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The role of endophytes in plant health and resistance against biotic and abiotic stresses

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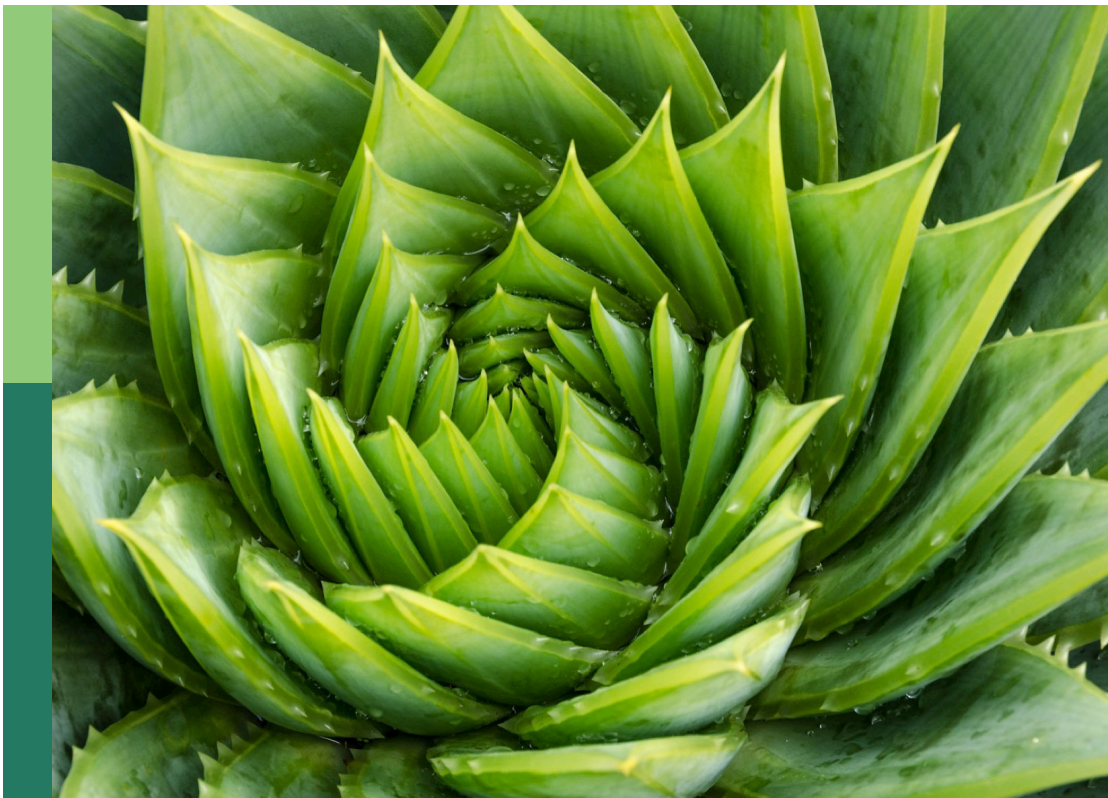
Published in
Frontiers in Plant Science



ISSN 1664-8714
ISBN 978-2-8325-4408-2
DOI 10.3389/978-2-8325-4408-2

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The role of endophytes in plant health and resistance against biotic and abiotic stresses

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Citation

Rauf, M., Arif, M., Ud-Din, A., eds. (2024). *The role of endophytes in plant health and resistance against biotic and abiotic stresses*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-4408-2

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 10 November 2022
DOI 10.3389/fpls.2022.1029836

OPEN ACCESS

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

RECEIVED 27 August 2022
ACCEPTED 06 October 2022
PUBLISHED 10 November 2022

CITATION
Naz F, Hamayun M, Rauf M, Arif M, Afzal Khan S, Ud-Din J, Gul H, Hussain A, Iqbal A, Kim H-Y and Lee I-J (2022) Molecular mechanism of Cu metal and drought stress resistance triggered by *Porostereum spadiceum* AGH786 in *Solanum lycopersicum* L. *Front. Plant Sci.* 13:1029836
doi: 10.3389/fpls.2022.1029836

Molecular mechanism of Cu metal and drought stress resistance triggered by *Porostereum spadiceum* AGH786 in *Solanum lycopersicum* L.

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Rapid industrialization and global warming have threatened the plants with multiple abiotic stresses, such as heavy metals and drought stress. For crop cultivation, the conventional approach of cleaning the soils by excavation is very costly and not feasible for large scale. Establishing toxin-free and drought-resistant crops is a major challenge in the environment under natural and anthropogenic pressure. In the past decades, copper contamination of agricultural land has become an emerging concern. For dry land reclamation, several new strategies, including bioremediation (phytoremediation and microbial remediation), have been used. Owing to the potential of Cu hyperaccumulators, the current project aims to enhance the drought tolerance and the phytoremediation potential of *Solanum lycopersicum* L. with the inoculation of copper and 12% polyethylene glycol (PEG)–induced drought stress–tolerant endophytic fungus *Porostereum spadiceum* AGH786 under the combined stress of copper heavy metal and PEG-induced drought stress. When *S. lycopersicum* L. was watered with individual stress of copper (Cu) concentration (400 ppm) in the form of copper sulfate ($CuSO_4.5H_2O$), 12% PEG–induced drought stress and the combined stress of both negatively affected the growth attributes, hormonal, metabolic, and antioxidant potential, compared with control. However, the multistress-resistant AGH786 endophytic fungus ameliorated the multistress tolerance response in *S. lycopersicum* L. by positively affecting the growth attributes, hormonal, metabolic, and antioxidant potential, and by restricting the root-to-shoot translocation of Cu and inducing its sequestration in the root tissues of affected plants. AGH786-associated plants exhibited a reduction in the

severity of copper (Cu) and drought stress, with higher levels of *SlCOPT (Cu transporters)* and *SlMT (metallothionine)* gene expressions in root and shoot tissues, indicating that AGH786 contributed to resistance to copper metal toxicity and drought stress in the host *S. lycopersicum* L.

KEYWORDS

Cu toxicity, heavy metal stress, metallothionine, drought stress, bioremediation, endophytic fungi

Highlights

- The *P. spadiceum* AGH786 endophytic fungus has been identified as a heavy metal stress–resistant, Cu hyperaccumulator, and drought stress–tolerant fungus in previous research.
- The *P. spadiceum* AGH786 endophytic fungus promoted growth and alleviated the combined stress of Cu and drought in *S. lycopersicum* L.
- The *P. spadiceum* AGH786 association enhanced the level of growth-promoting hormones, metabolites, and antioxidants under the combined stress of Cu and drought in *S. lycopersicum* L.
- The gene expressions of *SlCOPT (Cu transporters)* and *SlMT (metallothionine)* were strongly induced by *P. spadiceum* AGH786 inoculation in *S. lycopersicum* L. plants under the combined stress of Cu and drought.
- With the induction of *SlCOPT (Cu transporters)*, *P. spadiceum* AGH786 restricted the uptake and translocation of Cu from root to shoot tissues and sequestered the toxic Cu ions in fungal biomass and root tissues of *S. lycopersicum* L.
- The AGH786–*S. lycopersicum* association proved to be an effective combination of myco- and phytoremediation strategies for quickly reclaiming heavy metal–contaminated soils in drought-prone areas.

