters indicate significant differences in the same environmental status between two different plants. Different lowercase letters indicate significant differences in colonization status on water gradients.

rhizosphere was *Kamienskia*, and the dominant genus in the root was *unclassified_p__Glomeromycota*.

The diversity of AMF species inside and outside the roots of *P. euphratica* was greater than that in *H. ammodendron*. Only three of the seven AMF genera were detected inside and outside the roots of *H. ammodendron*, while *P. euphratica* detected all seven genera including *unclassified_o__Glomerales*, *Rhizophagus*, *Glomus*, and *Dominikia*, with a total number greater than that of *H. ammodendron*. Among all AMF-detected samples, the dominant genus was *unclassified_f__Glomerraceae*. The AMF in the rhizosphere soil of *P. euphratica* was approximately six times the amount of *H. ammodendron*, whereas the amount of AMF in *H. ammodendron* is 2 times more than that of *P. euphratica*.

In total, 25 DSE genera were detected in the rhizosphere soil and roots of *P. euphratica*, and more than one DSE genus coexisted in all 27 samples (Supplementary Table S2). The dominant genera were *Cyphellophora*, followed by *Fusarium* (Figure 7). The composition of DSE genera of *P. euphratica* varied among the different water

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TABLE 2 Differences in DSE colonization rates in the roots of *Populus euphratica* and *Haloxylon ammodendron* under different water gradients.

Endophyte	Category	Species	W1	W2	W3
DSE	F%	*Populus euphratica*	54.24bA	48.89bA	92.41aA
		Haloxylon ammodendron	67.10aA	23.75cB	45.06bB
	M%	*Populus euphratica*	28.15aA	11.98aA	33.99aA
		Haloxylon ammodendron	11.07aA	0.32aA	3.43aB
	MS%	*Populus euphratica*	10.90bA	46.56aA	23.89bA
		Haloxylon ammodendron	31.82aA	35.44aA	17.69aA

Same as Table 2.
Different uppercase letters indicate significant differences in the same environmental status between two different plants. Different lowercase letters indicate significant differences in colonization status on water gradients.

gradients. *Cyphellophora* and *Fusarium* have been widely detected in low water gradients. However, *Cadophora*, the dominant genus in the high-water gradients were rarely detected in the low water gradients.

In total, 25 DSE genera were detected in the rhizosphere soil and roots of *H. ammodendron*, and more than one DSE genus coexisted in all 27 samples. The three most abundant genera were *Neocamrosporium*, *Cyphellophora*, and *Preussia*. *Neochamrosporium* was widely distributed in high water gradients in roots, whereas *Scytalidium* was mainly detected in the low water gradient rhizosphere soil. Other genera of DSE were detected in samples from multiple water gradients. The abundance of DSE in the roots of each water gradient was lower than that in the rhizosphere. But the quantities of DSE in the rhizosphere were greater than that in the rhizosphere.

The TOP 10 DSE genera inside and outside the roots of the two plant species were also different: *Scytalidium* and *Microascus* were not dominant in the roots of *P. euphratica*, and *Cadophora, Phialophora*, and *Embellisia* were not dominant in the collected samples of *H. ammodendron* roots.

3.4 Effects of soil physical and chemical factors on AMF and DSE colonization

A redundancy analysis was conducted on the relationship between AMF and DSE colonization status of the roots of *P. euphratica* and *H. ammodendron* and soil physical and chemical factors at the plots. The results showed that the eigenvalues of the first and second sorting axes were 0.5842 and 0.1294, respectively. The two axes explained 71.36% of the changes in endophytic fungi, indicating that the first and second sorting axes could better reflect the changes between endophytic fungi and soil physical and chemical factors (Figure 8). The first axis was positively correlated with soil physical and chemical factors TP, negatively correlated with other soil physical and chemical factors, such as pH, AP, NO_3-N, ALP, UA, SOC, SWC, NH_4-N, TN and EC. It was positively correlated with AA, AM, AF, DF, and DM and negatively correlated with MS. The second axis was negatively correlated with MS, DF, DM, and all environmental factors, and positively correlated with AM, AF, and AA.

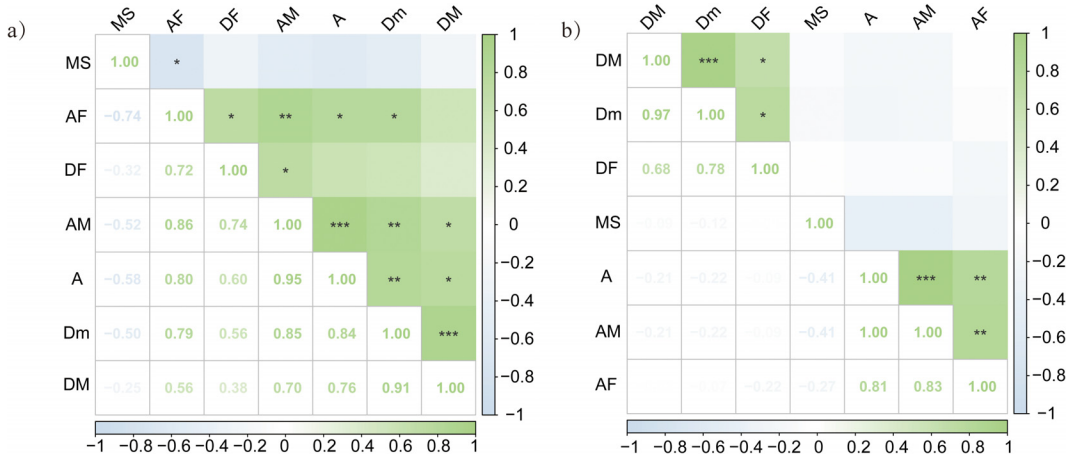

FIGURE 5
Correlation between DSE and AMF colonization status of **(A)** *Populus euphratica*, **(B)** *Haloxylon ammodendron*. MS, the microsclerotia colonization rate; AF, the colonization rate of AMF; AM, the colonization density of AMF; A, the richness of root hyphae; DF, the colonization rate of DSE; DM, the colonization density of DSE; Dm, the colonization density of DSE in the infected root segment. The asterisk indicates significance p-value. No mark when $P \geq 0.05$; when $0.01 < P < 0.05$, marked * ; when $0.001 < P < 0.01$, marked ** ; when $P \leq 0.001$, marked ***.

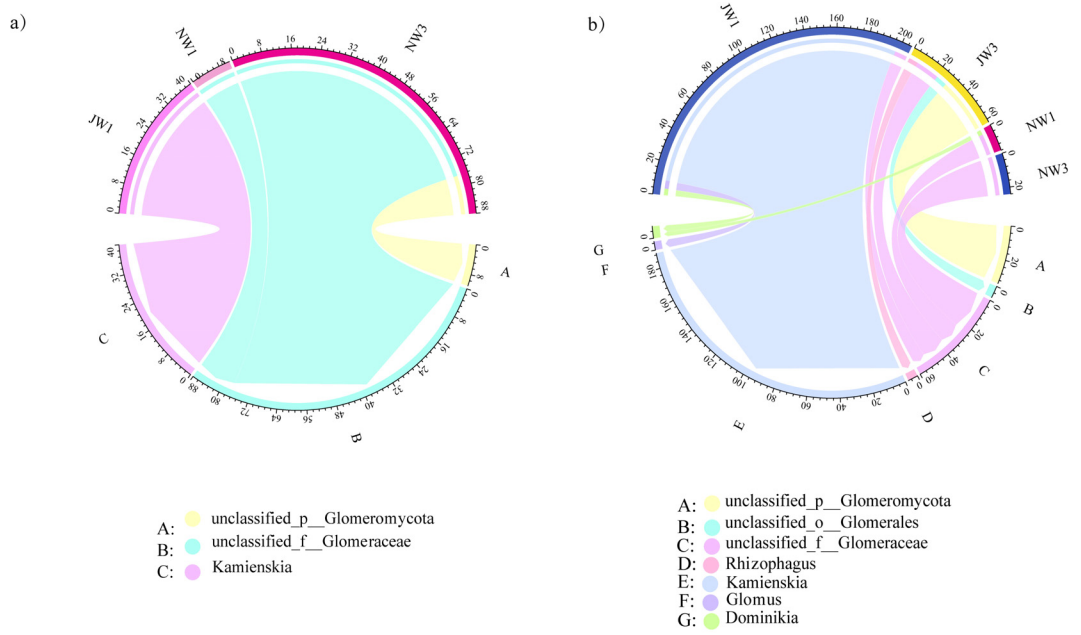

FIGURE 6
Differences in AMF diversity between *Haloxylon ammodendron* and *Populus euphratica* under different water gradients **(A)** *Haloxylon ammodendron*, **(B)** *Populus euphratica*. J, the sample taken from the rhizosphere; N, the sample taken from the plant roots.

The results indicated (Supplementary Table S1) that SWC, AP, TP, and TN were significantly and positively correlated with AM ($P<0.01$). UA levels were significantly positively correlated with AM ($P<0.01$), and significantly negatively correlated with AF ($P<0.05$). ALP levels were significantly positively correlated with AM ($P<0.01$). There was a significant negative correlation between NH4-N and AF ($P<0.05$). There was a highly significant negative correlation between NO3-N and AF ($P<0.01$) and a significant positive correlation with DM ($P<0.05$). PH was significantly and positively correlated with DF ($P<0.05$) and DM ($P<0.05$).

4 Discussion

4.1 Differences and correlation of colonization rates between AMF and DSE in roots

Soil moisture can directly affect the growth and development of mycorrhizal fungi and the formation of mycorrhizal symbioses (Liu et al., 2018). Under low water gradient, for example, endophytic fungi can more effectively connect the roots of *H. ammodendron* to

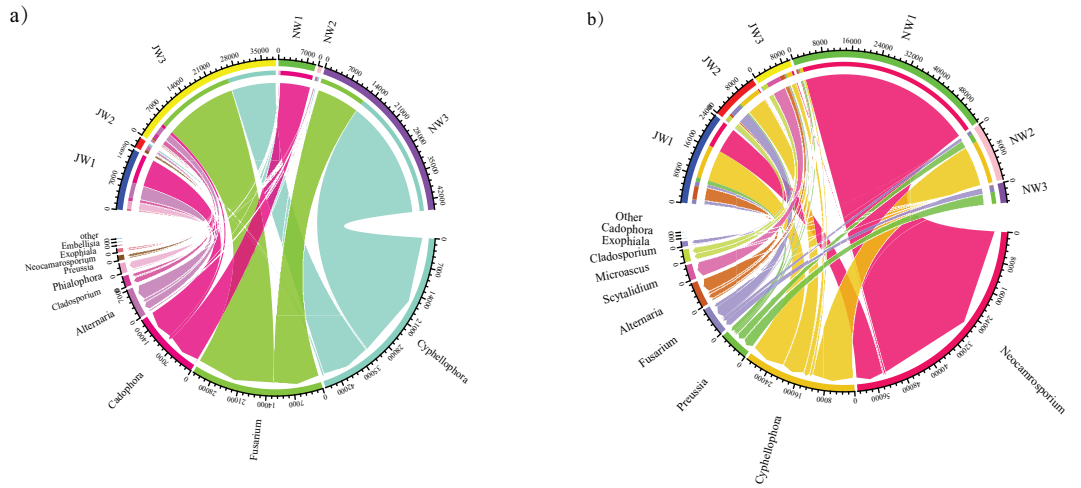

FIGURE 7
Differences in TOP 10 DSE genera diversity between *Haloxylon ammodendron* and *Populus euphratica* under different water gradients **(A)** *Populus euphratica*; **(B)** *Haloxylon ammodendron*. Same as Figure 6. J, the sample taken from the rhizosphere; N, the sample taken from the plant roots.

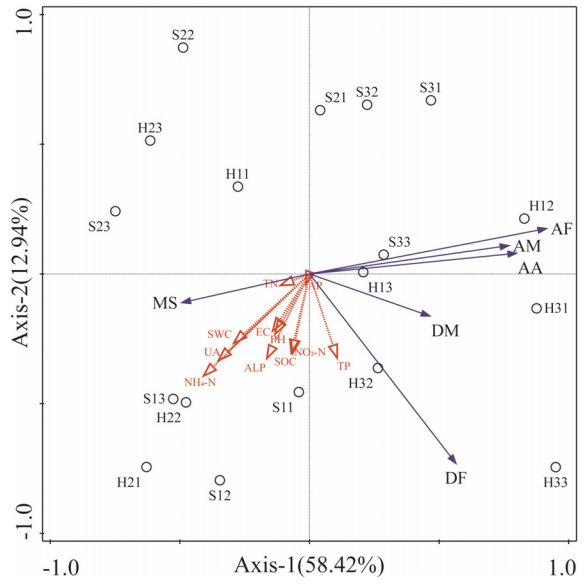

FIGURE 8
Redundancy analysis of environmental factors and colonization status of *Populus euphratica* and *Haloxylon ammodendron*. AM, the colonization density of AMF; A, the richness of root hyphae; DF, the colonization rate of DSE; DM, the colonization density of DSE; MS, the microsclerotia colonization rate. S, *Haloxylon ammodendron*; H, *Populus euphratica*; the number represents the moisture gradient and sampling number, for example, S11, the No.1 *Haloxylon ammodendron* sample collected at a high moisture gradient.

the surrounding soil microenvironment, thus exhibiting more significant functions in absorbing and transporting water and nutrients. In the present study, the DSE colonization rate of *H. ammodendron* decreased with decreasing water content. We speculate that this may be due to the drought stress exceeding a certain limit, which may have resulted in the production of certain substances by fungal cells (Pegler and Allen, 1993), leading to changes in the cell membrane permeability of *H. ammodendron* (Dick et al., 1996) and affecting the growth of fungi and plants. The colonization rate of AMF in the roots of *P. euphratica* and *H. ammodendron* was negatively correlated with soil water content, consistent with previous findings (Shen and Zhu, 2021). It has been speculated that appropriate level of humidity is beneficial for AMF colonization and spore germination (Frew, 2023). In this study, it was also found that under the same drought stress conditions, host plant species have an impact on the biomass and colonization rate of mycorrhizal fungi (Ilyas et al., 2024). For example, in contrast to the changes in the DSE colonization rate of the roots of *H. ammodendron* mentioned earlier, the DSE colonization rate of *P. euphratica* was negatively correlated with soil moisture content. This may be because the soil moisture content of the W1 plot in this study was more suitable for DSE colonization of the roots of *P. euphratica*. Under these conditions, DSE can have a relatively tight colonization of the roots of *H. ammodendron* (Ge et al., 2018). In our study, we also found that the total colonization rate of DSE under a low water gradient was higher than that of AMF, which not only indicates that DSE has a stronger colonization ability than AMF, but also indicates that DSE has greater colonization advantages under extreme environments (Alberton et al., 2010; Postma et al., 2007). The changes in the colonization structure that occurred in AMF and DSE with increasing drought severity may be an adaptation of endophytic fungi to environmental influences (Xie et al., 2017).

Zhao et al. (2021) found a positive correlation between the colonization rate of DSE microsclerotia and the content of AMF groups, which is consistent with the results of this study. In the present study, we also found a correlation between DSE colonization and AMF in the roots of the two plants. The colonization rate and density of AMF in *P. euphratica* were significantly positively correlated with the colonization rate of DSE, whereas the colonization rate and density of AMF were negatively correlated with the DSE colonization rate and density in *H. ammodendron*. This indicates that AMF and DSE can form different colonization relationships in the roots of different species, and that the interaction between the two mycorrhizal fungi is complex and greatly influenced by the host plant and environmental conditions. This is consistent with previous research results. The study of Shang et al. (2021) study showed that the colonization of AMF and DSE in the roots of cultivated *Vaccinium uliginosum* in Guizhou is highly significantly negatively correlated. Seerangan and Thangavelu, (2014) found that there was no clear relationship between AMF colonization and DSE colonization when studying plants growing in aquatic and wetland habitats, and the co-occurrence of AMF and DSE is more common in terrestrial habitats.

4.2 Diversity and abundance differences of AMF and DSE in the root and rhizosphere

In this study, we found that the majority of AMF and DSE were not unique to *P. euphratic*a or *H. ammodendron* but were shared by both, indicating that AMF and DSE strains do not exhibit strong host specificity in arid areas (Liu et al., 2017; Bi and Xie, 2021). For example, *unclassified_f:Glomeraceae*, an unclassified member of the *Glomeraceae* family, was found in the roots of *both H. ammodendron* and *P. euphratica*. *Glomeraceae* is a widely distributed community in the AMF population currently discovered, and was found in both soil and plant roots (Stürmer et al., 2018). Unlike AMF, DSE exhibits host preference (Stroheker et al., 2021). For example, *Cyphellophora*, the dominant genus of DSE, had a significantly greater amount of colonization in the rhizosphere soil and roots of *P. euphratic*a than *H. ammodendron*, whereas another genus, *Neocamrosporium*, had a higher number in the rhizosphere soil and roots of *H. ammodendron*, which also reflects the varying degrees of host preference for different mycorrhizal fungi (Joshee et al., 2009). Under the same drought conditions, there was a significant difference in the colonization status of AMF between the roots and rhizospheres of *P. euphratica*. The diversity and quantity of AMF in the rhizosphere soil were higher than in the roots, which is consistent with the results of Tian et al (2018). on AMF in the rhizosphere of potato roots. Differences in AMF communities between the soil and roots may be determined by the

heterogeneity of the rhizosphere soil (Liu et al., 2009). Shi et al. (2022) and Wang et al. (2010) conducted a diversity survey of AMF in the Xinjiang region and found that *Glomus* was the dominant genus. In this study, *Rhizophagus* and *Kamienskia* were more dominant than *Glomus*, which may be due to differences in hosts.