1 Introduction

Heavy metal contamination is increasingly becoming an environmental problem and causes great adverse effects around the world in the form of inorganic pollutants, which are discarded in our soil and water and into the atmosphere because of increased population growth and demands on rapidly growing metal industries, agriculture, fertilizers, pesticides, and improper waste disposal (Briffa et al., 2020). The exposure of plants to soil contamination by metal stress aggravates drought stress in an additive manner, making the plants more vulnerable to drought. Moreover, drought and heavy metal stress undesirably disturb soil fertility too, which retards the growth and development of plants (de Silva et al., 2012).

Copper (Cu) is an essential metal for normal plant development but becomes rapidly toxic in excess. For example, when the soil materials have been rich in copper and the pH of the soil offers metal availability if the soil has been contaminated by coal mining and waste deposits, or when agricultural soils have been heavily fertilized with manure or sewage, a high Cu content in the soil, which is toxic, may occur (Rehman et al., 2020; Srivastava et al., 2021). Cu is an essential micronutrient required for plant growth and is a good component of enzymatic activity, protein synthesis, and several biochemical processes in the cell. For example, it is the cofactor of enzymes involved in many biochemical processes, including photosynthesis, respiration, detoxification of peroxide anions, ethylene perception, and cell wall metabolism. The natural soil's Cu content ranges from 60 to 125 mg kg^{-1} (Kabata-Pendias, 2010).

The average content of Cu in plant tissues ranges from 2 to 50 μg g^{-1} dry weight (Cohu and Pilon, 2007). Cu is highly toxic as the redox cycling between Cu(I) and Cu(II) catalyzes the production of hydroxyl radicals *via* Fenton's reaction (Drążkiewicz et al., 2004). Symptoms of toxicity usually appear when the Cu concentration exceeds 20 μg g^{-1} dry weight in vegetative tissues (Marschner, 1995). The more typical symptoms of copper toxicity are leaf chlorosis and reduced growth, which are mainly caused by nutrient uptake inhibition or actual contact with plant metabolism (Kumar et al., 2021; Angulo-Bejarano, et al., 2021). Cu is a heavy metal anthropogenic contaminant that causes major health problems and affects plants; its toxic level affects their growth and productivity. Plant reaction to metal-induced stress may involve the synthesis of various secondary metabolites (Chrysargyris et al., 2021). Crop cultivation on such contaminated types of soil affects plant growth and productivity by damaging photosynthesis and inhibiting transpiration and water uptake.

Plants have been known to adopt different strategies under multiple stressful growth environments to enhance their

tolerating potential by evolving various physiological, morphological, biochemical, and molecular mechanisms. Physiologically, plants can reorganize their root system architecture by inducing primary root growth inhibition and an increase in the lateral root density. Although the morphological changes are generic, they may not be induced through the same signaling pathway. Plant hormones, mainly auxin, cytokinin, and ethylene, control root system architecture and remodel characteristics of the root, including primary root and lateral root growth as well as root hair formation (Juraniec et al., 2016). Moreover, another adaptive mechanism is root colonization, which is a competitive process and a vital step in the creation of plant–microbe relationships, and both host plants and their associated microbes' characteristics may affect it (Reinhold-Hurek and Hurek, 2011).

For dry, contaminated land reclamation, several new strategies, including bioremediation (phytoremediation and microbial remediation), have been used. Phytoremediation emerged as a promising cost-effective and environmentally friendly technology to render metals less bioavailable and less toxic (phytostabilization); clean up metal-polluted soils (phytoextraction); and/or uptake and release metals in methylated, volatile forms to the atmosphere such as mercury, selenium, and arsenic (phytovolatilization). The most employed strategies are phytoextraction and phytostabilization (de Silva et al., 2012; Nascimento et al., 2021). However, for phytoextraction (natural and chemical-assisted phytoextraction), several hyperaccumulator crops uptake and overaccumulate the heavy metal in their edible parts and medicinally used plant tissues, which is a major limitation and a serious health concern for human and animals. However, researchers have also used microbial remediation of the contaminated soils.

More recently, phytoextraction with aided microbial remediation has proven as more effective strategy for the remediation of heavy metals from the environment, as microbes not only self-accumulate metals but also help host plants in metal accumulation in root tissues by restricting the uptake and translocation by binding them to extracellular and intracellular molecules. For example, plant growth-promoting bacteria *Kluyvera intermedia*, *Klebsiella oxytoca*, and *Citrobacter murliniae* isolated from a site contaminated by gold ore processing activities to assist the phytoremediation of As, Cd, and Pb by *Sorghum bicolor* and mitigate the metal toxicity in plants. (Boechat et al., 2020). In recent years, the absorption of copper and other heavy metals through filamentous fungi has received a great momentum as an evolving technology for the elimination of the heavy metals from mining and industrial waste (Dhankhar and Hooda, 2011; Ahemad and Kibret 2014), for example, Cu heavy metal–tolerant *Rhizopus microspores* (Oladipo et al., 2018), *Aspergillus niger* and *Penicillium citrinum* (Sazanova et al., 2015), *Postia placenta, Meruliporia incrassate, Wolfiporia cocos*, and *Antrodia vaillantii* (Clausen and Green, 2003), *Laccaria bicolor* (Reddy et al., 2014), and Cd heavy metal–tolerant *Cerrena unicolor* (Jarosz-Wilkołazka, 2006).

The heavy metal toxicity inhibits enzymatic activity, plant growth, and yield (Nematshahi et al., 2012). To withstand heavy metal stress and metal toxicity, plants also have evolved various defense mechanisms, such as (1) reduced heavy metal uptake; (2) metal sequestration in vacuoles, both extracellular and intracellular; (3) detoxification by enzymes; (4) regulating excessive metal ion homeostasis; (5) binding to phytochelatins/metallothioneins (MTs); (6) activation of various antioxidants, enabling them to survive in the presence of a high concentration of copper; (7) upregulation of copper-induced genes through Cu signaling; and (8) overaccumulation of Cu-resistant proteins (Juraniec et al., 2016; Kramer et al., 2020).

Napoli et al. (2019) found that *Solanum lycopersicum* L. appears to be one of the efficient phytoremediator plants in the removal of Cu concentration from the soil, considering the total uptake by the plant and the remarkably accumulated Cu in fruits and roots. However, being an edible food crop, an alternative strategy must be used for the cultivation of *S. lycopersicum* L. in heavy metal–contaminated, multistress-prone regions. So that soil can be eliminated side by side, contamination-free crops must be produced by farmers.

Combining plants and their associated microorganisms to eliminate contaminants has proven to be a cost-effective, *in situ*, and promising technology (Tiodar et al., 2021), as genetically and physiologically resistant endophytic fungal microbes have shown the dominant potential to increase the remediation of heavy metals and stress tolerance in plants (Aziz et al., 2021a; Aziz et al., 2021b). Given the serious challenges posed by global industrialization to crop cultivation, as well as the risk of phytoremediation by major edible crops such as tomatoes, in a multistress environment, the current study was initiated to investigate a novel strategy for mitigating the harmful effects of combined heavy metal (Cu) and drought stress. Therefore, the present research also deciphers the exploitation of the plant–microbe interaction for multistress alleviation to grow a contamination-free, healthy crop of *S. lycopersicum* L. *under* drought stress.

Endophytes can help the host plant species withstand multiple difficulties, such as heavy metals, drought, high temperature, and salinity, in addition to inducing stress-responsive genes (Rauf et al., 2021; Javed et al., 2022; Ali et al., 2022a; Aziz et al., 2022a; Aziz et al., 2022b; Rauf et al., 2022). Endophytes have sparked a lot of interest in recent years because of their function in host seedlings and defense.

Amin and Ahmad (2015) found that the *S. lycopersicum* L. crop is susceptible to multiple impositions of stresses, such as heavy metal, as well as drought stress. However, research has not been done so far for mitigation of such combined stresses in *S. lycopersicum* L.

Here, we aimed to exploit the combination of microbial extraction, along with phytoextraction, by taking advantage of the endophytic fungus (*Porostereum spadiceum* AGH786) and the host plant *S. lycopersicum* L. as hyperaccumulator of Cu metal. Hence,

the current study was rationalized to unravel the multistress-tolerant endophytic fungi *P. spadiceum* AGH786 for the alleviation of the combined stress of Cu and drought in *S. lycopersicum* L. Hence, the present research aimed to explore the effect of *P. spadiceum* AGH786 on the physiological, morphological, hormonal, biochemical, and molecular parameters of *S. lycopersicum* L. grown under the combined stress of Cu metal toxicity and drought. The current investigation enabled us to unravel the dual potential of the *P. spadiceum* AGH786–*S. lycopersicum* symbiotic association as mycoremediation, as well as the phytoremediation of Cu toxicity in dry, contaminated lands, with the growth promotion of the host plant.

2 Methodology

2.1 Requisition of *P. spadiceum* AGH786

P. spadiceum (AGH786, Accession No 786) (Hamayun et al., 2017) was obtained in the form of slants from the Department of Botany, Plant–Microbe Interactions (PMI) Lab, Abdul Wali Khan University Mardan.

2.1.1 Assessment of growth and tolerance response of the *P. spadiceum* AGH786 strain under the stress of Cu metal and polyethylene glycol–mediated drought

Fungal strain *P. spadiceum* AGH786 was refreshed according to the method of Hamayun et al. (2009) and Khan et al. (2009). For subculturing, a section of the fungal colony was transferred on media containing copper (II) sulfate ($CuSO_4.5H_2O$) salt (CAS No. 7758-99-8; Sigma-Aldrich, Deisenhofen, Germany); supplemented with various concentrations of 0, 100, 500, and 1,000 ppm; incubated at 25°C; and kept overnight at 28°C in the dark; growth was evaluated phenotypically compared with control (0 ppm), as described by Shen et al. (2013). The *P. spadiceum* AGH786 strain was grown in a liquid medium by the method (Hamayun et al., 2017) supplemented with various concentrations of 0, 100, 500, and 1,000 ppm. After incubation, the filtrate was used for the analysis of metabolites and metal concentrations, subsequently. The drought tolerance response was also evaluated on PDA plates supplemented with 12% polyethylene glycol (PEG 8000), as described by (Praveen Kumar et al., 2014).

2.2 Response of *P. spadiceum* AGH786 to 12% polyethylene glycol–induced drought stress

The drought tolerance response was evaluated as described by (Kumar et al., 2014) using 12% polyethylene glycol (PEG 8000) amended on PDA plates.

2.3 Determination of hormones and metabolites in the fungal cultural filtrate

Primary and secondary metabolites (carbohydrates, proteins, and lipids), as well as indole-3-acetic acid (IAA), gibberellic acid (GA), salicylic acid (SA), and abscisic acid (ABA), were determined in the cultural filtrate of *P. spadiceum* AGH786. IAA was estimated by using the Salkowski reagent as described earlier (Hussain and Hasnain, 2011). GA and ABA were determined by the method of Ergün (2002). SA was estimated by using the technique of Warrier et al. (2013). For the determination of the total flavonoid content, the method of El Far and Taie (2009) was used. The method of Malik and Singh (1980) was adapted for the determination of the total phenolic content in fungal culture filtrate. The proline content was determined according to the method of Bates et al. (1973). Total soluble sugars were estimated as described by Nayer and Reza (2008).

2.4 Soil experiment

2.4.1 Preparation of soil for the inculcation

Soil (sandy loam) suitable for local cultivation of tomato crops was collected from the Mardan district of Khyber Pakhtunkhwa, Pakistan, for physicochemical analysis. The sand content of the soil mixture ranged from 71% to 74%. Silt content ranged from 11% to 13%. Clay content was 11%–16%. Soil pH ranged from 7.3 to 7.8. The electrical conductivity of the soil mixture ranged from 0.7% to 6%. Organic matter was 1.5%, carbonate was 1.32 meq/L, bicarbonate was 2.8 meq/L, and Cl^{-1} was 15 meq/L.

The sterilized soil was supplemented with fungal mycelium (2 g/100 g of soil), and plastic pots were prepared with 500 g of soil mixture, sufficient enough for growing tomato plants for up to 5 weeks. Pots without fungal biomass were used as control, and the soil pots were kept for 1 week to grow fungal hype uniformly in a growth chamber in the lab at 28°C.

2.4.2 Sowing of *S. lycopersicum* L. seeds

A non-hybrid variety of *S. lycopersicum* L. (Rio Grande) seeds was obtained from the National Agricultural Research Centre, Islamabad. Healthy, mature, and uniform-sized seeds were selected by physical appearance. Seeds were washed three times with autoclaved distilled water. Ethanol (70%) was applied for the sterilization of *S. lycopersicum* L. seeds. The seeds were washed with distilled water thrice and sown in the soil premixed with fungal biomass (2 g/100 g of soil). Then, *S. lycopersicum* L. seed pots were shifted to a growth chamber (day/night cycle: 14 h 28°C ± 0.3°C, 10 h 25°C ± 0.3°C; relative humidity, 70%; six plants per treatment) for 1 month, in November 2019, in the lab at Abdul Wali Khan University Mardan. The experiment was designed with a completely randomized design; there were eight treatments, and each treatment has six replicates.