Many of the AMF and DSE genera detected in this study are highly beneficial to the host. For example, *Glomus* genera are used for Se biofortification (Ye et al., 2020). The *Phialocephala* genus can promote the biological adaptability of *Saussurea involucrata* and *Rhodiola rosea* to extremely cold and strong radiation environments (Chen et al., 2018), and improve the gold tolerance properties of host plants. *Thielavia* in the soil provides plants with highly active endoglucanases, increases the accumulation of plant glucose, and promotes the absorption of soil nutrients by plants, thereby increasing the yields of *Medicago sativa* (Medicago sativa Linn) and *Zea mays* (Badhan et al., 2014; Shi et al., 2019). These findings demonstrated that endophytic fungi play an important role in improving plant adaptability to the environment.

4.3 Effects of soil physical and chemical factors on the colonization status of AMF and DSE

Studies have shown that the distribution of endophytic fungi that colonize plant roots is related to soil physical and chemical factors (Wang et al., 2015). Both redundancy and correlation analyses in this study indicated that soil physical and chemical factors have different effects on fungal colonization within roots. Nitrogen deposition is one of the most important global change factors (Tang et al., 2023), and nitrogen content plays a role in AMF colonization. When the distribution ratio of nitrogen, phosphorus, and potassium in the soil is unbalanced, the infectivity of AMF is improved, helping plant roots absorb more nutrients from the soil to cope with adversity (Zhou et al., 2021). Increasing nitrogen fertilizer application can promote the proliferation of bacteria and actinomycetes in the rhizosphere soil of *Oryza sativa*, and the nitrogen application level is positively correlated with the number of bacteria and actinomycetes. TP and ALP were highly significantly positively correlated with AMF colonization density, which is consistent with the results of Li et al. (2021). Soil phosphatase can promote the hydrolysis of organic phosphorus compounds into inorganic phosphorus, thereby increasing the available phosphorus content in the soil (Luo et al., 2022). However, under high phosphorus levels, the colonization rate of AMF significantly decreases (Salmeron-Santiago et al., 2023), which is not conducive to plant growth. Therefore, controlling and managing soil phosphorus levels are beneficial for improving the richness and diversity of soil AMF, thereby facilitating the establishment of sustainable ecosystems (Qin et al., 2020). It is obvious that the soil moisture content was not the reason for the lack of AMF colonization in the roots of *P. euphratica* under a moderate water gradient, and the soil moisture content did not cause AMF colonization in the other two gradients. We speculate that this may be due to the aforementioned soil physical and chemical factors (Yan et al., 2023). Under nitrate-nitrogen conditions, endophytic fungal inoculation can promote seedling growth (Sun et al., 2022). Nitrate-nitrogen can reflect soil nitrogen levels, and nitrogen can accelerate the growth metabolism of soil microorganisms (Qu et al., 2022), strongly supporting the results of our study. In this study, there was a significant positive correlation between pH and the colonization status of the DSE communities, which is consistent with previous studies. Hou (2020) found that the colonization rate of DSE was positively correlated with pH in his research on various desert plants. This may be because the pH affects the soluble salt content in the soil and the material exchange capacity of DSE, thus affecting its growth status of DSE (Zhang et al., 2015).

5 Conclusion

Our study investigated the effects of soil factors on the colonization of AMF and DSE in the rhizosphere and roots of the desert plants *P. euphratica* and *H. ammodendron* in Xinjiang. This was achieved by observing their colonization status, community composition, morphological characteristics, and differences in morphological colonization structure. The results showed that drought stress increased the colonization rate of DSE in the roots of *P. euphratica* and AMF in the roots of *P. euphratica* and *H. ammodendron* but decreased the colonization rate of DSE in the roots of *H. ammodendron*. The DSE was more dominant in extreme drought environments. The AMF diversity decreased with increasing drought severity, whereas the DSE diversity increased. DSE showed stronger adaptability to drought-stressed environments. The dominant and endemic species of DSE vary in different environments and hosts. AMF were more susceptible to soil factors such as SWC, soil nitrogen and phosphorus content (including AP, TN, TP, NH_4-N, and NO_3-N), and UA, whereas DSE was more affected by pH. The results of this study are conducive to the development of targeted and biased protection strategies for desert plants, especially *P. euphratica* and *H. ammodendron*, and provide a basis for endophytic fungal species selection in ecological restoration.

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/, PRJNA1046269.

Author contributions

HMW: Writing – original draft, Data curation, Visualization. HFW: Writing – review & editing, Conceptualization, Funding acquisition, Methodology, Visualization. SW: Data curation, Visualization, Investigation, Validation, Writing – original draft. LS: Data curation, Investigation, Supervision, Visualization, Writing – original draft. LC: Data curation, Writing – original draft.

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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.2024.1504650/full#supplementary-material

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The regulation of tobacco growth under preceding crop planting: insights from soil quality, microbial communities, and metabolic profiling

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Introduction: Specific microorganisms and metabolites in soil play key roles in regulating organismal behavior. Currently, the effects of different preceding crops on the rhizosphere soil quality of flue-cured tobacco remain unclear.

Methods: Four treatments were compared in the study: fallow + tobacco (CK), maize + tobacco (T1), rapeseed + tobacco (T2), and wheat + tobacco (T3).

Results and discussion: Results showed that preceding crops significantly enhanced soil nutrient levels and improved tobacco growth by altering rhizosphere metabolites and microbial community structure. Previous cultivation of maize and rapeseed significantly promoted tobacco growth, rapeseed and wheat cultivation enhanced the diversity of soil bacterial communities, and notably decreased the abundance of urea-degrading bacteria. In contrast, the preceding crop of maize reduced plant pathogenic fungi and promoted positive microbial interactions. Metabolomics analysis showed that different preceding crops altered lipids, organic acids, flavonoids, alkaloids, and terpenoids, enhancing secondary metabolite synthesis pathways in soil. Preceding crops regulated rhizosphere metabolites which potentially participated in soil carbon and nitrogen cycling, balancing soil nutrients, and improving tobacco yield. Overall, the three preceding crops altered the

composition and function of metabolites and microbial community structures in rhizosphere soil, thereby increased soil nutrient concentration. Both maize and rapeseed cultivation significantly boosted tobacco growth and biomass. These findings offer new insights into the potential interactions between rhizosphere metabolites and microbial communities and strategies of comprehensively regulating tobacco growth.

KEYWORDS

tobacco, preceding crops, soil quality, rhizosphere metabolites, soil microbial diversity

1 Introduction

Tobacco is a significant economic crop in China's agricultural sectors. However, in areas with limited arable land, such as Yunnan, tobacco cultivation often results in continuous cropping obstacles (Tan et al., 2021), leading to soil nutrient imbalances, reduced enzyme activity, decreased microbial diversity, and the accumulation of allelopathic substances (Ku et al., 2022), which negatively affected tobacco growth (Wang et al., 2020b). To mitigate these adverse effects, we have experimented with planting different crops during the tobacco fallow period. Diversified crop rotation has emerged as a promising approach for sustainable agriculture (Kang et al., 2020). Suitable crop diversity is crucial for enhancing soil conditions (Wang et al., 2022a). Selecting an appropriate previous crop can improve nutrient cycling, boost enzyme activity, stabilize microbial community structure and functional diversity (Trinchera et al., 2022), optimize rhizosphere metabolite composition and abundance, and enhance soil metabolic pathways (Wang et al., 2023b). Therefore, choosing suitable previous crops can effectively address the challenges of continuous cropping (Qin et al., 2017).

Corn, rapeseed, and wheat are common crops in rotation systems, contributing to soil nutrients. Maize, preceding rapeseed, enriched organic matter and available K in the rhizosphere soil (Hirzel et al., 2021). Rapeseed, as a preceding crop, significantly influences the nitrogen content of rice rhizosphere soil, thereby improving rice yields (Fang et al., 2021). Compared to other crops, wheat, with its long growth period and root residues, significantly increases soil organic matter content and urease activity (Woźniak, 2019; Chiotta et al., 2021; Neupane et al., 2021; Silva et al., 2022).

Additionally, numerous studies showed that preceding crops affect soil microbial species and abundance. For instance, maize rotation increases the relative abundance of specific soil bacteria, while rapeseed cultivation enhances microbial diversity and species symbiosis in rice rhizosphere soil (Zhang et al., 2023). Wheat cultivation supports rhizosphere-derived bacteria in soybeans, protecting them against soil-borne diseases (Yin et al., 2023). Soil microorganisms are essential for nutrient cycling, particularly carbon, nitrogen, and phosphorus (De Graaff et al., 2010), and they promote plant growth by decomposing organic matter and enhancing plant disease resistance (Adekunle et al., 2021).

The rhizosphere, a zone of complex ecological interactions among plants, soil, and microbes, is significantly influenced by root exudation (Yuan et al., 2024). Root exudation enhances plant growth by recruiting beneficial microbes, suppressing soil-borne diseases, and improving soil ecosystem multifunctionality (Seitz et al., 2022). Despite this, the combined regulatory effects of rhizosphere metabolites and soil microbes remain understudied. To address this, we propose the following hypotheses based on previous studies: (1) preceding crops influence rhizosphere metabolites via root exudates, altering the soil biochemical environment.; (2) preceding crops recruit specific microbial populations, shaping microbial community structure and function.; (3) preceding crops enhance soil nutrients and tobacco growth by modulating rhizosphere metabolites and microbial interactions. The effects of metabolites and microbial diversity on soil nutrient concentration and crop growth across various rotation systems are unclear. We aimed to determine the relative importance of edaphic factors and metabolites in shaping the microbial community. Therefore, establishing a stable and healthy soil microbiota community is crucial for plant growth, as it balances the soil environment and promotes sustainable agricultural development.

2 Materials and methods

2.1 Experimental site and soil agrochemical properties

The methods for growth regulation were conducted in a potted greenhouse at Yunnan Agricultural University (102.7479865° E, 25.1320219° N), where the average daily temperature is 17.5°C and the average daily light duration is 11.73 hours. Uncultivated red soil was utilized for the experiment. The agrochemical properties of the soil were as follows: available nitrogen was 86.4 mg/kg, available phosphorus was 22.5 mg/kg, rapid potassium was 328.57 mg/kg, and organic matter was 16.0 g/kg.

2.2 Experimental design and implementation

The experimental soil was sieved through a 2 mm sieve and transferred into plastic pots (42 cm × 28 cm × 40 cm), each containing 25 kg. Four treatments were established: control group (CK) with fallow + tobacco, treatment T1 with corn + tobacco, treatment T2 with rapeseed + tobacco, and treatment T3 with wheat + tobacco. Each treatment had 10 replicates. The pre-crop was sown in September of the previous year, and tobacco seedlings were transplanted in April of the following year. Each pot contained two maize or rapeseed plants, 20 wheat seeds, and 1 tobacco plant. The crop varieties used were: Yunrui 121 (corn), Youzha 50 (rapeseed), Ximai 6 (wheat), and Yunyan 87 (tobacco). Fertilization was applied using the ring-pit method (10 cm away from the plant, 10 cm deep). Fertilization was conducted twice: 60 g of compound fertilizer per pot during the previous cropping season (excluding the fallow treatment) and 60 g of compound fertilizer (N-P_2O_5-K_2O = 10-10-24) per pot during the flue-cured tobacco growing season. The soil moisture was maintained at about 50%, with the same treatment applied to the fallow.

2.3 Soil and tobacco plant sampling

Samples were collected at the tobacco maturation stage (August of the following year), after removing the surface soil, the entire root system was extracted, and the rhizosphere soil sample was collected by gently shaking the roots. The two potting soils were combined into one sample, with five duplicate samples for each treatment. Some samples were quick frozen in liquid nitrogen for 30 min, then stored at -80°C for microbial diversity and metabolic product analysis. The remaining samples were naturally dried and sieved through a 2 mm sieve to remove impurities. Mature tobacco plants were harvested, and their roots, stems, and leaves were weighed to calculate the fresh weight of the whole plant. These parts were briefly heated at 105°C for 15 min, then dried to a constant weight at 70°C. Three replicates were collected for each treatment.

2.4 Soil chemical properties test

Air-dried soil samples are sieved through a 1 mm sieve, and the pH was determined using the immersion method. Organic matter was measured with potassium dichromate titration. Available nitrogen, available phosphorus, rapidly available potassium, available zinc, available boron, and available molybdenum were determined by alkali diffusion, molybdenum antimony colorimetry, flame photometry, flame atomic absorption spectroscopy, curcumin colorimetry, and potassium thiocyanate colorimetry, respectively (Tan et al., 2022). Enzyme activities (sucrase (G0302W, 540nm), urease (G0301W, 578nm), acid phosphatase (G0304W, 405nm), catalase (G0303W, 510nm)) are measured using their corresponding standard assay kits (Suzhou Grace Biotechnology Co., Ltd., China) (Borase et al., 2020), The model of the microplate reader used in the detection was KAIAO 6700FLA (Beijin, China).

2.5 Rhizosphere soil microbial community genomic sequencing and analysis

The E.Z.N.A.™ kit was used to extract soil microbial genomic DNA, which was then analyzed via 0.8% agarose gel electrophoresis and quantified using a NanoDrop 2000. The 16S rRNA gene's V3-V4 region (Dai et al., 2022), corresponding to the complete bacterial community, was amplified using 338F/806R primers. Simultaneously, the fungal ITS1 gene was amplified using ITS1a and ITS1b primers (Usyk et al., 2017). After purifying the PCR products (Vazyme Biotech Co., Ltd, Nanjing, China), they were quantified using a BioTek FLx800. Paired-end sequencing was performed using the Illumina NovaSeq platform and NovaSeq 6000 SP Reagent Kit (500 cycles) (Parsortix, Shanghai, China). The QIIME 2 2019.4 platform was used for microbiome bioinformatics analysis (Ji et al., 2022), which included primer trimming, quality filtering, denoising, merging, and chimera removal.

2.6 Extraction and analysis of rhizosphere soil metabolites

Metabolite extraction for targeted metabolomic analysis was performed by Metware Biotechnology Co., Ltd., in Wuhan, China. Fresh soil samples (50 mg) were homogenized in 500 µl of ice-cold methanol/water (70%, v/v) for 15 minutes, then incubated and centrifuged at 4°C, 12000 rpm for 10 min. The resulting supernatant (400 µl) was transferred to a new centrifuge tube. The original tube was treated with ethyl acetate/methanol (1:3, v/v), shaken for 5 minutes, incubated on ice for 15 minutes, and then centrifuged at 4°C, 12000 rpm for 10 min, with 400 µl of the supernatant collected. The two supernatants were combined and concentrated. The dried concentrate was reconstituted with 100 µl of 70% methanol-water, sonicated for 3 min, and centrifuged at 4°C, 12000 rpm for 3 min before extraction. The soil secretions of the final extract (60 µl) underwent LC-MS/MS analysis using an Agilent 7890 high-performance liquid chromatography system (Santa Clara, CA, USA) coupled with a QTOF/MS-6545 mass spectrometer (LECO, St. Joseph, MI, USA). Data processing involved the use of ProteoWizard software and the XCMS program for data conversion, peak extraction, alignment, retention time correction, and filtering to obtain metabolic identification information. Subsequently, the R package ropls (Version 1.6.2) was employed for data analysis.

2.7 Data processing and analysis

The sequence data analysis primarily utilized QIIME2 and R packages (v3.2.0). A network analysis of the top 50 abundant bacterial and fungal communities was conducted using microbial

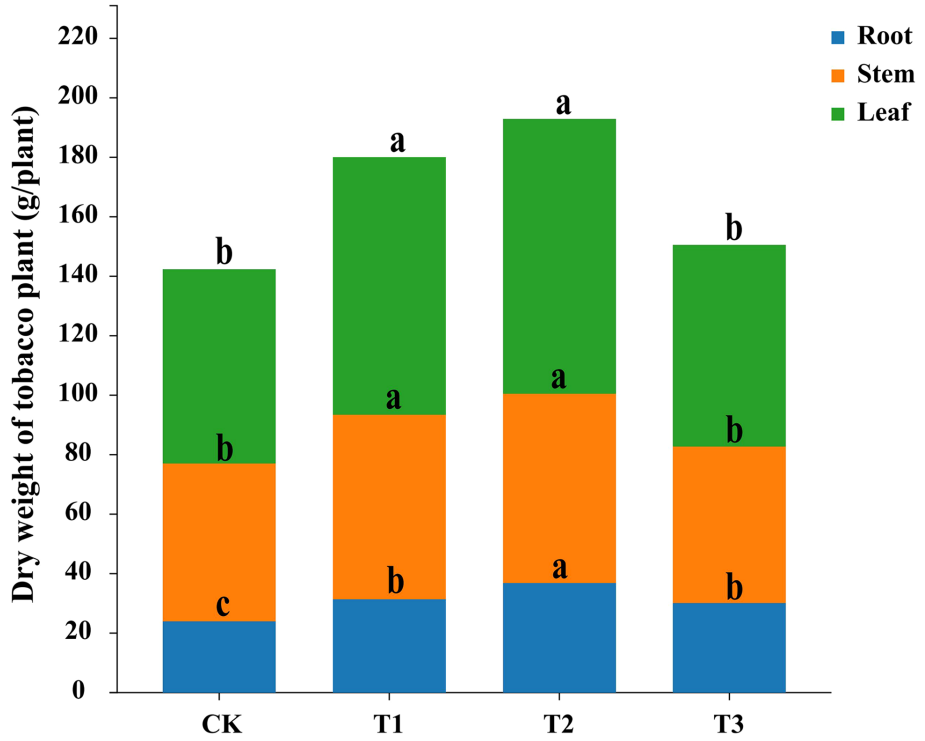

FIGURE 1
Impact of three different preceding crops on tobacco biomass accumulation in different organ. fallow + tobacco (CK), maize + tobacco (T1), rapeseed + tobacco (T2), and wheat + tobacco (T3), different letters indicated significant differences between treatments at $p < 0.05$ level, the same below.