2.4.3 Experimental design

Treatment 1. Control (distal water)
Treatment 2. AGH786 (2 g/100g) (endophytic fungus)
Treatment 3. PEG (12%) (drought stress)
Treatment 4. Cu (400 ppm)
Treatment 5. Cu (400 ppm) + PEG (12%)
Treatment 6. AGH786 (2 g/100 g) + PEG (12%)
Treatment 7. Cu (400 ppm) + AGH786 (2 g/100 g)
Treatment 8. Cu (400 ppm) + AGH786 (2 g/100 g) + PEG (12%)

Napoli et al. (2019) reported 400 ppm of Cu supplementation for evaluation of Cu uptake and accumulation response of *S. lycopersicum* L. from the Cu-contaminated soil. Consistently, in the current research, *S. lycopersicum* L. plants were selected as an efficient Cu accumulator and supplemented with the copper (II) sulfate ($CuSO_4.5H_2O$) salt (CAS No. 7758-99-8; Sigma-Aldrich, Deisenhofen, Germany), at the concentration of 400 ppm Cu/pot at 14, 21, and 28 days after germination. After 5 weeks, growth parameters of *S. lycopersicum* L. seedlings, including total chlorophyll content, shoot–root length, and fresh and dry weight of shoot–root, were measured. Seedlings were harvested, and fresh leaves grind in liquid nitrogen for total carbohydrates; proteins; lipids; flavonoids; phenols; proline; total antioxidants such as ascorbic oxidase (AAO), catalase (CAT), and peroxidase (POD); and different plant hormones such as GA, IAA, SA, and ABA. Chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Fluka (Buchs, Switzerland), and Merck (Darmstadt, Germany).

2.4.4 Drought stress on *S. lycopersicum* L.

For induction of drought stress, PEG 8000 was used at 12%, and three doses of 300 ml of the PEG solution (12%) were given to each pot for 3 days (Hamayun et al., 2010).

2.5 Growth parameters

At the end of the experiment, the total yield was recorded by measuring the shoot, root length, and fresh and dry weight of *S. lycopersicum* L. seedlings.

2.5.1 Chlorophyll and carotenoids content

For determination of chlorophyll and carotenoid contents, Maclachlan and Zalik's (1963) method was used.

2.5.2 Determination of endogenous indole-3-acetic acid, gibberellic acid, salicylic acid, and abscisic acid

For the determination of endogenous IAA contents of grained fresh leaves (0.1 g) in liquid nitrogen, purification and extraction of IAA were performed as described above (Hussain and Hasnain, 2011). GA and ABA were determined by the method of Ergün (2002). SA was estimated by using the technique of Warrier et al. (2013).

2.5.3 Determination of secondary metabolites

The total flavonoid content was estimated by the $AlCl_3$ method, as mentioned earlier (El Far and Taie, 2009). The phenolic content was determined by the method discussed above. Proline contents were analyzed according to the protocol of Bates et al. (1973). The total soluble sugar estimation was performed according to Nayer and Reza (2008), as discussed above. Optical Density (OD) was noted at 485 nm. For extraction of total lipid, we used the method of Van Handel (1985). The determination of malondialdehyde (MDA) was done as mentioned earlier (Schmedes and Hølmer, 1989).

2.5.4 Determination of antioxidant activities

With minor modifications, the DPPH (1,1-diphenyl-2-picryl hydroxyl) scavenging activity was measured using the method of Abbasi et al. (2011), Plant matter (0.1 g) was mixed in 1 ml methanol, along with a 0.004% methanol solution of DPPH. About 1 ml of the DPPH solution was then added to 0.5 ml of the samples and then blended and kept for 30 min at room temperature in the dark. The intensity of the DPPH staining was estimated to be 517 nm. The decline in absorption by the sample suggested an elevated scavenging of free radicals according to the equation

$$\%DPPH = \left(\frac{1 - AE}{AD}\right) \times 100$$

where AE = absorption with extract and AD = absorption of DPPH solution only.

CAT activity was used for H_2O_2 cleavage (Guo et al., 2006). The decrease in H_2O_2 is followed by a decrease in absorption at 240 nm, which was measured as M H_2O_2 min^{-1} cleavage.

After dehydrogenating guaiacol as a substratum, the generation of PODs was calculated (Malik and Singh, 1980). In 3 ml of the phosphate buffer (pH 7.0), the enzyme was extracted from the plant. First, take 0.1 g of leaves and grind in 1 ml Tris buffer, centrifuged for 15 min at 5°C at 12,000 rpm. Within 2–4 h, the obtained supernatant was employed as an enzyme source. Pipet 3 ml phosphate buffer (0.1 M), 0.03 ml H_2O_2 (12.3 mM or 0.04%) solution, 0.05 ml guaiacol solution (20 mM), 0.1 ml plant extract, and 0.03 ml H_2O_2 (12.3 mM or 0.04%) solution into a cuvette. The resulting mixture was properly shaken, and OD was recorded at 436 nm.

2.5.5 Reactive oxygen species accumulation through 3,3′-diaminobenzidine

For examining the H_2O_2 biosynthesis and the accumulation of the 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO,

USA), a staining assay was performed using leaf disc, as described by Rauf et al. (2022).

2.6 Heavy metal content analysis in fungal biomass and plant tissues

The bioavailability of Cu was assessed using atomic absorption spectrometry, as described earlier (Li et al., 2014). For determining the Cu content in fungus grown on Cu supplemented media, fungal biomass was retrieved by filtering. While plant samples were washed in water to remove surface element traces, then divided into leaf, root, and shoot segments, and oven-dried at 65°C for 48 h until the weight was constant. The samples were then crushed to powder form with a mortar and pestle, then 0.2 g roots/shoots powder was added for digestion with 5 ml HNO_3 (65% w/w) at 110°C for 2 h, then cooled and mixed with 1 ml H_2O_2 (30% w/w), and heated for 1 h. Next, the digests were diluted with deionized water in a conical flask with triple deionized water (Shen et al., 2013).

2.7 RNA isolation and cDNA synthesis

Total RNA was extracted from *S. lycopersicum* L. seedlings using the Gene JET Plant RNA Purification Kit (Thermo Scientifics), as specified by the manufacturer. During the isolation procedure, the DNase treatment was carried out using RNase-free DNase that was obtained from the TURBO DNase Kit by Ambion (Cambridge, United Kingdom). Around 2 µg of total RNA was reverse-transcribed using the Revert Aid First Strand cDNA Synthesis Kit by Invitrogen (Karlsruhe, Germany), as described earlier (Rauf et al., 2021).

qPCR primers were designed utilizing Primer 3.0 (Untergasser et al., 2012) for gene expression analysis of the heavy metal stress–related molecular marker genes *copper transporters (SlCOPTs)* and *metallothionein (SlMTs)*. As an internal control, *ACTIN2* was used. All primers were synthesized from Bio Basic (Korea), and sequences with gene accession numbers have been mentioned in Table 1. Amplification of each gene was performed in triplicate by using an ABI PRISM 7900HT sequence detection system (Applied Biosystems Applera, Darmstadt, Germany), and the amplification product was visualized using SYBR Green (Applied Biosystems Applera, Darmstadt, Germany). Amplification curves were analyzed with a normalized reporter (Rn: the ratio of the fluorescence emission intensity of SYBR Green to the fluorescence signal of the passive reference dye). Reverse transcription–quantitative PCR (RT-qPCR) expression analysis was performed by using three independent biological replicates with at least three technical replicates as described earlier (Rauf et al., 2022).

2.8 Statistical analysis

Each experiment was performed in triplicates, the data were analyzed using ANOVA through SPSS-20, and the means that differed from one another in a significant way were further examined using the Duncan Multiple Range Test at the p-value of 0.05 (SPSS, Inc., Chicago, IL, USA).

3 Results

3.1 Drought stress and Cu metal toxicity response of *P. spadiceum* AGH786

Tolerance response of *P. spadiceum* AGH786 against drought stress and Cu toxicity has been shown in Figures 1A, B, which revealed the differential tolerance potential of the *P. spadiceum* AGH786 strain growing on media (PDA and Czapek), supplemented with the different concentrations of copper salt at 100, 500, and 1,000 ppm and 12% PEG. In the current results, the *P. spadiceum* AGH786 strain showed the highest tolerance potential in terms of sustainable biomass production at 100 ppm copper supplemented media compared with the higher concentrations (500 and 1,000 ppm). In addition, 12% PEG–treated media also showed sufficiently sustainable biomass production (Figure 1C). Quantification of bioavailable Cu content revealed that *P. spadiceum* AGH786 mycelium efficiently absorbed the Cu supplemented in growth media, in a dose-dependent manner (Figure 1D).

3.2 Determination of hormonal, metabolic, antioxidant, and H_2O_2 content in *P. spadiceum* AGH786 culture filtrate

After the assessment of the tolerance response of *P. spadiceum* AGH786 against drought stress and Cu toxicity, plant growth-promoting hormones (IAA, GA, SA, and ABA) were quantified. Significantly enough of these plant growth-promoting hormones were quantified in AGH786 fungal culture growing on media (Czapek), supplemented with the different concentrations of copper salt at 100, 500, and 1,000 ppm and 12% PEG (Figure 2).

Moreover, hormonal contents were differentially upregulated in various concentrations of copper salt at 100, 500, and 1,000 ppm, with the highest increase in IAA (AGH786-treated media), GA (Cu 1,000 ppm), SA (Cu 1,000 ppm, followed by Cu 500 ppm), and ABA (12% PEG and Cu 1,000 ppm) upon supplementing the Cu 1,000 ppm compared with the control. However, IAA (Cu 1,000 ppm) contents were significantly ($p< 0.05$) decreased with an increase in SA (Cu 1,000 ppm) and ABA (Cu 1,000 ppm) contents upon

TABLE 1 Primers used for reverse transcription–quantitative PCR (RT-qPCR).

Primers used for RT-qPCR			
Gene name	**Gene accession**	**Gene code**	**Primer sequences**
Copper transporter	*Solyc08g006250*	*SlCOPT1_F*	*ATTCTCTTCTCCGGTTGGCC*
		SlCOPT1_R	*CTAACTCCGTACAACGCCGT*
Copper transporter	*Solyc06g005820*	*SlCOPT2_F*	*GGCCAACCTGAGAAGAGAATC*
		SlCOPT2_R	*ATGAAGAACGACGGCCACAT*
Copper transporter	*Solyc09g011700*	*SlCOPT3_F*	*ACAAAAGGCCCATAGGTGCT*
		SlCOPT3_R	*TCTCAACCGCGACAAGTTCA*
Copper transporter	*Solyc10g084980*	*SlCOPT4_F*	*AAGCCGGAATACAAGCGGTT*
		SlCOPT4_R	*CTGCATGACCAACAACAGCC*
Copper transporter	*Solyc02g082080*	*SlCOPT5_F*	*GCTGTGAATGCTCCCCTTCT*
		SlCOPT5_R	*TGACATCATCCTCATCGCCG*
Copper transporter	*Solyc09g014870*	*SlCOPT6_F*	*TGACATGCCAGGAATGGGAG*
		SlCOPT6_R	*AGGACATACATGCCCGTTCG*
Actin	*Solyc05g054480*	*SlACTIN_F*	*AGATCCTCACCGAGCGTGGTTA*
		SlACTIN_R	*GAGCTGGTCTTTGAAGTCTCGA*
Metallothionein	*NM_001247117.2*	*SlMT1-F*	*CTAGCTGCAAGTGCGACAAC*
		SlMT1-R	*ACCCCAAGCACCAAAGTCTC*
Metallothionein	*EU884310*	*SlMT2-F*	*GCTGTGGATCTAGCTGCAAGTGCG*
		SlMT2-R	*AAGGGTTGCACTTGCAGTCAGATCC*
Metallothionein	*NM_001247125.2*	*SlMT3-F*	*ATGTCTTGCTGTGGTGGAAG*
		SlMT3-R	*TAGCAATTGCAAGGGTCACA*
Metallothionein	*NM_001247362.2*	*SlMT4-F*	TGTGGGATGTACCCCGACTT
		SlMT4-R	TCTGTTGCTTTCTCAGCCACT

supplementation of 12% PEG compared with the control culture of *P. spadiceum* AGH786 (Figures 2A-D).

After the assessment of the tolerance response of *P. spadiceum* AGH786 against drought stress and Cu toxicity, primary and secondary metabolites were also estimated in the *P. spadiceum* AGH786 culture filtrate grown under PEG-induced drought stress and Cu supplementation. Significantly ($p< 0.05$), higher concentration of soluble sugars was recorded in various concentrations of copper salt (100, 500, and 1,000 ppm), with the highest increase (Cu 1,000 ppm) upon supplementing the Cu 1,000 ppm compared with the control (Figure 2E).

The total soluble sugar content was also significantly increased (Cu 1,000 ppm) in *P. spadiceum* AGH786 culture grown in PEG-induced drought stress

A differentially higher concentration of total flavonoids was also recorded in various concentrations of copper salt (100, 500, and 1,000 ppm) with the highest increase (Cu 500 ppm) upon supplementing the Cu 500 ppm compared with the control. The total flavonoid content was also significantly increased in the *P. spadiceum* AGH786 culture grown in PEG-induced drought stress (Figure 2F).

A differentially higher concentration of total phenolics was also recorded in various concentrations of copper salt (100, 500, and 1,000 ppm) with the highest increase upon supplementing the Cu 1,000 ppm compared with the control. However, the total phenolic content was significantly decreased (12% PEG) in the *P. spadiceum* AGH786 culture grown in PEG-induced drought stress (Figure 2G).

The proline quantification test also showed an increase in the *P. spadiceum* AGH786 culture grown on media having various concentrations of copper salt (100, 500, and 1,000 ppm), with the highest increase upon supplementing the Cu 500 ppm compared with the control. However, the proline content was also significantly increased in the *P. spadiceum* AGH786 culture grown in PEG-induced drought stress (Figure 2H).