ASVs to examine interspecies relationships. Gephi visualization (v 0.10.1) was employed to explore symbiotic patterns in soil microbial communities based on strong ($p > 0.6$) and significant ($p < 0.01$) correlations. Node sizes corresponded to connectivity with other nodes, and coloration reflected genus classification. Additionally, Spearman analysis of soil properties, bacterial and fungal abundances, and α-diversity indices was performed using SPSS 26.0 software. KEGG pathway enrichment analysis was applied to analyze the differential metabolites. Pearson correlation analysis was used to investigate relationships among rhizosphere soil nutrients, metabolites, and microbial diversity.

3 Results

3.1 Impact of three different preceding crops on tobacco biomass accumulation

Compared to fallow (CK), preceding crops maize (T1), rapeseed (T2), and wheat (T3) all enhanced tobacco growth. Former crop planting of maize and rapeseed significantly increased the dry weights of tobacco roots, stems, and leaves ($p < 0.05$), the dry weight of tobacco was not significantly affected by former crop planting of wheat, but it significantly increased the dry weight of tobacco root ($p < 0.05$). Among the preceding crops, rapeseed had the greatest impact on tobacco biomass accumulation (Figure 1).

3.2 Effect of three different preceding crops on agrochemical properties and enzyme activities of tobacco rhizosphere soil

Previous crops significantly influenced soil nutrient concentration and enzyme activities compared to fallow conditions ($p < 0.05$). Both T1 and T3 treatments significantly increased available phosphorus and potassium content compared to fallow (Figures 2A–E). All three treatments demonstrated notably higher effective zinc and boron content, urease, and acid phosphatase activities than the fallow group (CK). Additionally, the T2 and T3 groups exhibited significantly higher catalase activities than CK (Figures 2F–L). These results indicate that cultivation of maize and rapeseed promoted phosphorus and potassium accumulation in the soil. A decrease in soil organic matter was noted after cultivating maize and canola. Notably, all three preceding crops positively improved the availability of zinc and boron and the activities of carbon and nitrogen-related enzymes.

3.3 Influence of three preceding crops on soil microbial diversity in the tobacco rhizosphere

Rapeseed cultivation significantly enhanced the Chao1 and Shannon indices of soil bacteria compared to fallow ($p < 0.05$, $p <$

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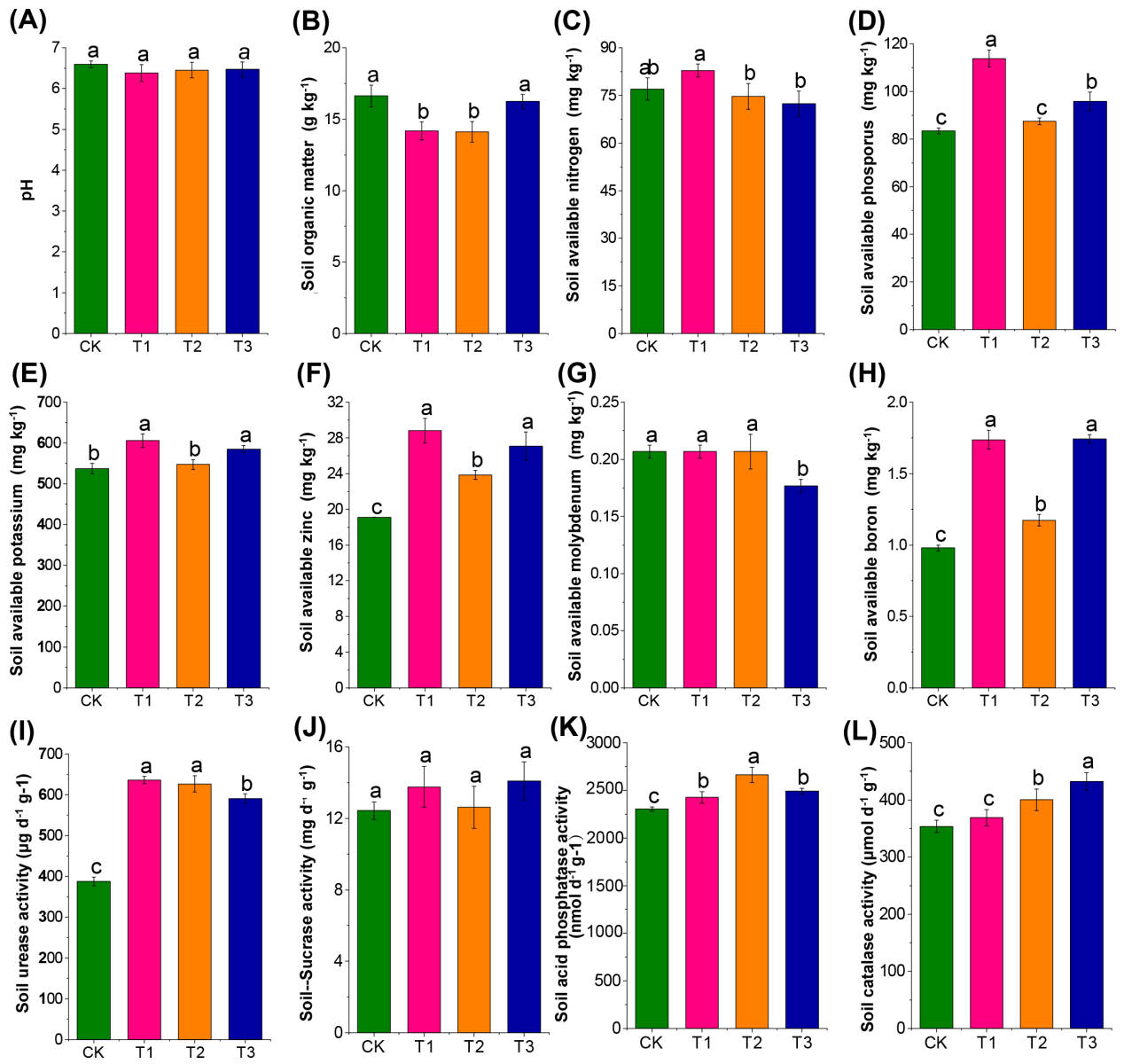

FIGURE 2
Effect of three different preceding crops on soil agrochemical properties and enzyme activities in the tobacco rhizosphere. **(A)** pH, **(B)** Soil organic matter, **(C)** Soil available nitrogen, **(D)** Soil available phosphorus, **(E)** Soil available potassium, **(F)** Soil available zinc, **(G)** Soil available molybdenum, **(H)** Soil available boron, **(I)** Soil urease activity, **(J)** Soil sucrase activity, **(K)** Soil acid phosphatase activity, **(L)** Soil catalase activity, with different letters indicated significant differences between treatments at $p < 0.05$ level.

0.01), with wheat also significantly increasing the Shannon index ($p < 0.05$). These results indicate that preceding crops notably increased the abundance of soil bacteria in tobacco rhizosphere soil. PCoA analysis showed distinct bacterial communities in tobacco rhizosphere soil following different preceding crops and fallow. In contrast, fungal communities exhibited greater clustering across treatments (Figures 3A, B), with maize as a preceding crop differing significantly from fallow. This suggests that preceding crops significantly impact bacterial and fungal communities in tobacco rhizosphere soil, with maize preceding notably affecting fungal communities.

3.4 Impact of three preceding crops on the composition of soil microbial communities in the tobacco rhizosphere soil

The predominant bacterial phyla in tobacco rhizosphere soil across all treatment groups were Proteobacteria, Gemmatimonadota and Actinobacteria, along with eight others, comprising over 90% of the total abundance. The T2 treatment group showed the highest relative abundance of Acidobacteriota. Notably, Chloroflexi and Patescibacteria were more abundant in T1 treatment compared to other treatments. Similarly, the dominant fungal phyla including Ascomycota,

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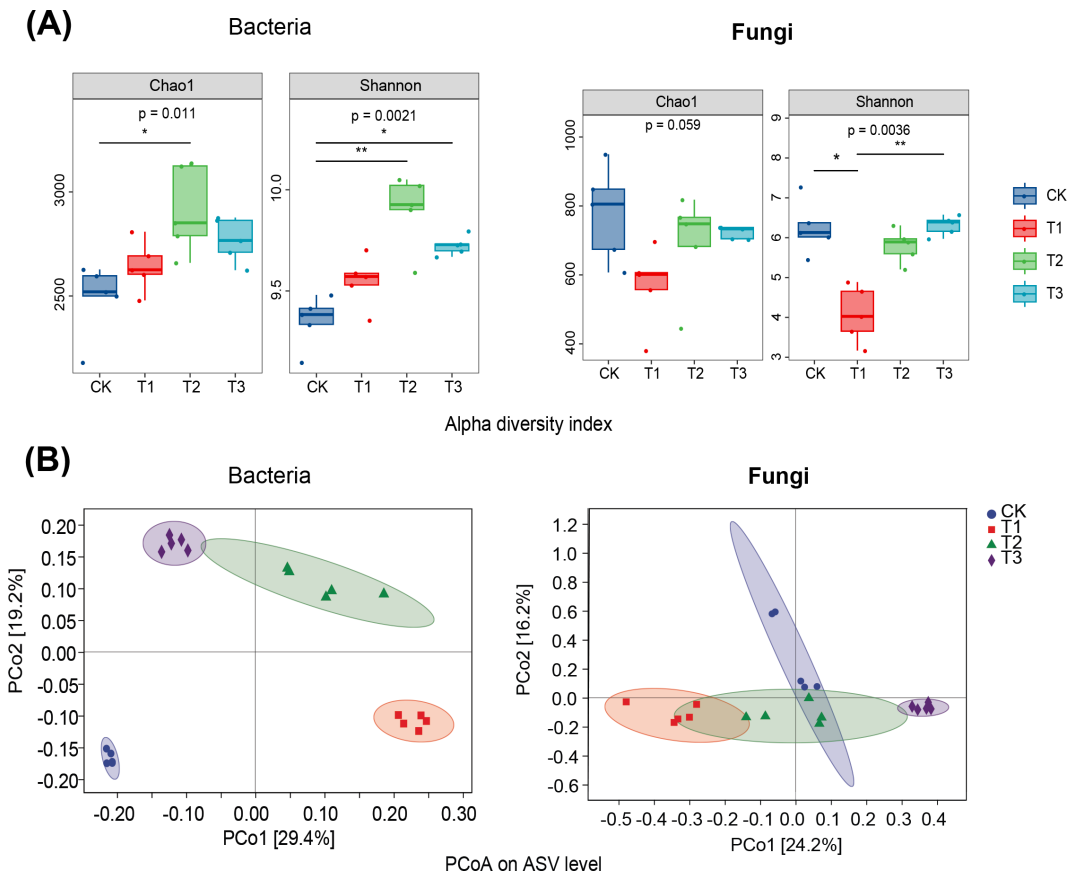

FIGURE 3
Effect of three preceding crops on soil microbial diversity of Chao1 index, Shannon index and Principal Coordinate Analysis (PCoA) in the tobacco rhizosphere. **(A)** α diversity, **(B)** Principal Coordinate Analysis.

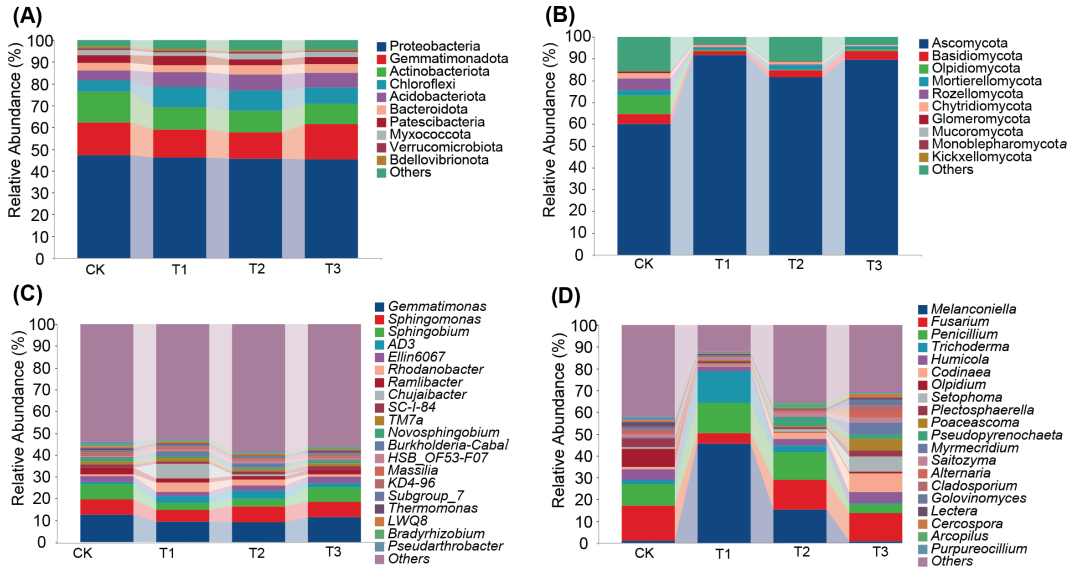

FIGURE 4
Impact of three preceding crops on the soil microbial community composition at the phyla and genus levels in the tobacco rhizosphere. relative abundance of **(A)** bacteria at phyla level, **(B)** fungi at phyla level, **(C)** bacteria at genus level, **(D)** fungi at genus level.

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Basidiomycota, Olpidiomycota, Mortierellomycota, and Chytridiomycota constituted over 80% of the total abundance. The T1 treatment demonstrated a significantly higher relative abundance of Ascomycota compared to other treatments (Figures 4A, B).

At the genus level, the top 20 relatively abundant bacterial genera in tobacco rhizosphere soil included *Gemmatimonas*, *Sphingomonas*, *Sphingobium*, *AD3*, and *Ellin6067*. Notably, *Rhodanobacter* and *Chujaibacter* showed the highest relative abundance in T1 treatment. Among fungal genera, *Melanconiella*, *Fusarium*, *Penicillium*, *Trichoderma*, and *Humicola* were predominant. *Melanconiella* was dominant in T1 and T2, *Trichoderma* was most abundant in T1, and *Codinaea* exhibited the highest relative abundance in T3. The variation of fungal communities among different treatment in rhizosphere soil exceeded bacterial communities (Figures 4C, D). These findings suggest that maize as a preceding crop had a more significant impact on the relative abundance of soil bacteria, whereas all three preceding crops substantially influenced the relative abundance of soil fungi.

3.5 Impact of three preceding crops on microbial functions and co-occurrence networks in tobacco rhizosphere soil

The FATROTAX database was utilized to predict soil bacteria functions, revealing an assessment of 62 categories (Figure 5A). After cultivating three distinct preceding crops, a significant decrease in the relative abundance of chemolithotrophic bacteria was observed, along with an increase in symbiotic animal functional bacteria compared to fallow groups. Notably, the presence of urea-degrading bacteria in tobacco soil decreased significantly with rapeseed and wheat as predecessors, while nitrate-reducing bacteria increased significantly with maize and rapeseed as predecessors ($p < 0.001$). For soil fungal communities, functional predictions during various preceding crop maturity stages were made using the FUNGuild database (Figure 5B), which categorizes nutritional modes into pathotrophs, saprotrophs, and symbiotrophs. Maize as a predecessor led to a significant reduction in lichen parasites, plant pathogens, and soil saprotroph

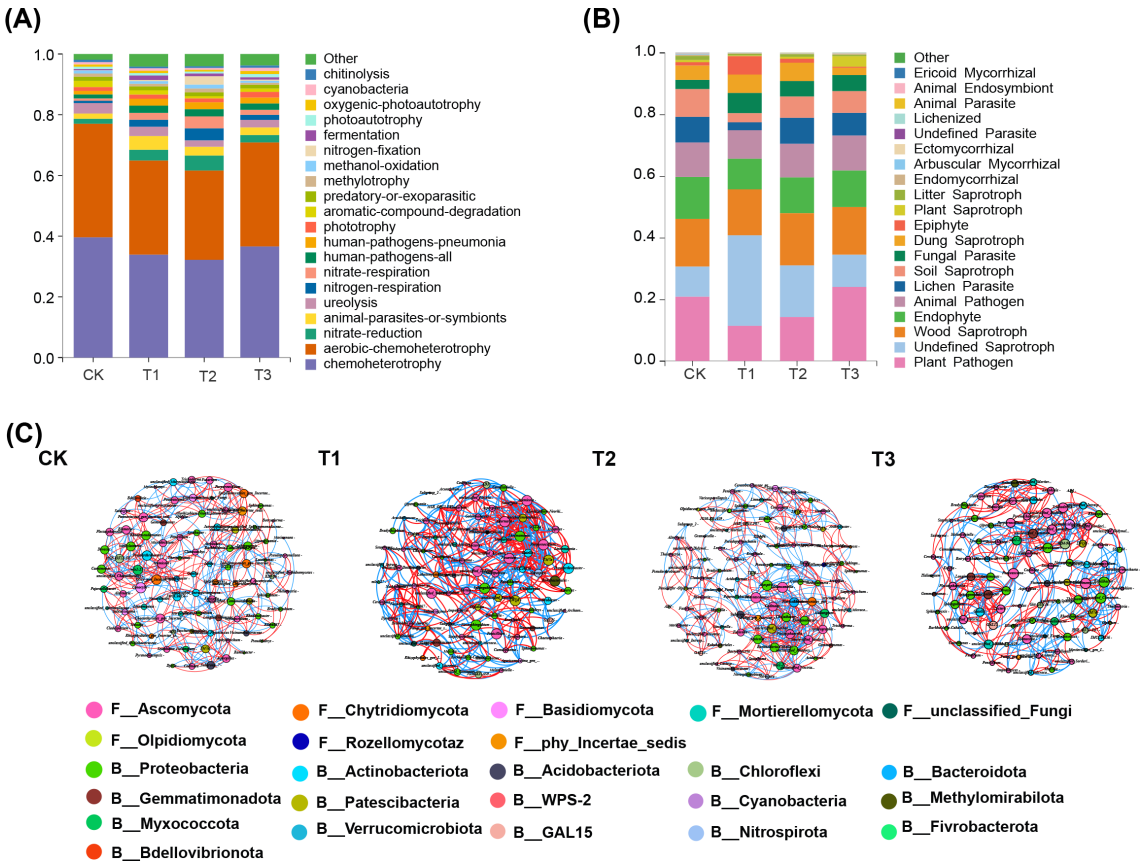

FIGURE 5
Impact of three preceding crops of microbial functions prediction and co-occurrence networks of bacteria and fungi in the tobacco rhizosphere soil. **(A)** bacterial function prediction based on the FATROTAX database, **(B)** fungal function prediction based on the FUNGuild database, **(C)** bacteria and fungi co-occurrence network in the tobacco rhizosphere soil. Lines between different genus indicates a significantly ($p < 0.01$) strong positive association (red, Spearman's $\rho > 0.6$) or negative association (blue, Spearman's $\rho < -0.6$). The size of each node is proportional to the number of connections, with same color nodes belonging to the same phyla.