After the assessment of the tolerance response of *P. spadiceum* AGH786 against drought stress and Cu toxicity, antioxidant enzymes (CAT, POX, and AAO) and reactive oxygen species (ROS) (H_2O_2) were also quantified. Results showed a significant ($p< 0.05$) increase in POX and AAO activities detected in the *P. spadiceum* AGH786 fungal culture growing on media (Czapek), supplemented with the different concentrations of copper salt at 100, 500, and 1,000 ppm, with the highest increase in POX activity (Cu 500 ppm), AAO activity (Cu 500 ppm), and CAT activity (Cu 500 ppm) compared with the control. The H_2O_2 content was also increased at Cu 500 and 100 ppm supplementation compared with the control.

PEG-induced drought stress triggered a significant ($p< 0.05$) increase in H_2O_2 content and antioxidant activity of POX, CAT, AAO enzymes in the *P. spadiceum* AGH786 culture (Figures 2I-L).

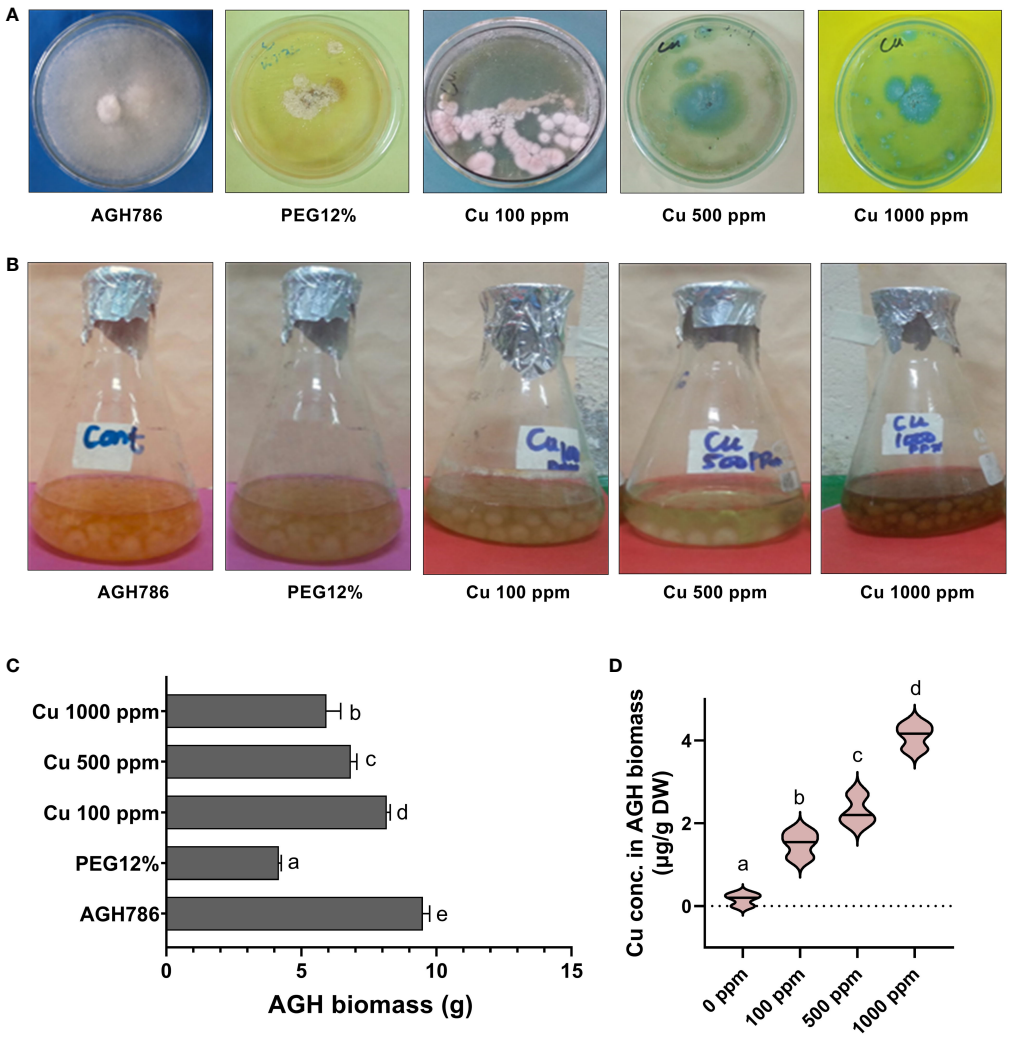

FIGURE 1
(A) Growth of *P. spadiceum* AGH786 on different concentrations of Cu and 12% PEG on solid media. **(B)** Growth of *P. spadiceum* AGH786 on different concentrations of Cu and 12% PEG on liquid media. **(C)** Fresh weight of *P. spadiceum* AGH786 on different concentrations of Cu and 12% PEG on liquid media. **(D)** Bioavailable Cu concentration in fungal biomass. Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

3.3. Effect of *P. spadiceum* AGH786 on growth attributes of *S. lycopersicum* L. under Cu and polyethylene glycol stress

The effect of *P. spadiceum* AGH786 on *S. lycopersicum* L. plants supplemented with copper salt (400 ppm) and PEG (12%) was investigated in comparison to control, in terms of shoot–root fresh, dry weight, and shoot–root length (Figure 3). Root colonization by *P. spadiceum* AGH786 with *S. lycopersicum* L. was also assessed by lactophenol cotton blue staining, which confirmed the successful plant microbial interactions with the root tissue of *S. lycopersicum* L. plants under observations (Figure 3B).

P. spadiceum AGH786 inoculation significantly promoted the growth parameters in comparison to non-inoculated plants. Moreover, *S. lycopersicum* L. plants supplemented with copper salt (400 ppm) and PEG (12%) and inoculated with *P. spadiceum* AGH786 also exhibited a significant increase in the shoot length, root length, shoot fresh weight, and dry weight, compared with non-inoculated plants under stress (Figures 3C-H).