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TABLE 1 Topological characteristics of co-occurrence networks of bacteria and fungi in the rhizosphere soil of flue-cured tobacco under three preceding crops.

Index	Modularity (MD)	Average clustering coefficient	Average path length	Network diameter	Grath density	Average degree (AD)	Positive	Negative	Nodes	Edges
CK	0.581	0.542	3.694	8	0.083	8.041	205	189	98	394
T1	0.535	0.575	3.592	8	0.106	10.634	304	245	100	549
T2	0.589	0.585	4.134	11	0.098	9.74	249	238	100	487
T3	0.703	0.615	4.067	9	0.088	8.74	222	215	100	437

fungi compared to fallow conditions ($p < 0.05$), and a significant increase in fungal parasites and undefined saprotroph fungi ($p < 0.01$). However, there was also an increase in fungal parasites in treatments using rapeseed and wheat as precursors, but no significant difference was observed.

The rhizosphere soil of tobacco was analyzed to identify the top 50 bacterial and fungal genera, investigating co-occurrence networks under different predecessor conditions (Figure 5C; Table 1). Microbial community structure and co-occurrence patterns varied, with rapeseed and wheat showing more complex networks compared to other planting patterns. Key nodes in the co-occurrence networks included *Conocybe*, *Humicola*, *Pseudarthrobacter*, *TM7a*, *Thermomonas*, *Sphingobium*, *Purpureocilliu*, *Poaceascamom*, *mle1-27*, *Lectera*, and *Gaiella*. The proportions of positive and negative correlations differed among networks: maize had 55.37% positive and 44.63% negative, rapeseed had 51.13% positive and 48.87% negative, wheat had 50.80% positive and 49.2% negative, and fallow had 52.03% positive and 47.97% negative. Mutualistic relationships prevailed over competitive ones, with maize showing the strongest mutualistic interaction, followed by rapeseed, while wheat exhibited the weakest mutualistic interaction.

3.6 Impact of three preceding crops on differential metabolite analysis in the tobacco rhizosphere soil

Volcano plots were employed to classify differential metabolites, compare with CK, identifying 124, 127, and 223 differential metabolites in T1, T2, and T3 treatments, respectively. These metabolites included lipids, flavonoids, alkaloids, amino acids and derivatives, terpenoids, and other compounds. Compared to fallow conditions (CK), T1, T2, and T3 treatments exhibited 60, 86, and 215 upregulated metabolites, and 64, 41, and 8 downregulated ones (Figures 6A, C, E; Supplementary Table S1). Using the OPLS-DA model, VIP values were calculated, compared to fallow conditions, cluster analysis (Top30) showed significant upregulation and downregulation of 20 and 10 metabolites in T1, 19 and 11 in T2, and 26 and 4 in T3 treatment, respectively, (CK) (Figures 6B, D, F). These results suggest that both rapeseed and wheat previous crops enhance the upregulation of differential metabolites in soil, with wheat demonstrating the most pronounced upregulation trend.

3.7 Differential metabolite enrichment in KEGG pathways and key metabolic pathways

The fallow group (CK) differed significantly from T1 treatment, which exhibited enrichment in biosynthesis of secondary metabolites, pantothenate and CoA biosynthesis, terpenoid, pyridine, and piperidine alkaloids ($p < 0.05$) (Figure 7A). In contrast, T2 treatment demonstrated significant enrichment in gluconasturtiin, valine, leucine, and isoleucine amino acids, 2-oxocarboxylic acid metabolism, and cyanogenic amino acid metabolism, among others ($p \leq 0.05$) (Figure 7B). T3 treatment showed significant enrichment in gluconasturtiin, 2-oxocarboxylic acid, valine, leucine, isoleucine, aminoacyl-tRNA, cyanogenic amino acid metabolism, and other pathways ($p \leq 0.05$) (Figure 7C). Furthermore, compared to the fallow planting pattern, the rhizosphere differential metabolites were significantly enriched in secondary metabolite biosynthesis after planting three different crops. Therefore, the degree of enrichment in secondary metabolite biosynthesis under the four treatments was presented. Soil under both rapeseed and wheat as preceding crops showed a significant increase in the abundance of L-valine, 3-methyl-2-oxo-butanoic acid, nicotine, and nicotinic acid (Figure 7D).

3.8 Conjoint analysis

The relationship between soil environmental variables and microbial community was assessed using RDA analysis. In the bacterial community structure of tobacco soil, RDA1 and RDA2 explained 23.71% and 22.17% of the variance, respectively. Organic matter (OM), available nitrogen (AN), zinc (Zn), and boron (B) had significant effects on bacterial community composition ($p < 0.01$) (Figure 8A). Similarly, in the fungal community structure of tobacco soil, RDA1 and RDA2 accounted for 19.6% and 18.33% of the variation, respectively. Environmental factors including OM, Zn, B, molybdenum (Mo), and AN significantly influenced fungal community composition ($p \leq 0.05$) (Figure 8B).

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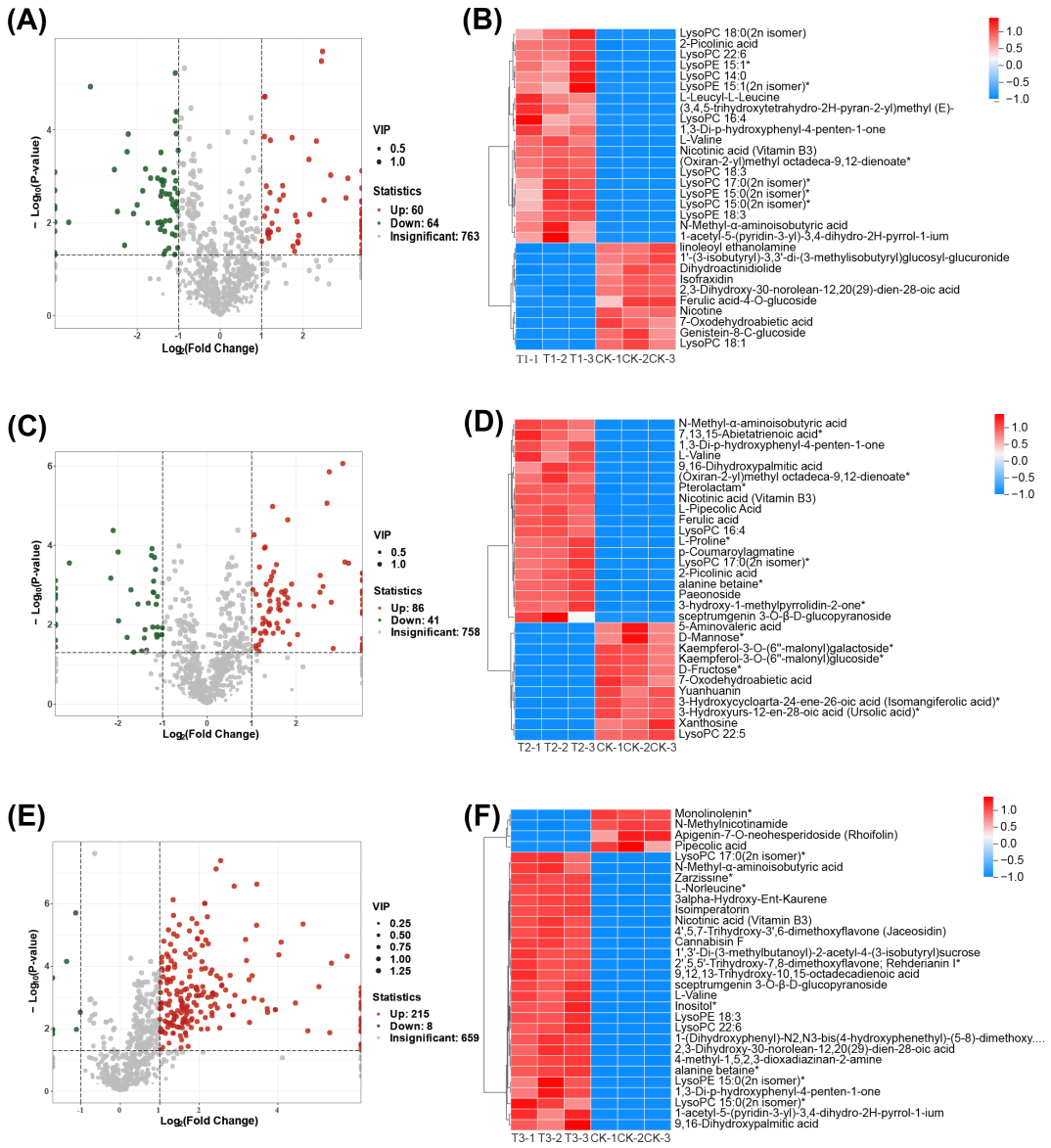

FIGURE 6
Differential metabolite analysis. **(A, C, E)** are the volcano plots of up and down-regulated metabolites of T1 vs CK, T2 vs CK, and T3 vs CK, respectively. **(B, D, F)** are differential metabolite cluster analysis charts (Top30) of T1 vs CK, T2 vs CK and T3 vs CK, with the color indicating the relative metabolite expression in the group of samples.

A correlation analysis was performed between soil environmental factors and the peak area of differential metabolites in key metabolic pathways (Figure 8C). This analysis showed that certain key metabolites such as Nicotine, Anabasine, significantly affected the levels of available nitrogen, effective molybdenum and zinc, and organic matter, the metabolites demonstrated a positive correlation with effective zinc and organic matter content, and a negative correlation with available nitrogen and effective molybdenum content.

To investigate the relationship between differential metabolites in rhizosphere soil and microbial communities, a correlation analysis was conducted focusing on the top 10 phyla by relative abundance in the microbial community and the peak area of differential metabolites in key metabolic pathways. Significant correlations were identified between the bacterial phyla GAL15, Chloroflexi, Acidobacteriota, Gemmatimonadota, Myxococcota, and Actinobacteria with more than five key differential metabolites, including nicotinic acid and 3,7-Di-O-methylquercetin (Figure 8D). Similarly, the fungal phyla Ascomycota and Basidiomycota also showed significant correlations with over five key differential metabolites, such as 3,7-Di-O-methylquercetin, nicotinic acid, and anatabine (Figure 8E). These findings indicate that microbial phyla are influenced by changes of key metabolic substance and pathways of various preceding crops.

4 Discussion

This study demonstrated that all three preceding crops increased tobacco biomass compared to fallow conditions. Another study noted

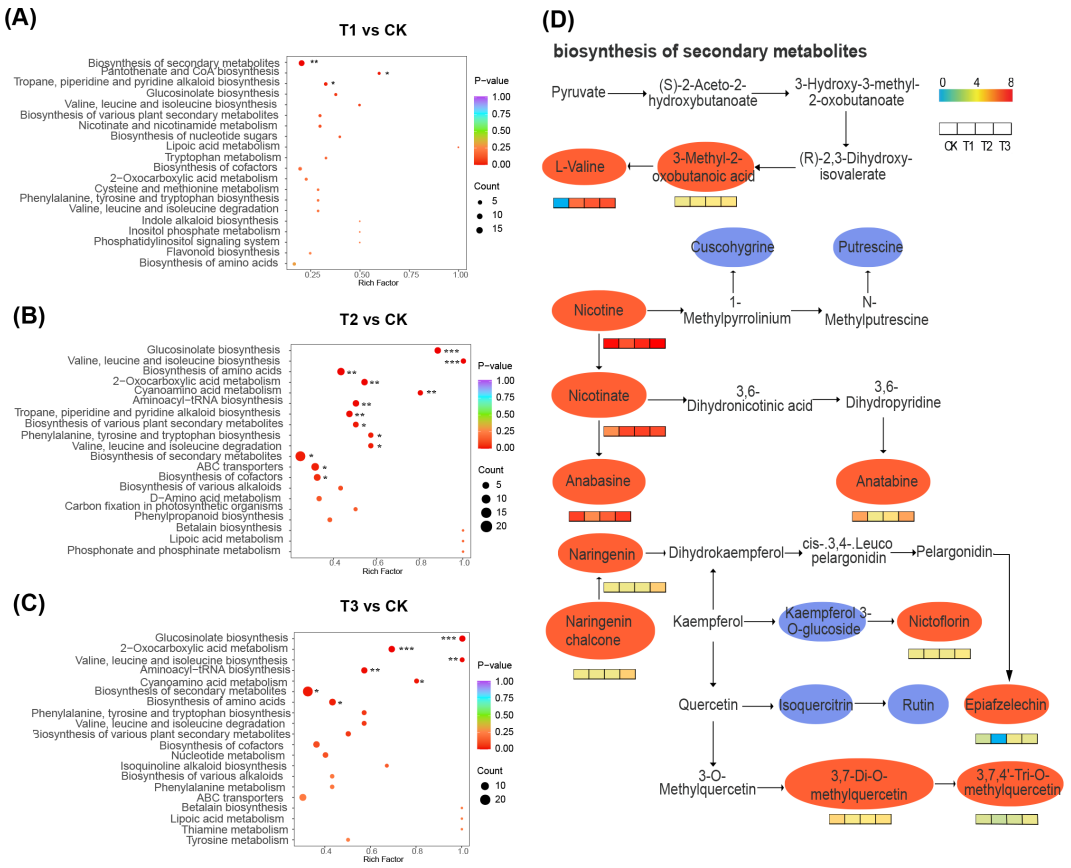

FIGURE 7
Differential metabolite enrichment in KEGG pathways and key metabolic pathways. **(A-C)** are the differential metabolite enrichment in KEGG pathways of T1 vs CK, T2 vs CK, and T3 vs CK, respectively. **(D)** is the highly enriched pathway in the key metabolic pathways (secondary metabolic pathway), with the ellipse representing the metabolite in the pathway. Red indicates a significant difference, while blue indicates no significant difference.

that different preceding crops can enhance plant growth by improving nutrient availability until maturity (Zhang et al., 2022), resulting in superior tobacco growth compared to fallow conditions. Specifically, tobacco cultivated after rapeseed showed the highest biomass, highlighting the substantial influence of different preceding crops on planting patterns (Chichongue et al., 2022). Soil nutrients directly influence crop growth in agriculture (Wulanningtyas et al., 2021; Vaziritabar et al., 2024). This study found that soil with maize and wheat as preceding crops exhibited significantly higher levels of available phosphorus and potassium compared to fallow conditions and rapeseed predecessors. The increase in available phosphorus may be attributed to excessive phosphorus fertilizer application (Yan et al., 2023), while the decrease in available potassium in soils with rapeseed as a predecessor due to the high utilization rate of rapeseed for potassium absorption (Gu et al., 2024). Zinc acts as a catalyst for protein synthesis and activates numerous enzymes, while boron contributes to tobacco carbon metabolism and transport (Vera-Maldonado et al., 2024; Singh et al., 2024). Different predecessor treatments significantly boosted soil available zinc and boron levels, partially fulfilling the demands of both previous crops and tobacco for trace elements (Brodowska et al., 2022; Palmer et al., 2023). Predecessor planting could enhance soil enzyme activity, modify nutrient cycling, and influence matter and energy metabolism (Wang et al., 2022b). Soil urease and acid phosphatase activities significantly increased under all three predecessor treatments compared to fallow conditions, with soil catalase activity particularly rising under rapeseed and wheat predecessor treatments. These changes improve soil fertility and nitrogen nutrition, mitigate the harmful effects of hydrogen peroxide, and enhance available phosphorus accumulation (Borase et al., 2020), thus benefiting the growth of subsequent crops.