3.4 Effect of *P. spadiceum* AGH786 on photosynthetic pigments of *S. lycopersicum* L. under Cu and 12% polyethylene glycol stress

The effect of *P. spadiceum* AGH786 on *S. lycopersicum* L. plant's photosynthetic potential, supplemented with copper salt

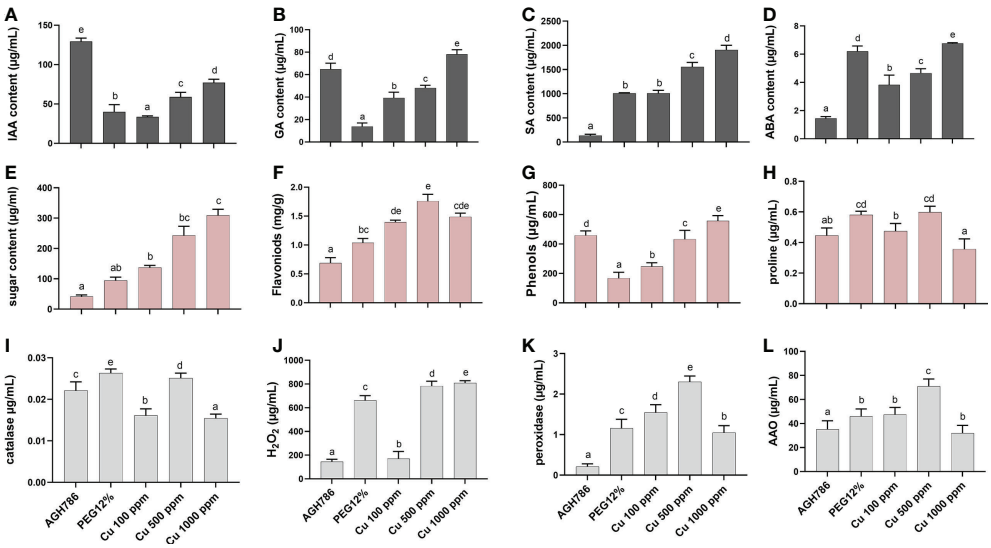

FIGURE 2
Effect of *P. spadiceum* AGH786 on hormonal content of *S. lycopersicum* L. under heavy metal (Cu) toxicity and drought stress (12% PEG). **(A)** Indole-3-acetic acid (IAA) level, **(B)** gibberellic acid (GA) level, **(C)** salicylic acid (SA) level, **(D)** abscisic acid (ABA) level, **(E)** total soluble sugars, **(F)** total flavonoids, **(G)** total phenolics, **(H)** proline content, **(I)** catalase activity, **(J)** H_2O_2 content, **(K)** peroxidase activity, and **(L)** ascorbate oxidase activity under different concentrations of Cu and 12% PEG in liquid media. Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

(400 ppm) and PEG (12%), was investigated in comparison to control. The photosynthetic potential was evaluated in terms of the production of chlorophyll a and b, total chlorophyll, and carotenoids.

P. spadiceum AGH786 inoculation significantly promoted the production of chlorophyll a and b, total chlorophyll, and carotenoids in comparison to non-inoculated plants. Moreover, *S. lycopersicum* L. plants supplemented with copper salt (400 ppm) and PEG (12%) and inoculated with *P. spadiceum* AGH786 also showed a significant promotion in the production of chlorophyll a and b, total chlorophyll, and carotenoids, compared with non-inoculated plants under stress (Figures 4A-D).

3.5 Effect of *P. spadiceum* AGH786 on hormonal contents of *S. lycopersicum* L. under Cu and polyethylene glycol stress

The effect of *P. spadiceum* AGH786 on *S. lycopersicum* L. plant's phytohormonal contents, supplemented with copper salt (400 ppm) and PEG (12%), was investigated in comparison to control.

P. spadiceum AGH786 inoculation significantly promoted the production of IAA, GA, and SA, while a reduction in ABA levels was observed in comparison to non-inoculated plants under normal growth conditions. Moreover, *S. lycopersicum* L. plants supplemented with copper salt (400 ppm) and PEG (12%) and inoculated with the *P. spadiceum* AGH786 showed a significant promotion in the production of IAA, GA, and SA, while ABA content was also increased, compared to non-inoculated plants under stress (Figures 5A-D).

3.6. Effect of *P. spadiceum* AGH786 on metabolic attributes of *S. lycopersicum* L. under Cu and polyethylene glycol stress

The effect of *P. spadiceum* AGH786 on *S. lycopersicum* L. plant's primary and secondary metabolic contents, supplemented with copper salt (400 ppm) and PEG (12%), was investigated in comparison to the control.

P. spadiceum AGH786 inoculation significantly promoted the production of total flavonoids, tannins, total proteins, and total lipids in comparison to non-inoculated plants under normal growth conditions, whereas total soluble sugar and proline levels were reduced by *P. spadiceum* AGH786 in *S. lycopersicum* L. Moreover, *S. lycopersicum* L. plants supplemented with copper salt (400 ppm) and PEG (12%) and inoculated with *P. spadiceum* AGH786 showed a significant promotion in the total flavonoids, tannins, total proteins, total soluble sugar, and total lipids in comparison to non-inoculated plants under stress, whereas proline level was reduced by *P.*