Soil microbes play a critical role for sustainable agriculture by influencing soil fertility through following processes, such as organic carbon decomposition, humus formation, and soil nutrient transformation cycles (Xu et al., 2024). Modularization of microbial interactions is crucial for maintaining stability and resilience of microbial communities, influenced by resource allocation, habitat heterogeneity, phylogenetic status, and ecological niche overlap (Qiao et al., 2024). A recent study has identified a modular structure in rhizosphere soil microbial networks, which varies with different predecessor crops (Liu et al., 2020). This finding underscores that different crops influence the rhizosphere soil's microbial diversity (Kowalchuk et al., 2002; Chaparro et al., 2014; Li et al., 2014). Additionally, predecessor

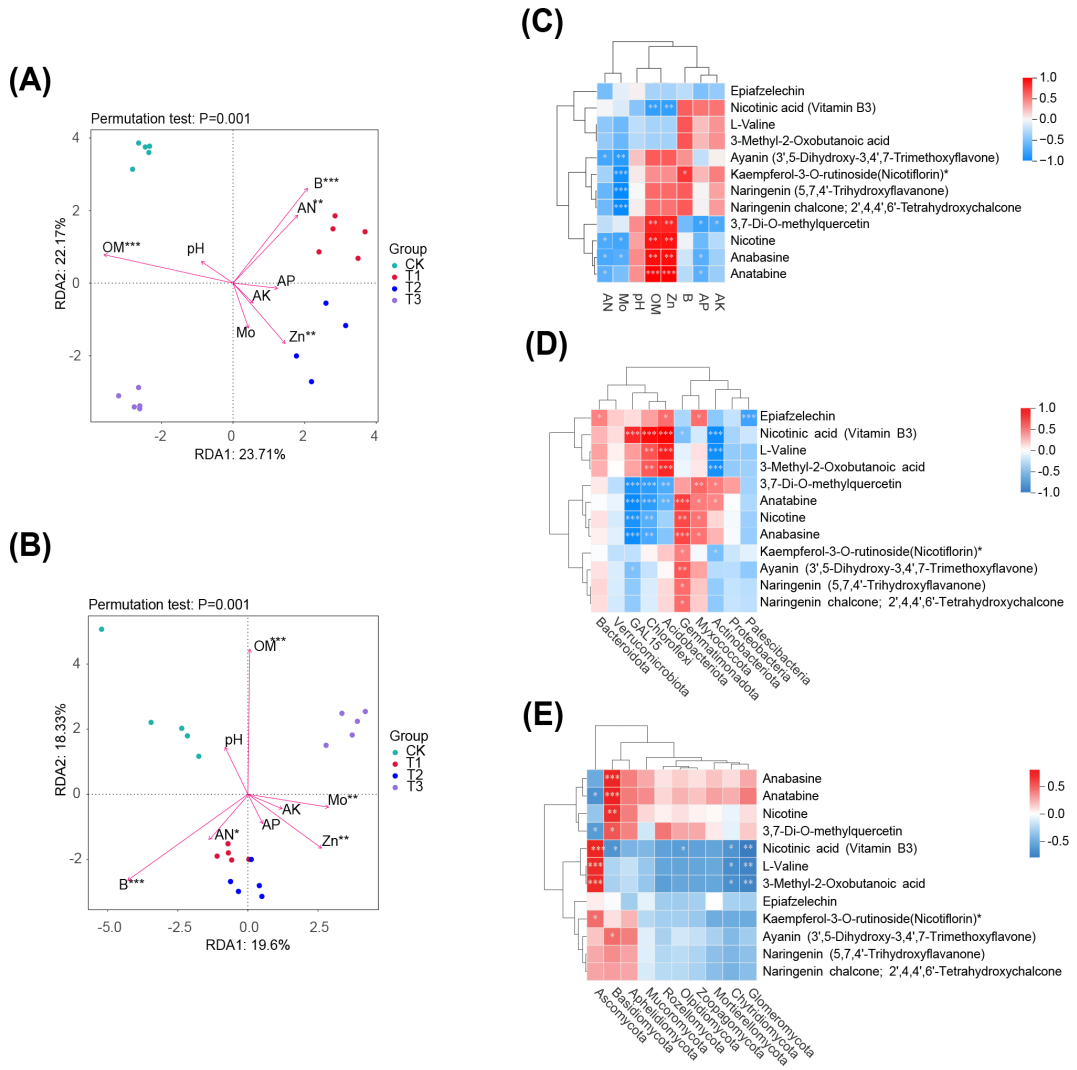

FIGURE 8
Conjoint analysis. **(A, B)** are the redundancy analysis between soil environmental variables and microbial community structure of bacteria and fungi, respectively. **(C)** indicates the correlation analysis between differential metabolites of key metabolic pathways and soil environmental indexes, **(D)** and **(E)** are the correlations between soil microbial community structure (bacteria and fungi) and differential metabolites in key metabolic pathways, respectively. Significance level: *** $p < 0.001$, ** $0.001 \leq p < 0.01$, * $0.01 \leq p < 0.05$.

crops can enhance soil bacterial abundance and diversity, thereby promoting crop growth (Xi et al., 2021).

Redundancy analysis demonstrated a significant correlation between soil physicochemical properties and soil microbial composition. Different predecessor crops primarily affect the bacterial phyla as Acidobacteriota, Chloroflexi, and Patescibacteria. Acidobacteriota decompose soil plant litter and utilize carbohydrates from root exudates (De Chaves et al., 2019). After rapeseed cultivation, Acidobacteriota abundance increased, facilitating carbohydrate transformation in the rhizosphere. Chloroflexi are crucial for soil organic matter metabolism and community establishment (Nabi et al., 2022). In contrast, Patescibacteria, which have minimal known functions in soil environments, require further study (Wang et al., 2023a). The abundances of Chloroflexi increased after maize cultivation, enhancing plant photosynthesis and carbon metabolism. Ascomycota, which often comprise over 90% of fungal species (Wu et al., 2020), also significantly increased after maize cultivation. Ascomycota are key in degrading lignin-rich organic matter and releasing nutrients for plant growth. Root exudates strongly influence the diversity and types of active fungal populations (Jin et al., 2022). Different predecessor crops alter the rhizosphere microbial community in tobacco, potentially affecting plant-microbe interactions and nutrient cycling, which benefits tobacco growth.

Metabolite analysis of plants and rhizospheric organisms has revealed that specific metabolites detected in the rhizosphere soil of crops are primarily plant root exudates (Shi et al., 2023). The biosynthesis of secondary metabolites in the soil is significantly influenced by previous crops such as maize, rapeseed, and wheat, with plants playing a more important role than microorganisms (Bi et al., 2021; Li et al., 2022). Variations in rhizospheric metabolites

primarily come from exudates of preceding crops, impacting plants growth, soil characteristics, and microbial communities (Tiziani et al., 2022). Importantly, this study indicated that maize and wheat cultivation resulted in increased available phosphorus concentration in soil compared to planted fallow, however, acid phosphatase activity was highest in soils where the previous crop was rape, the phosphorus efficiency of the three preceding crop treatments was improved, possibly due to the release of organic acids and enzymes in root secretions, enhancing the solubility of phosphorus compounds and thereby increasing their bioavailability (Chai and Schachtman, 2021). Alkaloids, nitrogen-containing compounds, are closely associated with nitrogen (Wang et al., 2023b), with three key alkaloid metabolites nicotine, anabasine, anatabine showing a negative correlation with mineral nitrogen levels in soil.

The study established a interdependent relationship between the microbial community and metabolites, with microbial community stability and diversity significantly influencing metabolite types and abundance. Conversely, plant root exudate metabolites shape the rhizosphere soil microbiota and drive plant-soil feedbacks on plant growth (Adamczyk et al., 2021). However, the impact of rhizosphere metabolites from varying predecessor crops on microbial community interactions in tobacco cultivation remains unclear. RDA and correlation analyses showed that soil environmental factors (e.g., OM, AN, Zn, B) significantly influence microbial community structure, while metabolites (e.g., nicotine, 3,7-Di-O-methylquercetin) are closely related to soil nutrient content. These metabolites may regulate microbial community composition, improving the rhizosphere environment and promoting tobacco growth. The correlation analysis revealed that Actinobacteria, the primary bacterial phylum, was influenced by rhizosphere metabolites. Actinobacteria often utilize flavonoids as substrates to produce diverse secondary metabolites (Wang et al., 2020a). Notably, six key flavonoid compounds in the metabolic pathways and L-valine were negatively correlated with Actinobacteria. Previous studies indicated that elevated amino acid levels, such as L-valine, may inhibit the growth of some bacteria (Moe, 2013). This inhibitory effect may indirectly improve the rhizosphere environment of tobacco by reducing the competitiveness of pathogens, thereby contributing to the enhancement of tobacco growth performance (Wu et al., 2024). In addition, high concentrations of amino acids may serve as a nutrient source for certain non-pathogenic microbes, promoting their proliferation and indirectly suppressing pathogens (Gong et al., 2023). This mechanism may provide a novel indirect protection strategy for tobacco growth. Notably, L-valine exhibited the most significant increase and variation under all three predecessor crops compared to fallow conditions, suggesting its potential role as a marker metabolite affecting soil bacterial diversity. In summary, the study demonstrated that different predecessor crops influence the composition of metabolic substances and the function of microbial communities in the rhizosphere through root exudates. These variations also affected soil nutrient concentrations and regulate tobacco growth. However, the study's limitations include the specific soil and management practices used for potting, necessitating further validation through field experiments (Yuan et al., 2024).

5 Conclusion

This study examines how different preceding crops affect rhizosphere soil nutrients, microbial diversity, and metabolite profiles of tobacco, and their combined impact on tobacco growth. The results indicate that preceding canola, wheat, and maize significantly increased available phosphorus, potassium, boron, and zinc in the rhizosphere soil. Both canola and wheat enhanced soil bacterial diversity while reducing nitrogen-transforming bacteria. Conversely, maize reduced fungal pathogens and positively influenced microbial populations. Notably, wheat cultivation had the greatest impact on rhizosphere metabolites, increasing the abundance of most differential metabolites. The changes in metabolites of tobacco following maize and canola cultivation promoted tobacco growth, whereas no effect was observed after wheat cultivation, indicating that shifts in metabolite profiles may not always benefit tobacco growth. This study confirms that changes in key metabolites influence microbial communities, shaping the rhizosphere microenvironment and enhancing tobacco growth. Overall, this research demonstrates that rhizosphere metabolites influenced by different preceding crops regulate subsequent crop growth and soil improvement, offering insights for sustainable ecological tobacco cultivation.

Data availability statement

Microbial data associated with this article can be found in the online version. Raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA, Bacteria: https://www.ncbi.nlm.nih.gov/sra/PRJNA1105736; Fungi: https:/www.ncbi.nlm.nih.gov/sra/PRJNA1105742) with Accession No. PRJNA1105736, No. PRJNA1105742. All other raw data was transferred to Mendeley data as Microsoft Excel files (https://data.mendeley.com/drafts/2c9vjc6s46).

Author contributions

PZ: Data curation, Formal analysis, Methodology, Writing – original draft. HZ: Data curation, Formal analysis, Methodology, Writing – original draft. XL: Data curation, Formal analysis, Methodology, Writing – original draft. LZ: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing. YXZ: Formal analysis, Methodology, Writing – review & editing. TX: Formal analysis, Methodology, Writing – review & editing. GZ: Formal analysis, Writing – review & editing, Methodology. SJ: Formal analysis, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Supervision. JW: Formal analysis, Methodology, Writing – review & editing. YH: Formal analysis, Methodology, Writing – review & editing. JL: Formal analysis, Methodology, Writing – review & editing. JZ: Formal analysis, Methodology, Writing – review & editing. YJZ: Formal analysis, Methodology, Writing – review & editing. YL: Formal analysis, Writing – review & editing, Methodology. FN:

Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing. DL: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

Authors HZ, YXZ, TX, JW, YH, JL, JZ, YJZ, and YL were employed by Lincang Branch Company of Yunnan Tobacco Company.

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

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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.2025.1530324/full#supplementary-material

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Weather parameters and biotic factors synergistically shape the phyllosphere microbiome of pomelo (*Citrus maxima* (Burm.) Merr.) across annual cycle

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Phyllosphere microbiome plays important roles in crop adaptation to the changing environments. Perennial woody crops undergo annual cycles with the changing weather parameters and the biological factors, which might shape the phyllosphere microbial community. In this study, we aimed to investigate the dynamics of phyllosphere microbiome of pomelo (*Citrus maxima* (Burm.) Merr.), an economically important horticultural crops worldwide, and to compare the respective contribution of the weather parameters and the biotic factors to the microbial community assembly, with special focus on the amino acids in leaves. Hi-Seq analysis revealed that both bacterial and fungal communities showed annual cycle dynamics, and the bacterial community in summer was much different from those in other seasons probably due to high temperature and precipitation. However, contribution of the biotic factors (e.g., leaf traits) (12%-29%) to microbial community assembly was higher than that of the weather parameters (4%-15%). Redundancy analysis indicated that the leaf amino acids significantly affected bacterial community while sugars significantly affected fungal community, highlighting the differential patterns of bacterial and fungal community as affected by the biotic factors. Finally, structure equation model showed that the weather parameters influenced microbial community colonizing pomelo leaves both in a direct way and in an indirect way via leaf traits (mainly amino acids). These results demonstrate the primary role of weather parameters and the key role of leaf amino acids in shaping phyllosphere microbiome.

KEYWORDS

phyllosphere microbiome, pomelo (*Citrus maxima* (Burm.) Merr.), annual dynamics, amino acids, weather parameters, leaf chemical traits

1 Introduction

Plants are the most important organisms on the Earth, whose leaves produce organic carbon (photosynthates) from CO_2 and water, and thus sustain the life on the planet. It is inspiring that the global leaf area has increased by 5.39×10^6 km^2 during the years of 2000-2017, reaching 1.71×10^8 km^2 (Chen et al., 2019). When plant leaves function as the primary productivity, they meanwhile serve as habitats for diverse microorganisms, which are collectively called phyllosphere microbiome. According Peñuelas and Terradas (2014), up to 10^{26} bacteria occupy the global plant leaves. Despite of its huge population size, phyllosphere microbiome has been less investigated, compared with rhizosphere microbiome which has received intense attention for decades (Zhu et al., 2022). However, increasingly accumulated evidence indicates that phyllosphere microbiome plays significant roles in benefiting plants with respect to stress tolerance, growth promotion, nutrient uptake, and disease suppression (Stone et al., 2018). For example, a 1-aminocyclopropane -1-carboxylate- (or ACC-) deaminase producing bacterial strain isolated from the leaves of tropical yam significantly promoted the plant growth of tomato after its colonization of the phyllosphere (Herpell et al., 2023). Epiphytic and endophytic N_2-fixers in phyllosphere can contribute greatly to plant N nutrition (Zhu et al., 2023). Considering the necessity of leaf disease control, nutrient supply with foliar spray, and aerial spray of stimulants for stress inhibition in most crops, especially in horticultural crops, it is applausive to apply phyllosphere microbiome-based microbiological technology to achieve sustainable development in agriculture industry.

Since phyllosphere microbime can be helpful in plant growth and development, deep insights into the bacterial or fungal community and their driving force are necessary. It is revealed that phyllosphere bacterial community is highly dynamic, with its composition and structure sensitive to the environments including biotic and abiotic factors (Thapa et al., 2017; Li et al., 2023; Wang et al., 2023; Kong et al., 2024). Among biotic factors, the functional traits of host plants have been intensively studied. Earlier studies focused on the foliar C:N:P stoichiometry, which was demonstrated to affect the phyllosphere nitrogen fixing bacterial community or other functional groups (Martirosyan and Steinberger, 2014; Rico et al., 2014). Thapa et al. (2017) indicated that 83% of the observed variance in phyllosphere microbiome could be assigned to the contents of iron, manganese, and chlorophyll b of leaves. Similarly, Yuan et al. (2023) suggested that the contents of isotope carbon and copper, and the leaf area were the main factors influencing the community structure of phyllosphere microbiome. More recently, by using genome-wide association studies (GWAS), Su et al. (2024) revealed that 4-hydroxycinnamic acid, a compound in the phenylpropanoid biosynthesis pathway and synthesized by a rice gene *OsPAL02*, was the main driver for the enrichment of Pseudomonadales, which was the key taxa maintaining phyllosphere microbiome homeostasis. These results strongly point out the importance of plant identity in shaping phyllosphere microbiome (Li et al., 2023).

Plant functional traits can be mediated by abiotic factors, which thus can further exert significant influences on phyllosphere microbiome. For example, light intensity modulated the phyllosphere bacterial community of garden lettuce by affecting the functional composition of leaves (Kong et al., 2024). By analyzing 16S rRNA gene sequences from 1453 leaf samples across China, Wang et al. (2023) revealed that phyllosphere microbiome was mostly explained by climate and host plant factors, with abiotic environmental cues more important at low latitudes. Meanwhile, a great deal of literature shows the involvement of soil physicochemical properties in regulating phyllosphere microbiome (Jia et al., 2020; Zhou et al., 2021), probably via their influences on plant growth performance and leaf chemical composition (Zhu et al., 2022). Taken together, it seems recognized that abiotic factors regulate phyllosphere nicrobiome in an indirect way by affecting host plant traits.

Our previous study on rhizosphere microbiome found that soil amino acids could profoundly regulate rhizosphere bacterial community (Feng et al., 2021), because amino acids can serve as both carbon source and nitrogen source for these soil organisms. Particularly, application of exogenous phenylalanine enriched functional groups promoting nitrogen cycling and plant growth (Feng et al., 2023a). In contrast, however, the regulation of phyllosphere microbiome by leaf-derived amino acids has not been fully elucidated yet. Moreover, perennial woody plants undergo seasonal changes in the plant traits as affected by the dynamics in weather parameters. Thus, we investigated the annual dynamics of phyllosphere microbiome and plant functional traits of pomelo (*Citrus maxima* (Burm.) Merr.) in this study, which will provide novel insights into the respective contribution of biotic factors and abiotic factors and the regulation of phyllosphere microbiome by amino acids in leaves. We aimed i) to explore the importance of leaf derived amino acids in shaping phyllosphere microbiome, ii) to compare the effects of abiotic environmental cues (weather parameters) and biotic factors on phyllosphere microbiome, and iii) to reveal the annual dynamics of phyllosphere microbiome in pomelo, which will facilitate the rational management and utilization of phyllosphere microbiome for plant growth and health in pomelo.

2 Materials and methods

2.1 Experimental sites and samplings

The sampling sites in this study are located in Meizhou, Guangdong Province, China, where 'Sanhong' pomelo (*Citrus maxima* (Burm.) Merr) is widely planted as cash crops. Leaf samples were taken from two pomelo orchards, namely site 1 (N 24.49207°, E 116.75385°) and site 2 (N 24.35575°, E 116.69304°), across annual cycle spanning four seasons. Briefly, sampling was conducted in Dec. 2022 (winter), Feb. 2023 (spring), May 2023 (summer), and Aug. 2023 (autumn). At each site, nine plants were randomly selected, then four mature and healthy leaves were sampled from each plant. Every twelve leaves from three plants

were pooled as one sample, thus producing three biological replicates at each sampling time for each site. The sampled leaves were stored in sterile bags in an icebox and transported to laboratory as quickly as possible. Finally, a total of 24 samples were collected for the analysis of phyllosphere microbiome.

2.2 Acquisition of climate data

The sampling sites are typical of subtropical monsoon climate, which is characterized by high temperature (annual average temperature 21.4°C) and concentrated precipitation from Apr. to Sep. (annual average precipitation 1370.26 mm). The climate data (air temperature, precipitation) during the sampling times were retrieved from China Meteorological Data Service Center (http://data.cma.cn/) (Figure 1), which was regarded as weather parameters shaping phyllosphere microbiome.

2.3 Determination of leaf chemical traits

A total of 24 nutritional constituents were quantified as biological factors (leaf traits) shaping phyllosphere microbiome, which were divided into two categories: amino acid group and non-amino acid group.

Total nitrogen (TN), total phosphorus (TP), and total potassium (TK) in leaves were extracted and quantified according to Belkhodja et al. (1998). N and P contents were determined with the Kjeldahl method and spectro-photometrically, respectively. K content was measured with flame emission spectroscopy. Iron (Fe) content was measured with atomic absorption spectro- photometry. The measurement of sugar contents in leaves was according to Ren et al. (2022) with some modifications. Briefly, fresh samples were ground with liquid N_2, and then 0.5 g leaf powder was extracted with 10 mL of distilled water at 100°C for 1 h. The extractant was subjected to filtration with 0.22 μm membrane, and the contents of sucrose and fructose were quantified with high pressure liquid chromatography (HPLC). Meanwhile, the contents of NO_3^- and NH_4^+ were determined spectro-photometrially at the absorbances of 410 and 625 nm, respectively (da Cunha et al., 2024).

Soluble amino acids in leaves were extracted and determined according to the Chinese National Standard GB/T 30987-2020 (Le et al., 2022). Briefly, 2.0 g fresh leaves were ground with liquid N_2, incubated with 200 mL boiling water for 30 min, and then filtered with 0.45 μm membrane. The free amino acids in the filtrate were determined using an automatic amino acid analyzer (L-8900, Hitachi) (Feng et al., 2021).

2.4 DNA extraction of phyllosphere microorganisms and Hi-Seq analysis

To characterize the phyllosphere (both epiphytic and endophytic) microbiome, the total DNA in leaves (0.25 g) was extracted using the OMEGA E.Z.N.A.® Soil DNA Kit (OMEGA Bio-Tek, Norcross, Georgia, US) according to manufacturer's instructions. DNA concentration and quality were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and 2% agar-gel electrophoresis.

For the bacterial community, the V5-V6 regions of the 16S rRNA genes were amplified with the specific primers of 799F and 1107R (F: 5′-AACMGGATTAGATACCCKG-3′, R: 5′-GGGTTGCGCTCGTTGCG-3′) (Chen et al., 2022). For the fungal community, ITS1 regions were amplified by PCR with the specific primers of ITS1F and ITS2 (F: 5′-CTTGGTCAT TTAGAGGAAGTAA-3′, R: 5′-GCTGCGTTCTTCATCGATGC-3′) (Li et al., 2022a). The raw

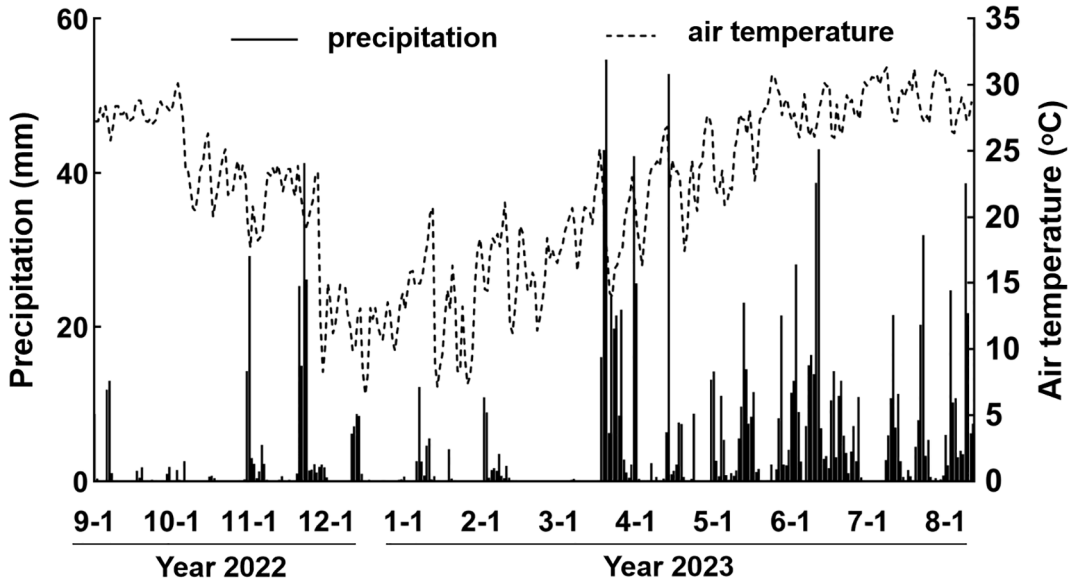

FIGURE 1
The air temperature and precipitation records during the sampling period.

image data files obtained by high-throughput sequencing were converted into the original sequence by Base Calling analysis, and the results were stored in the FASTQ file format. It contained the sequence information (Reads) and Reads quality information. Using FLASH software (version 1.2.11) (Magoč and Salzberg, 2011), the Reads of samples were assembled by overlap, and the obtained assembling sequences were the Raw Tags. Using the Trimmomatic software (version 0.3.3) (Bolger et al., 2014), the Raw Tags were filtered to obtain Clean Tags. We obtained the Effective Tags by using UCHIME software (version 8.1) (Caporaso et al., 2010) to identify and remove chimeric sequences. Then, we clustered the Tags to obtain operational taxonomic units (OTUs) at a 97% sequence similarity level by using UCLUST in QIIME (version 1.8.0) (Edgar et al., 2011) and classified OTUs based on the Silva (bacteria) and UNITE (fungi) taxonomic databases.

2.5 Bioinformatics analysis and statistics

The 'vegan' package was applied to calculate the microbial richness index (observed Chao1, ACE) and diversity index (Shannon-Wiener and Simpson diversity) (Feng et al., 2024). Principal coordinate analysis (PCoA) was performed using the 'PCoA' function in 'ape' and 'ggplot2' packages to visualize the microbial community structure. To determine whether there were significant differences in microbial community structure between seasons, the 'anosim' and 'adonis' functions in 'vegan' package were used for similarity analysis (ANOSIM) and replacement multivariate analysis of variance (PERMANOVA) respectively (Paradis et al., 2004; Wickham, 2011).

The core taxa were defined as the coexistent taxa in four seasons with the relative abundance (RA) >0.1%. Lefse was completed using the Wekemo Bioincloud (https://www.bioincloud.tech). Kruskal-Wallis test (P<0.05) and LDA threshold score >2.5 were used to identify biomarkers with significant differences between groups (Gao et al., 2024).

The networks between different subcommunities were analyzed to explore co-occurrence patterns. The Spearman rank coefficient (ρ) between OTUs of samples with occurrence rates greater than 50% was calculated using the R package 'picante' (Lv et al., 2022) in pairs. Only the robust and significant correlation between OTUs ($|r|$ >0.6, P<0.05) was selected for network construction. Then, the Gephi (http://gephi.github.io/) was used to visualize the network. In addition, the network topology was calculated in the package 'igraph' (Xiong et al., 2018).

The variation partitioning analysis (VPA) was performed using the 'varpart' and 'anova.cca' functions to measure the contribution of climatic and biological factors to the changes in microbial community structure (Zhao et al., 2020). Random forest (RF) analysis was performed using the 'RandomForest' package in R (Shibahara et al., 2017) to determine the importance ranking of each biological factor's contribution to the difference in alpha diversity indexes between groups. The 'varclus' function in the 'relaimpo' package was used to test the collinearity of biological factors. Spearman ρ^2>0.7 indicates that there was collinearity between the biological factors, and one of the representative variables needs to be selected. Redundancy analysis (RDA) was performed using the 'decorana' and 'rda' functions from the 'vegan' package to elucidate the influence of biological factors on the bacterial and fungal community structure (Feng et al., 2023b).

The structural equation model (SEM) in Package R 'lavaan' (Tenenhaus et al., 2005) was used to evaluate the effects of climatic and biological factors on microbial community diversity in leaves. The chi-square test, df and its associated P-values, goods-of-fit index (GFI), approximate root-mean square error (RMSEA), and Akaechi Information criteria (SRMR) were used to determine the fit between the model and the data (good fit when df<5, 0.05<P≤ 1.00, GFI>0.800, RMSEA ≤ 0.05, SRMR<0.08, lower chisq indicating a better fit) (Feng et al., 2021).

All data were the average of three biological replicates. Multiple range test and t test were performed with SPSS v21.0. All the R codes for analysis in this study are available in the following GitHub repository (https://github.com/vn0909/Codes-for-Analysis).

3 Results

3.1 Phyllosphere microbiome and the core taxa across annual cycle

The amplicon sequencing of 16S rRNA and ITS genes revealed diverse bacterial and fungal taxa associated with pomelo leaves. Totally, there were 20 bacterial phyla and 13 fungal phylla, or 58 bacterial genera and 59 fungal genera detected with RA > 0.1% (Supplementary Data Sheet S1). The dominant (RA>1.0%) bacterial genera included *Methylobacterium-Methylorubrum*, *Pseudomonas*, *Hymenobacter*, *Sphingomonas*, *Massilia*, *Methylocella*, *Acinetobacter*, *Amnibacterium*, and *Curtobacterium* (Figure 2A, Supplementary Data Sheet S1), while the dominant fungal genera included *Acrodontium*, *Hyphozyma*, *Inocybe*, *Uwebraunia*, *Nigrospora*, *Amphinema*, *Coniosporium*, *Zasmidium*, *Golubevia*, *Cyphellophora*, *Zeloasperisporium*, *Zymoseptoria*, *Strelitziana*, *Phaeosphaeria*, *Neonectria* (Figure 2B, Supplementary Data Sheet S1).

On the basis of the occupation and RA, we identified 13 bacterial core genera and 14 fungal core genera in the phyllosphere microbiome. For bacterial core taxa, *Methylobacterium-Methylorubrum* was the most abundant genus (32.08%), followed by *Pseudomonas* (16.23%), 1174_901_12 (7.08%), and *Hymenobacter* (5.44%). These 13 core genera totally occupied 82.32% of the phyllosphere bacterial community (Figure 3A). For fungal core taxa, *Acrodontium* was the most abundant genus (RA 5.86%), followed by *Hyphozyma* (4.60%), *Inocybe* (4.04%) and *Uwebraunia* (3.58%). These 14 core genera totally occupied 78.50% of the phyllosphere fungal community (Figure 3B).

In contrast to the core taxa occurring across all seasons, Lefse analysis reveals that there were some specific taxa indicative of each season (Figure 4). Four bacterial genera (*Amnibacterium*, *Methylobacterium-Methylorubrum*, *Spingomonas*, *Massilia*) and 5 fungal genera (*Acrodontium*, *Zymoseptoria*, *Zeloasperisporium*,

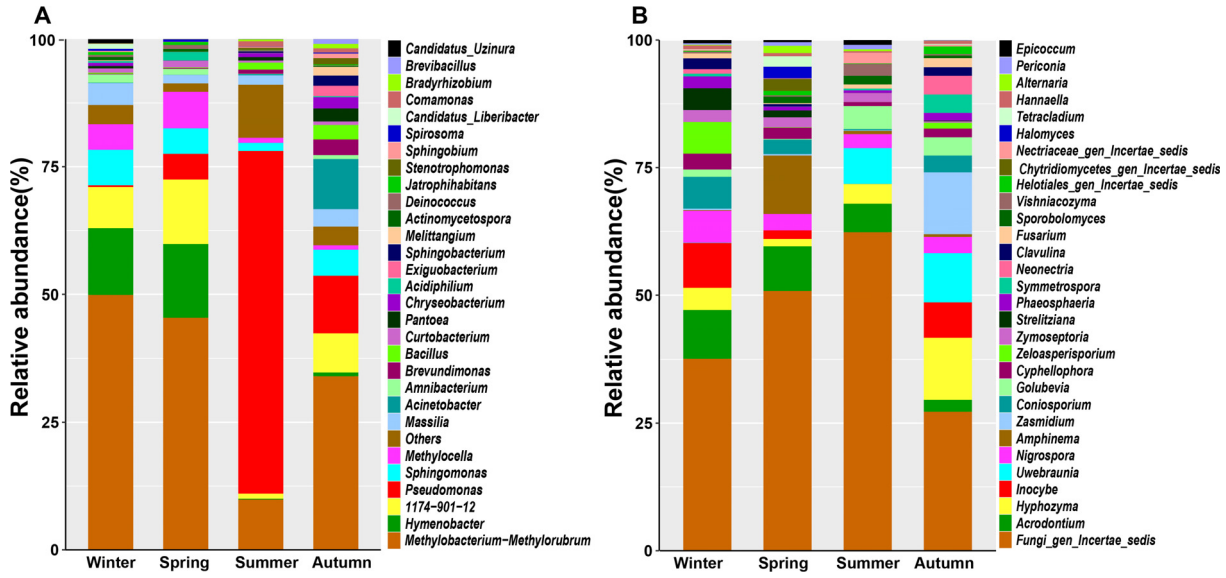

FIGURE 2
The composition of bacterial **(A)** and fungal **(B)** community at genus level associated with pomelo leaves.

Strelitziana, *Inocybe*, *Clavulina*) were significantly enriched in winter. Three bacterial genera (*Hymenobacter*, 1174_901_12, *Methylocella*) and 3 fungal genera (*Amphinema*, *Halomyces*, one unidentified genus) were significantly enriched in spring. Five bacterial genera (*Curtobacterium*, *Ralstonia*, *Delftia*, *Thauera*, *Pseudomonas*) and several unidentified fungal genera were significantly enriched in summer. One bacterial genera (*Pantoea*) and 3 fungal genera (*Uwebraunia*, *Zasmidium*, *Neonectria*) were significantly enriched in autumn (Figure 4). It is noteworthy that some core genera were also indicative of particular season, probably suggesting that their seasonal dynamics shaped the phyllosphere microbiome.

3.2 Diversity of bacterial and fungal community and their seasonal dynamics

We calculated the alpha diversity of phyllosphere microbiome in different seasons. The highest values of chao1 and richness were observed in winter for bacterial community, and in spring for fungal community. The highest values of shannon diversity were observed in autumn for bacterial community, and in spring for fungal community; but the highest values of simpson diversity were observed in autumn for both bacterial and fungal community (Table 1). These data indicate the difference in annual dynamics of bacterial community and fungal community colonizing pomelo leaves.

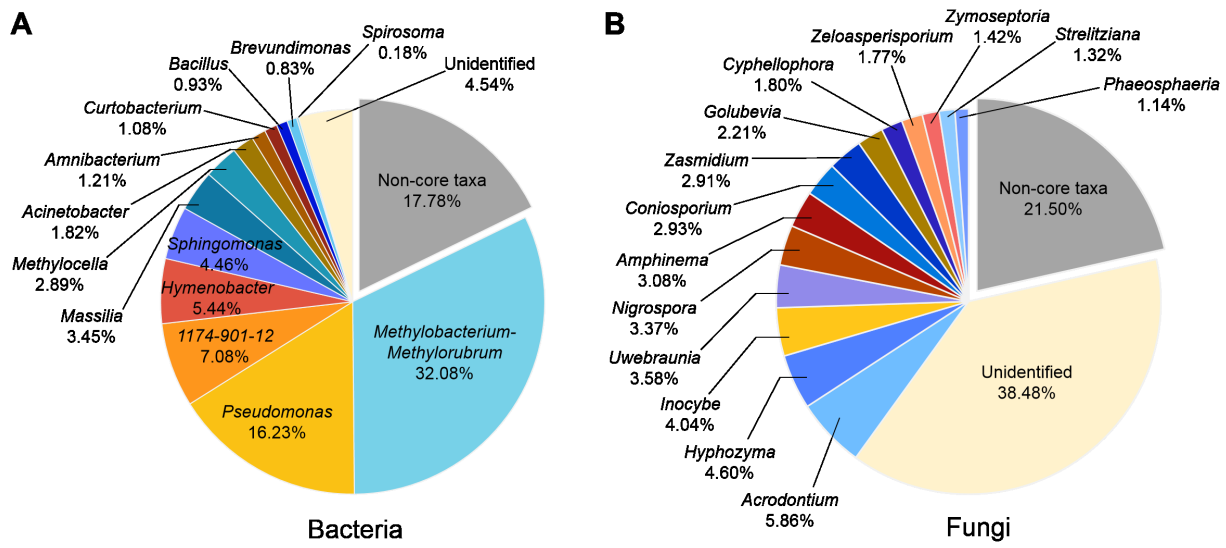

FIGURE 3
The core genera of bacterial **(A)** and fungal **(B)** community associated with pomelo leaves. The percentages indicate the relative abundance of the responding genus.

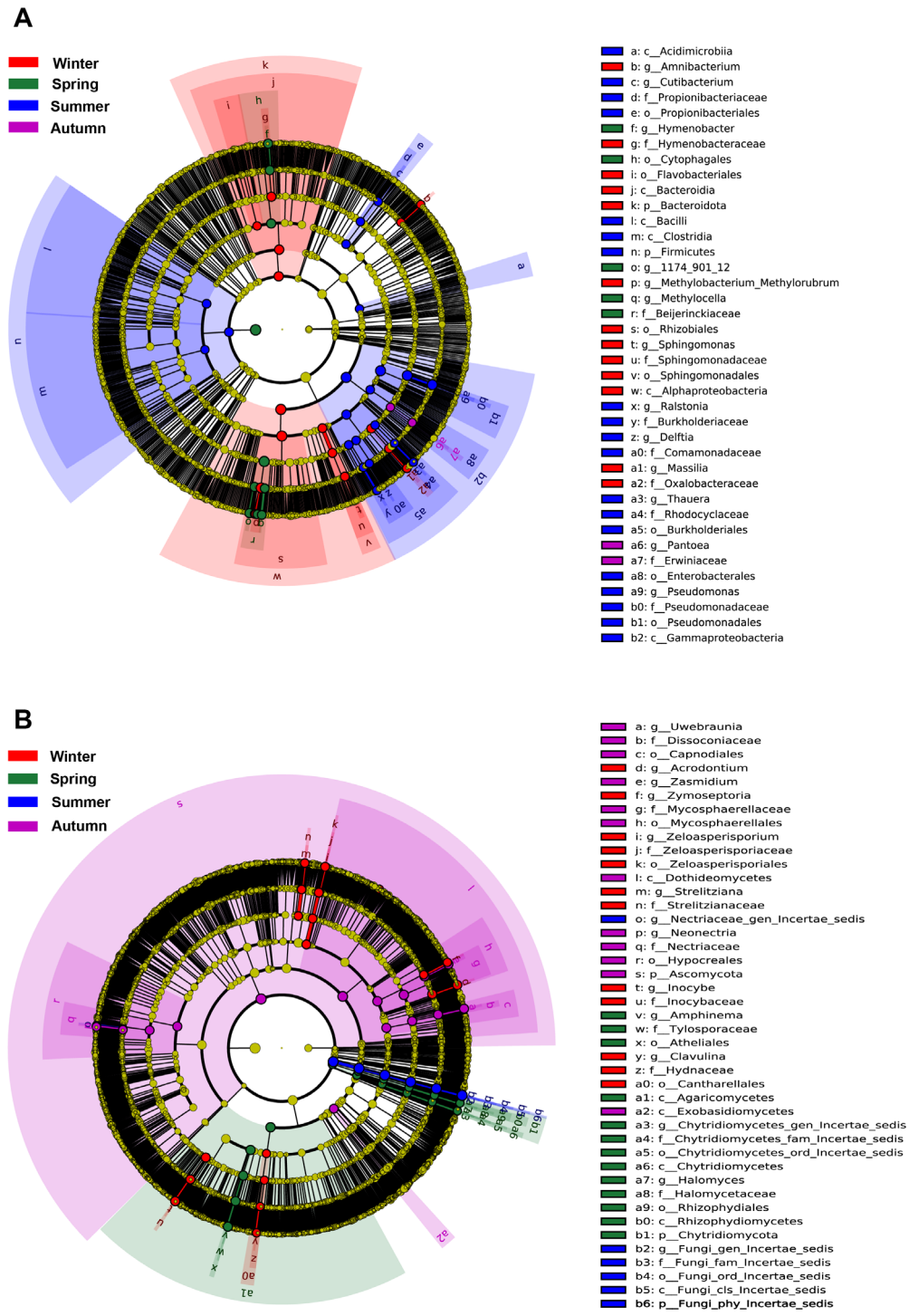

FIGURE 4
Lefse analysis demonstrating the bacteral **(A)** and fungal **(B)** biomarkers of each season.

PCoA clearly demonstrates that the bacterial community shifted from winter (2022) to autumn (2023), with the bacterial community in summer much different from those in other three seasons. We calculated the dissimilarity in bacterial community between two successional seasons, and observed a significant difference in spring-to-summer shift and in summer-to-autumn shift (Figures 5A, B). This reflects the distinctness of bacterial community in summer compared to other three seasons. In contrast, phyllosphere fungal community showed a different shifting pattern. PCoA plotting demonstrates that the fungal communities in four seasons were much different from each other, which is also confirmed with the significant dissimilarity of winter-to-spring, spring-to-summer, and summer-to-autumn shift. It seems that the fungal community in autumn was much different from that in other three seasons (Figures 5C, D).

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TABLE 1 The fluctuation of alpha diversity of bacterial and fungal community associated with pomelo leaves across annual cycle.

Sampling time	Chao1	Richness	Shannon	Simpson
Bacterial community				
Winter (Dec. 2022)	596.9 ± 75.35a	483.17 ± 87.45a	3.67 ± 0.48a	0.94 ± 0.03ab
Spring (Feb. 2023)	435.81 ± 46.75b	360.83 ± 43.66b	3.36 ± 0.25a	0.92 ± 0.01ab
Summer (May 2023)	447.46 ± 49.34b	323.5 ± 71.03b	3.36 ± 1.41a	0.77 ± 0.24b
Autumn (Aug. 2023)	438.64 ± 69.64b	387.33 ± 63.91b	3.82 ± 0.13a	0.95 ± 0.00a
Fungal community				
Winter (Dec. 2022)	901.79 ± 94.54b	706.17 ± 62.1b	3.33 ± 0.22a	0.89 ± 0.06a
Spring (Feb. 2023)	1467.68 ± 284.23a	1174.67 ± 243.7a	3.63 ± 0.3a	0.88 ± 0.03a
Summer (May 2023)	1390.5 ± 206.09a	1102.17 ± 194.82a	3.2 ± 0.22a	0.81 ± 0.04b
Autumn (Aug. 2023)	797.31 ± 103.33b	639.33 ± 106.6b	3.32 ± 0.43a	0.91 ± 0.04a

Different letters in each column indicate the significant difference among four seasons according to multiple range test ($P<0.05$, Tukey's).

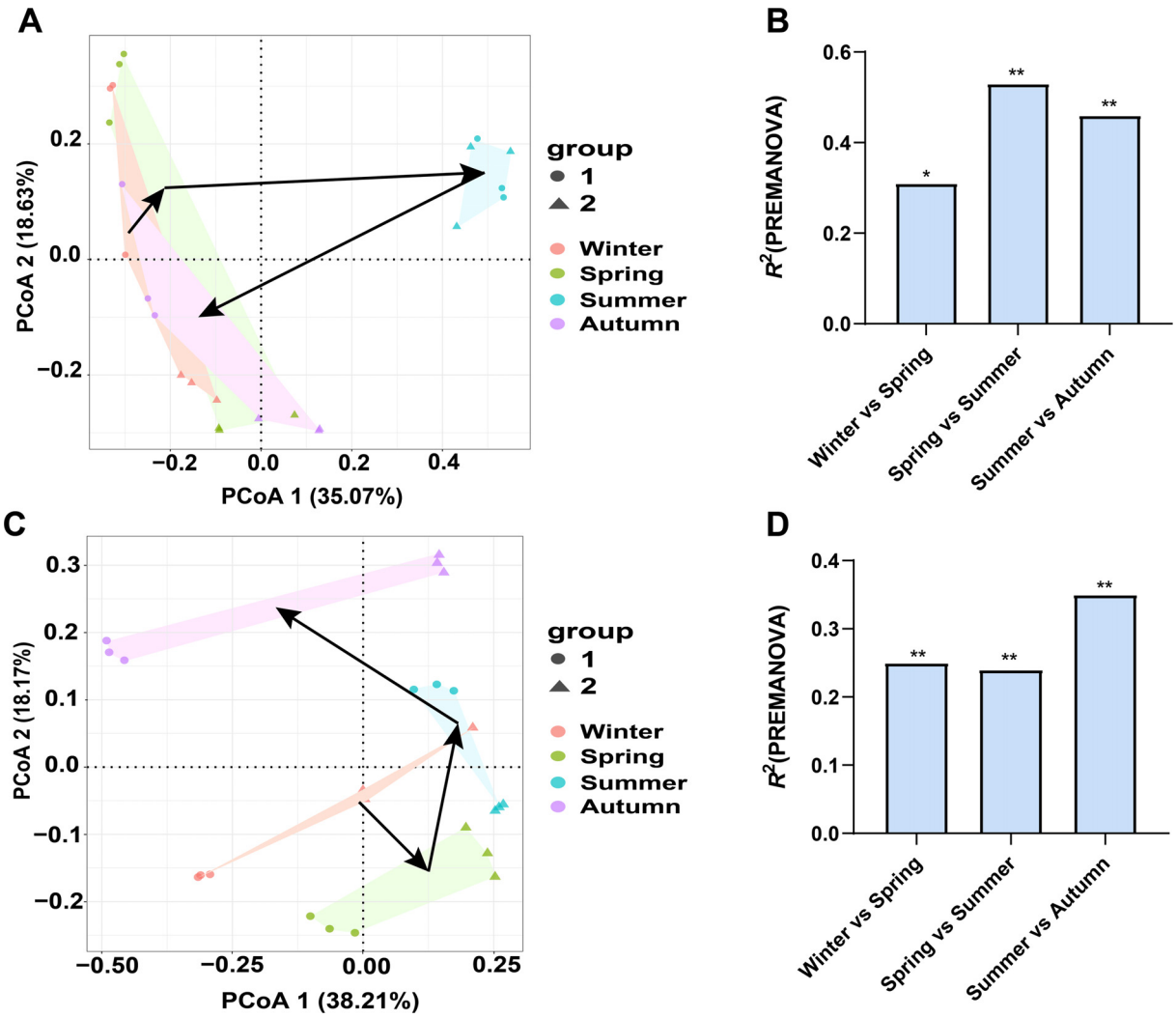

FIGURE 5
The annual dynamics of bacterial **(A, B)** and fungal **(C, D)** community associated with pomelo leaves. PCoA plotting **(A, C)** demonstrates the difference of microbial community in each season, while PREMANOVA **(B, D)** quantifies the difference in microbial community of two successive seasons. Group 1 and 2 in PCoA plotting **(A, C)** indicate the sampled orchards at site 1 and 2, respectively. * and ** indicate the significant difference in the microbial community between two paired seasons at $P<0.05$ and $P<0.01$ level.

3.3 Effect sizes of abiotic vs biotic factors and annual fluctuation of phyllosphere microbiome networks

To further explore the difference in phyllosphere microbiome across seasons, we performed network analysis of bacterial or fungal community in each season. The lowest values of node number, edge number, modularity were observed in summer and the highest values were observed in spring or autumn, for bacterial community; while the lowest values in node number, edge number, average degree, network density, and modularity were observed in summer, autumn or winter, and the highest values were observed in spring for fungal community (Figure 6, Table 2). This confirms the differential annual patterns between bacterial and fungal community of pomelo leaves as revealed in Figure 5.

According to beta diversity, it is clear that phyllosphere micobiome were strongly shaped by seasonality, which was closely associated with weather parameters. We probed into the annual dynamics of precipitation and air temperature, and found that winter and spring were characteristic of low precipitation and air temperature, while summer and autumn were characteristic of high precipitation and air temperature (Figure 1).

Considering the effects of biological factors (leaf traits) on phyllosphere microbiome (Li et al., 2022a; Yuan et al., 2023), we further measured 24 leaf nutritional constituents, including 16 amino acids, 2 carbohydrates, and 6 nutrients (Supplementary Data Sheet S2). Then we performed VPA to compare the effect size of weather parameters and biological factors. Leaf traits contributed 12% of the variation in bacterial community, much higher than climate (4%). Meanwhile, they had an overlap of 30%, indicating a strong interplay between climate and leaf traits (Figure 7). For fungal community, leaf traits and climate contributed 29% and 15%, respectively, with an interplay of 11% (Figure 7). In general, it seems that leaf traits exerted a greater effect on phyllosphere microbiome of pomelo than climate.

3.4 Phyllosphere bacterial and fungal community shaped by biological factors

Since leaf traits were more effective in shaping phyllosphere microbiome than climate, we focused on these nutritional constituents. Three rounds of collinearity analysis revealed that His was collinear with Leu, Ile, and Val, and Lys was collinear with Tyr and Phe (Supplementary Figure S1). Therefore, Leu, Ile, Val, Tyr, and Phe were excluded but only 19 nutritional constituents entered the following RDA. RDA revealed that 6 kinds of amino acids significantly shaped bacterial community, with Lys, Arg, and Ser ranking the top three, however, only 2 non-amino acid parameters (Fru and Glu) exerted significant influence (Figure 8A). For fungal community, only 3 constituents, including Fru, Met, and Suc exerted significant influences (Figure 8B). Similarly, RF analysis revealed that 8 kinds of amino acids and 5 non-amino acid constituents significantly affected the alpha diversity of bacterial community, while 5 kinds of amino acids and 3 non-amino acid constituents significantly affected the alpha diversity of fungal community (Figures 8C, D). SEM demonstrates that climate showed a positive effect on both the amino acids and the non-amino acid constitutes in leaves, which further positively affected the alpha diversity of bacterial community. In contrast,

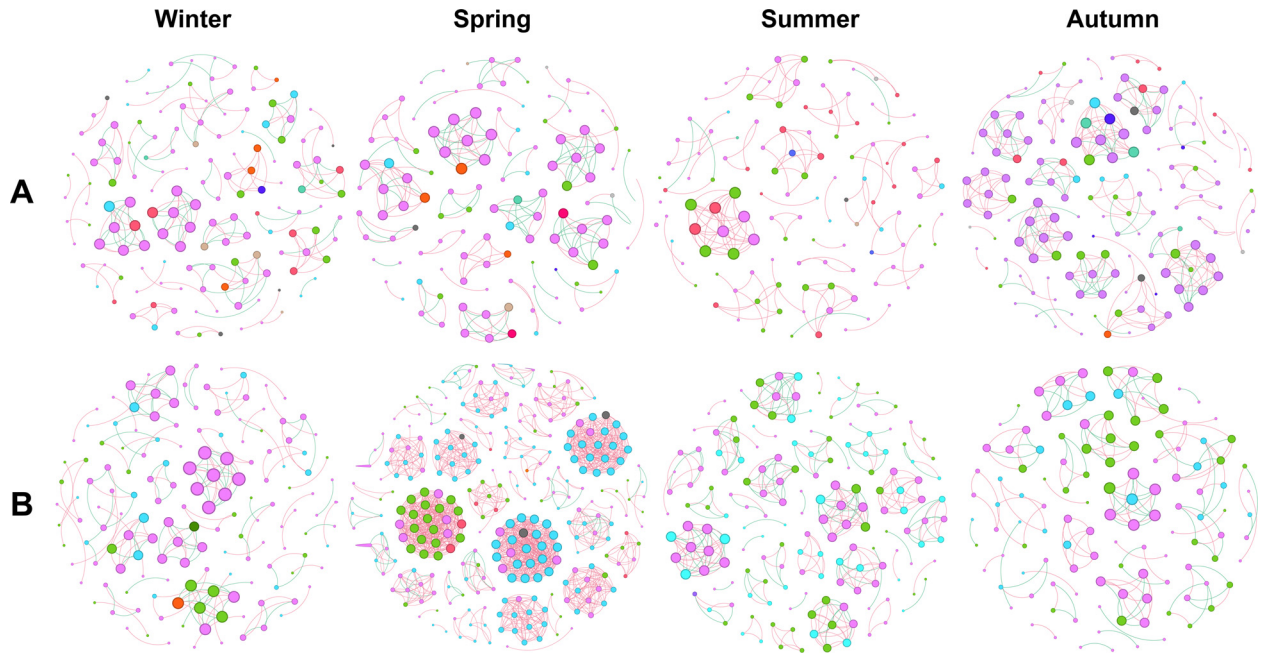

FIGURE 6
Co-occurrence network analysis of the bacterial **(A)** and fungal **(B)** community associated with pomelo leaves in each season. The different colors in each network indicate different phyla of bacteria **(A)** or fungi **(B)**.

TABLE 2 The network properties of microbial community on the pomelo leaves sampled across annual cycle.

Network properties	Winter (Dec. 2022)	Spring (Feb. 2023)	Summer (May 2023)	Autumn (Aug. 2023)
Bacterial community				
Node number	143	120	113	185
Edge number	189	200	141	164
Average degree	2.643	3.333	2.496	1.773
Network density	0.019	0.028	0.022	0.010
Modularity	0.949	0.927	0.908	0.977
Fungal community				
Node number	149	280	167	119
Edge number	190	1362	340	177
Average degree	2.550	9.729	4.072	2.975
Network density	0.017	0.035	0.025	0.025
Modularity	0.939	0.984	0.937	0.940

non-amino acid constitutes did not affected the alpha diversity of fungal community, but climate directly affected it (Figure 9). This indicates the more complicated influences of climate and leaf traits on fungal community than on bacterial community.

4 Discussion

Bacterial and fungal communities colonizing phyllosphere are critical components of plant microbiome, which play a essential role in maintaining plant health, nutrient acquisition and stress resistance (e.g. N) (Chen et al., 2020; Li et al., 2022b; Zhu et al., 2023; Li et al., 2024). However, phylosphere microbiome has been less explored so far compared to rhizosphere microbiome. Phyllosphere microbiome is highly dynamic in response to environmental cues, which include both biotic and abiotic factors (Thapa et al., 2017; Li et al., 2023; Wang et al., 2023; Kong et al., 2024). In this study, we demonstrate that both weather parameters (precipitation and air temperature) and biological factors (leaf traits) shaped the bacterial and fungal communities of pomelo leaves, with plant factors exerting a greater influence. This is similar to the results by Zhou et al. (2023), who indicated that environmental factors (geographic location and climatic conditions) and host genotype affected the epiphytic bacterial and fungal communities of wild soybeans across China. However, plant traits contributed 12%-19% to the variation of microbial community, much higher than weather parameters (4%-15%) in our study, while environmental factors contributed 19.9%-25.8% to the variation, much higher than host genotype (0.4%-3.6%) in the study by Zhou et al. (2023). This suggests that the relative importance of environmental factors and plant factors might depend on context, such as plant species, sampling area. It is well established that plant microbiome can be regulated both directly by plant traits (internal factors) and indirectly by environments (external factors) (Li et al., 2023; Kong et al., 2024), and external factors normally work via their influences on internal factors. The seasonal fluctuations of amino acids and sugars in citrus leaves have been reported, normally with low contents in the actively growing seasons (e.g. summer) (Yildiz et al., 2013; Xiong et al., 2024). Additionally, plant leaves can release volatile organic compounds (VOCs), which might possess antimicrobial activity and act as carbon sources, thereby regulating

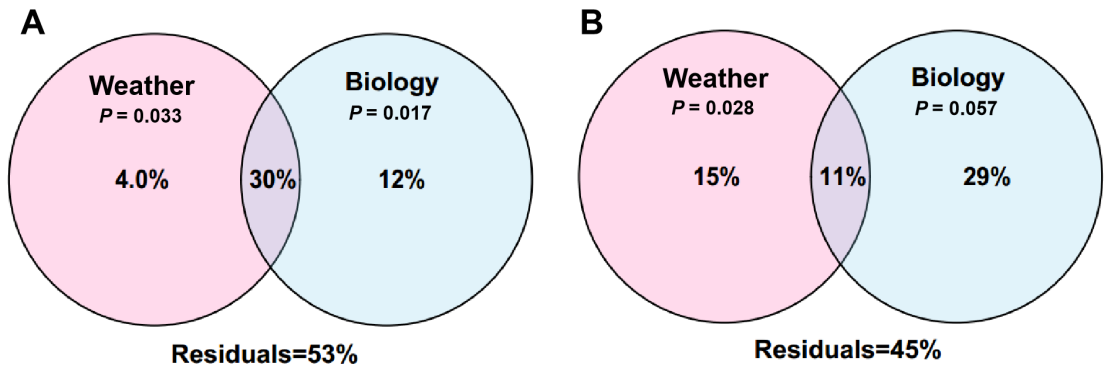

FIGURE 7
Variation partitioning analysis (VPA) showing the respective contributions of weather parameters and biological factors to bacterial **(A)** and fungal **(B)** community assembly.

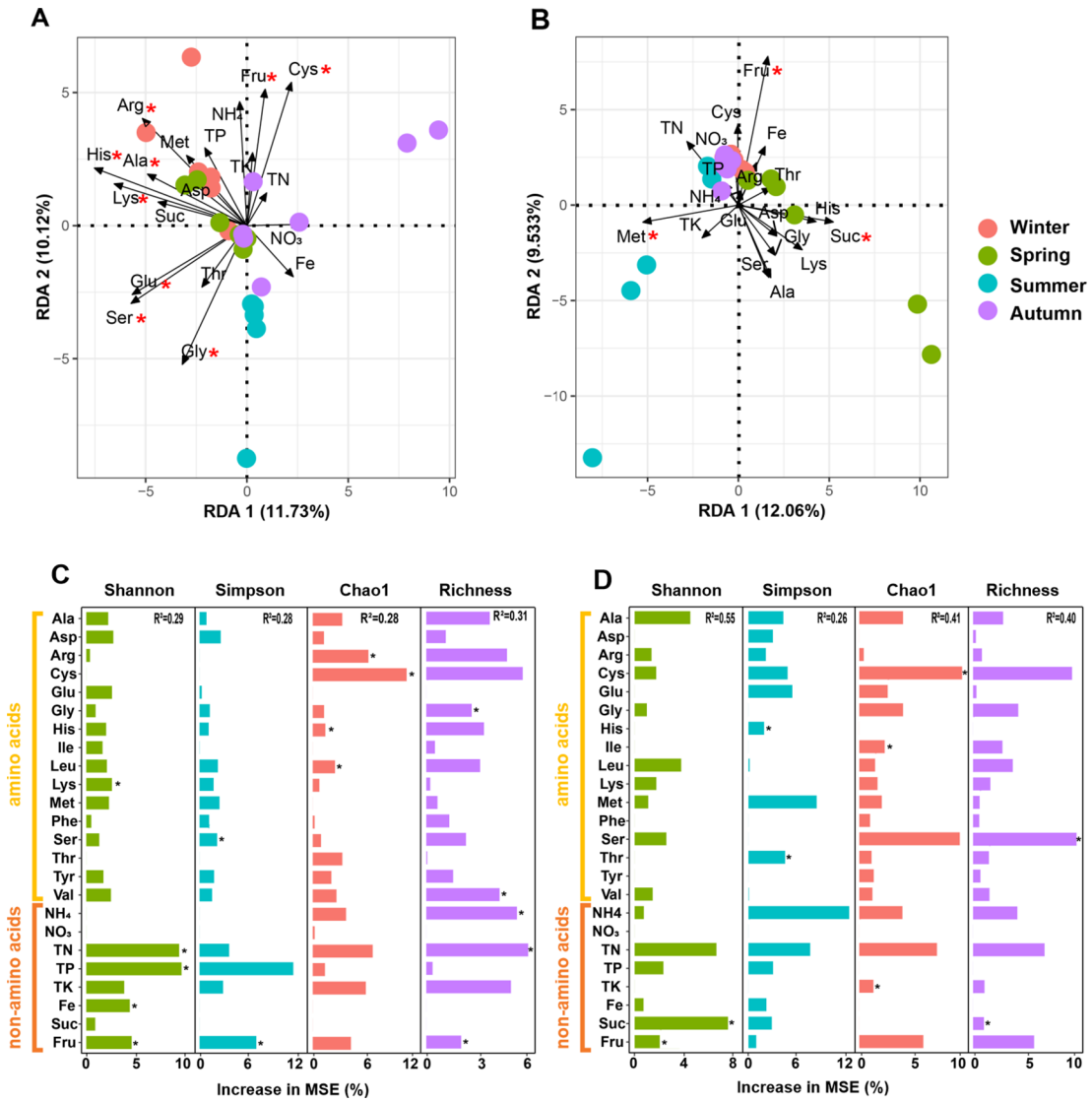

FIGURE 8
Redundancy analysis revealing the effects of leaf traits on the bacterial **(A, B)** and fungal **(C, D)** community associated with pomelo leaves. **(B, D)** indicate the quantitative effect of each leaf trait. Red aristers in **(A, B)** indicate significant influences. Black aristers in **(C, D)** indicate significant effects.

phyllosphere microbiome (Farré-Armengol et al., 2016). Specifically, *Citrus* plants are well recognized for their fragrance (namely VOCs), which was demonstrated to strongly structure their phyllosphere bacterial community (Wang et al., 2022). In this study, it is possible that the weather parameters greatly affected the plant traits (such as amino acids and sugars in leaves), especially in summer when the vegetative growth of pomelo plants was vigorous with both high temperature and high precipitation. Moreover, the VOC profile of *Citrus* plants varies much depending on seasonality (Lin et al., 2022), thereby probably contributing to the seasonal pattern of phyllosphere microbiome in this study. Considering the coupled effects of appropriate climate conditions and N fertilizers in promoting plant vegetative growth, N fertilizer application is necessary to regulate the phyllosphere microbiome even with appropriate temperature and precipitation in citrus production systems.

It is interesting that the contribution of weather parameters to bacterial community (4.0%) was much lower than that to fungal community (15%). It is possible that fungal community is more sensitive to weather parameters, especially to environmental moisture (monitored as precipitation in this study) than bacterial community (Kaisermann et al., 2015; He et al., 2023). Moreover, it is notable that the overlap of weather parameters and biological factors for bacterial community (30%) was much higher than that for fungal community (11%). This suggests that the influence of weather parameters on the bacterial community was more likely dependant on their effects on plant traits, while weather parameters influenced the fungal community in a relatively independant manner.

The core taxa of microbiome are defined as the members shared by all or most microbial communities with similar backgrounds, and play essential roles in the community functioning (Shade and Handelsman, 2012; Ren and Wu, 2016). For example, Shen et al. (2022)

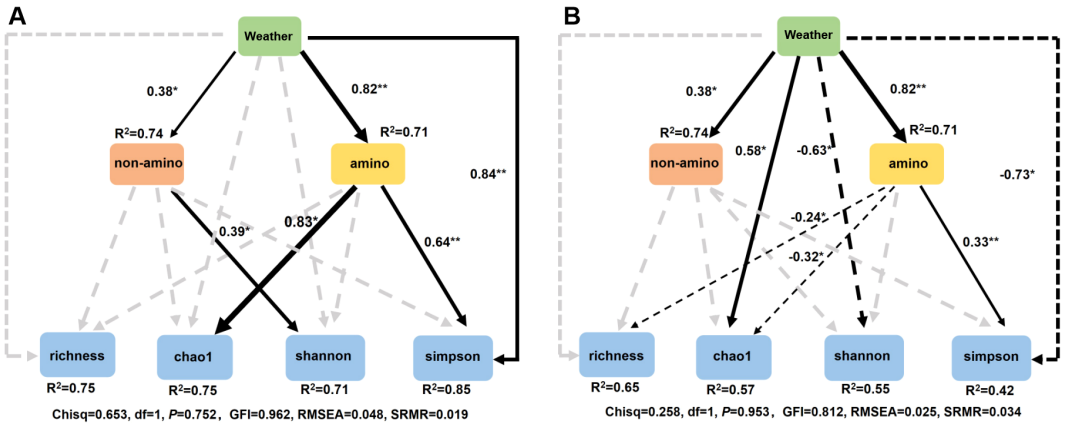

FIGURE 9
Structure equation model (SEM) analysis integrating the effects of weather parameters and biological factors on alpha diversity of bacterial **(A)** and fungal **(B)** community.

demonstrated that the core taxa (mainly belonging to Myxococcales, Pseudomonadales, Xanthomonadales) of suppressive soils from six banana plantation sites showed protective effects against banana *Fusarium* wilt disease, compared to the core taxa of conducive soils. In this study, we explored the core taxa of phyllosphere microbiome according to occupancy, and identified *Methylobacterium-Methylorubrum*, *Pseudomonas*, *Hymenobacter*, *Sphingomonas*, and *Massilia* as the top 5 core genera of bacterial community for pomelo, among which *Methylobacterium-Methylorubrum*, *Pseudomonas*, and *Sphingomonas* were also the core genera of wild soybean (Zhou et al., 2023). *Methylobacterium-Methylorubrum* is one of the most commonly reported phyllosphere bacteria promoting growth performance of many plant species, such as cucumber (Zhang et al., 2025) and rice (Oeum et al., 2024), which has been intensively investigated regarding its colonization capacity and functionality (Abanda-Nkpwatt et al., 2006; Yurimoto et al., 2021). The mechanisms underlying the plant growth promotion by *Methylobacterium-Methylorubrum* in phyllosphere mainly include nitrogen fixation, secretion of auxin, cytokinin, and 1-aminocyclopropane-1-carboxylate deaminase, and etc (Zhang et al., 2021). Recently, Zhang et al. (2024) demonstrated that the phosphoribosylpyrophosphate synthetase of *Methylorubrum extorquens* AM1 facilitated its superior colonization capability and functionality. It is possible that *Methylobacterium-Methylorubrum* bacteria assimilate methanol emitted from phyllosphere and then provide carbon sources to other members in the community. *Pseudomonas* is frequently recognized as beneficial member of phyllosphere community. Li et al. (2025) inoculated *P. fluorescens* to *Salix matsudana* and showed a increase of 90.51% in plant biomass. In detail, inoculation increased the asymbiotic nitrogen-fixation, improved photosynthetic traits (e.g. net photosynthetic rate, intercellular CO_2 concentration, stomatal conductance, transpiration rate) and the root traits (e.g. root length, root branching) and modified the phyllosphere microbiome beneficial for plant health, thereby promoting the plant nutrient uptake and biomass. Su et al. (2024) indicated that the compound 4-hydroxycinnamic acid synthesized by OsPAL02 in rice plants enriched Pseudomonadales in phyllosphere, while the reduced Pseudomonadales abundance in the knockout mutant of *OsPAL02* resulted in the dysbiosis of phyllosphere microbiome and higher susceptibility to disease. These studies suggest that *Pseudomonas*, either native or inoculated, might function via maintaining homeostasis of phyllosphere microbiome in most cases.

Our study reveals that *Acrodontium*, *Hyphozyma*, *Inocybe* were the top 3 core fungal genera of pomelo leaves. *Acrodontium* is the frequent colonizer of citrus leaves, which was enriched in healthy trees compared to HLB-infected trees and thus was regarded as the keystone taxa of phyllosphere fungal microbiome (Ginnan et al., 2020). It is interesting that a *Hyphozyma* species (*H. roseoniger*) can convert sclareol to ambradiol (Ncube et al., 2022), which might contribute to the production of fragrance compounds of pomelo leaves. Surprisingly, however, *Inocybe* has been frequently reported as dominant and ectomycorrhizal fungus (Nara, 2009; Bohorquez et al., 2021; Khan and Reshi, 2022), occasionally occurring in leaf litter (Liber et al., 2022).

The chemical properties of plant leaves are primary factors shaping phyllosphere microbiome (Yadav et al., 2005; Li et al., 2022a; Luo et al., 2023). When nutrient contents such as N, P, K and their stoichiometry have been explored for a long time, in this study, we focused on the amino acids in leaves and found that amino acids contributed much to the variation in microbial community, which has been less reported before. Our previous work on rhizosphere microbiome revealed that amino acids greatly regulated bacterial community in rhizosphere, and phenylalanine was the most effective in promoting soil N cycling (Feng et al., 2021; Feng et al., 2023a). This study reveals that amino acids played much more important roles in shaping bacterial community than non-amino acid constituents, such as several kinds of mineral nutrients, with Lys, Arg, and Ser more effective than others. Our results put the special importance on the amino acids in leaves for the first time although other chemical properties of leaves have been investigated. This importance can be attributed to the fact that amino acids can provide both N and C sources to the phyllosphere microbiome (Feng et al., 2021, 2023a). Similarly, several studies also shed lights

on amino acids. Proline, tyrosine, serine and phenylalanine showed important influence on the phyllosphere microbiome of nettle (*Urtica cannabina*), with the affected taxa including both bacteria (*Enterococcus*, *Hymenobacter*, *Sphingomonas*, *Sphingobacterium*, *Massilia*, *Ochrobactrum*, Oxalobacteraceae) and fungi (*Pezizella*, *Udeniomyces*, *Filobasidium*, Didymellaceae, Glomerellales, Helotiales) (Jia et al., 2023). Total fee AAs were one of the most outstandingly determined factors interacting with phyllosphere microbiome of garden lettuce, with the functional taxa (*Kinetoplastibacterium*, *Natronococcus*, *Bacillus*, *Bradyrhizobium*, *Methanococcus*) harboring *mdh* or *glyA* genes significantly affected (Kong et al., 2024). In contrast, fungal community (e.g. *Taphrina*, *Cylindrocladiella*, *Aspergillus*, *Boletus*, *Malassezia*, *Cladosporium*, *Xenocylindrocladium*, *Cordyceps*, *Pyrenochaeta*) in the phyllosphere of tea plants was more sensitive to sugars than bacterial community (Chen et al., 2024), which might be due to their differential trophism. Since the phyllosphere microbime is significantly regulated by the amino acids and sugars in leaves, the future research can focus on the fine regulatory patterns of these compounds on some specific taxa, such as *Methylobacterium-Methylorubrum*, *Pseudomonas*, *Sphingomonas*, which represent beneficial taxa for plant performance (Li et al., 2022b, 2024; Zhang et al., 2024). As such, it is possible to develop amino acid-based biostimulants enriching the beneficial phyllosphere microbial taxa, which supports the sustainability of agricultural production.

5 Conclusion

Phyllosphere microbime can promote plant disease resistance and growth performance. Therefore, the understanding of the annual dynamics and drivers of phyllosphere microbime is pivotal to the utilization of it. Therefore, we characterized the phyllosphere bacterial and fungal communities across annual cycle, and identified *Methylobacterium-Methylorubrum*, *Pseudomonas*, *Hymenobacter* and *Acrodontium*, *Hyphozyma*, *Inocybe* as the top core taxa of pomelo phyllosphere microbiome. The bacterial community in summer and the fungal community in autumn were much different from those in other seasons, respectively. Both biological factors (including 24 leaf traits) and weather parameters (temperature and precipitation) affected microbiome assembly, with the former (12%-29%) contributing more to the assemblage than the latter (4%-15%). Furthermore, we demonstrated for the first time that amino acids and sugars in leaves were the main drivers of bacterial and fungal communities, respectively, highlighting the importance of amino acids in manipulating phyllosphere microbiome. In general, our data suggest that biological factors (e.g. amino acids and sugars in leaves) and weather parameters regulate the phyllosphere microbiome in direct and indirect ways, respectively.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

WY: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. QYo: Formal Analysis, Investigation, Methodology, Writing – review & editing. WZ: Formal Analysis, Investigation, Methodology, Visualization, Writing – review & editing. WQZ: Data curation, Methodology, Writing – review & editing. GF: Methodology, Resources, Validation, Writing – review & editing. HZ: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. QYa: Conceptualization, Funding acquisition, Project administration, Resources, 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.

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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.2025.1532188/full#supplementary-material

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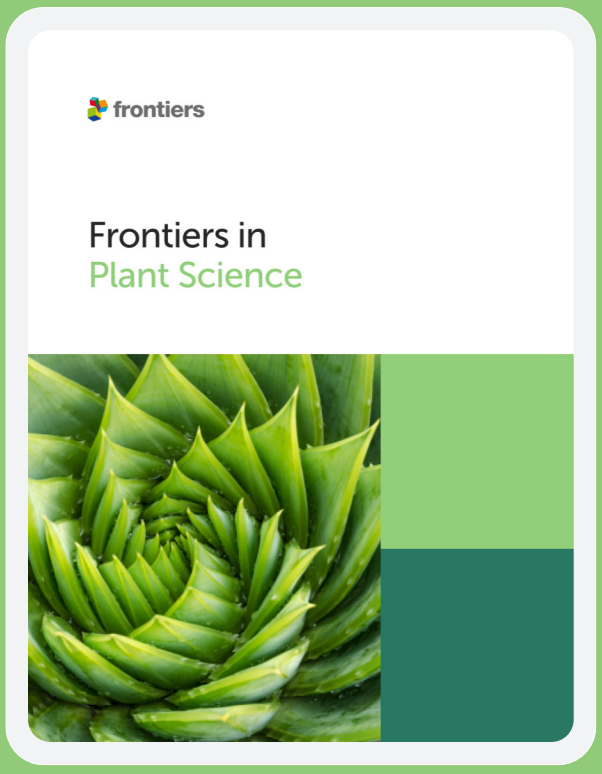

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