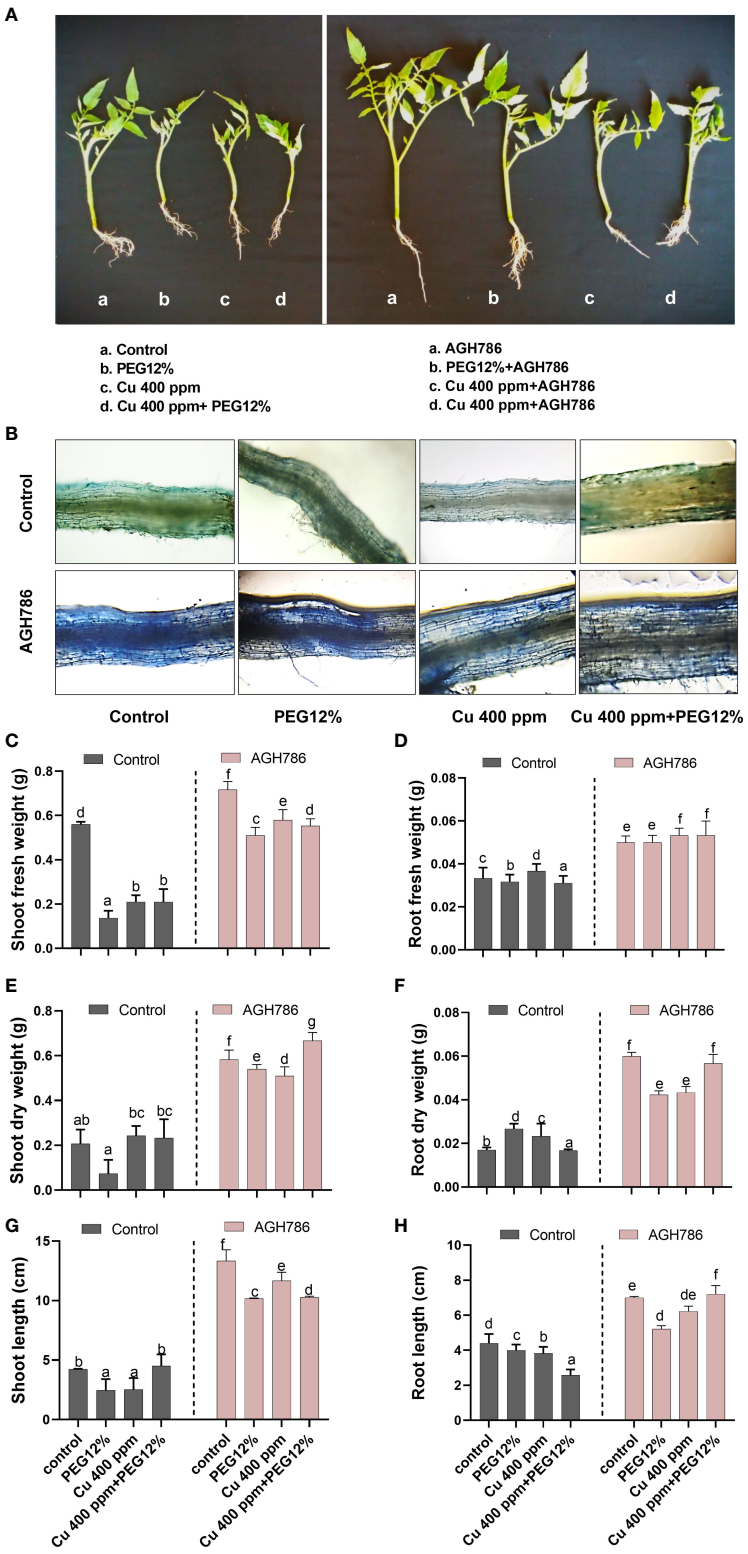

FIGURE 3
Effect of *P. spadiceum* AGH786 on growth attributes of *S. lycopersicum* L. under heavy metal (Cu) toxicity and drought stress (12% PEG). **(A)** Effects of *P. spadiceum* AGH786 on the growth of host seedlings. **(B)** Root colonization by *P. spadiceum* AGH786. **(C)** Effects of *P. spadiceum* AGH786 on shoot fresh weight. **(D)** Root fresh weight. **(E)** Shoot dry weight. **(F)** Root dry weight. **(G)** Shoot length. **(H)** Root length of host seedlings. Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

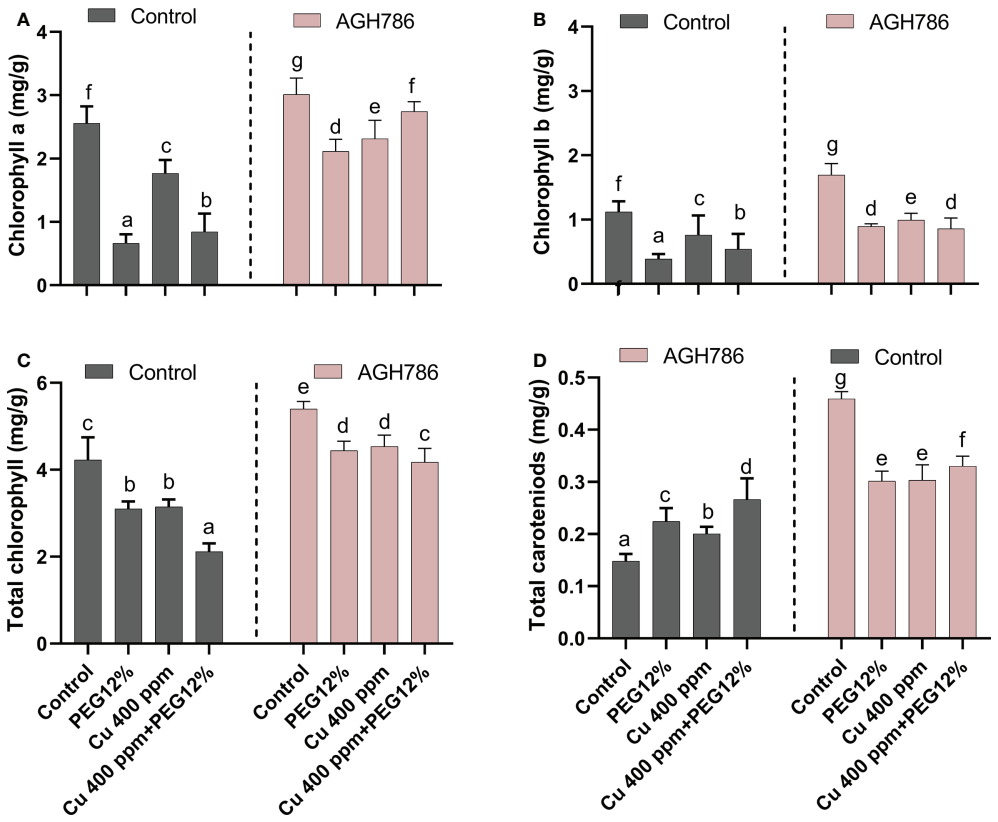

FIGURE 4
Effect of *P. spadiceum* AGH786 on the photosynthetic potential of *S. lycopersicum* L. under heavy metal (Cu) toxicity and drought stress (12% PEG). **(A)** Chlorophyll a, **(B)** chlorophyll b, **(C)** total chlorophyll, and **(D)** total carotenoids. Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

spadiceum AGH786 in *S. lycopersicum* L. compared with non-inoculated plants under stress (Figures 6A-F).

3.7 Effect of *P. spadiceum* AGH786 on reactive oxygen species generation and antioxidant potential of *S. lycopersicum* L. under Cu and polyethylene glycol stress

In response to heavy metal toxicity and drought stress, oxidative damage response in terms of ROS production was evaluated in *S. lycopersicum* L. upon inoculation of *P. spadiceum* AGH786. To this end, the amount of H_2O_2 was observed as brown spots by using DAB staining in the leaves of *S. lycopersicum* L. (Figure 7A). A higher amount of H_2O_2 accumulation was recorded in the individual treatment of Cu and 12% PEG in plant tissues, whereas the highest increase was found in plants treated with the combined treatment of Cu and 12% PEG, in comparison to control. The inoculation of *P. spadiceum* AGH786 induced a reduction in ROS production and H_2O_2 accumulation in plants under stress compared with the non-inoculated control (Figures 7A, B).

MDA content (product of lipid peroxidation in biomembranes degradation by ROS overproduction) was quantified in *S. lycopersicum* L. plants under stress upon inoculation of *P. spadiceum* AGH786. Results showed that *S. lycopersicum* L. plants under stress upon inoculation of *P. spadiceum* AGH786 exhibited lower MDA content compared with the non-inoculated control (Figure 7C).

The effect of *P. spadiceum* AGH786 on *S. lycopersicum* L. plant's antioxidant potential, supplemented with copper salt (400 ppm) and PEG (12%), was investigated in comparison to the control. Antioxidant potential was evaluated in terms of induction of enzymatic (CAT, POX, and AAO) and non-enzymatic antioxidants (AsA), free radical scavenging activity, and total antioxidant production.

P. spadiceum AGH786 inoculation significantly induced the enzymatic (CAT, POX, and AAO) and non-enzymatic antioxidants (AsA) of *S. lycopersicum* L. plants in comparison to non-inoculated plants. Moreover, *S. lycopersicum* L. plants supplemented with copper salt (400 ppm) and PEG (12%) and

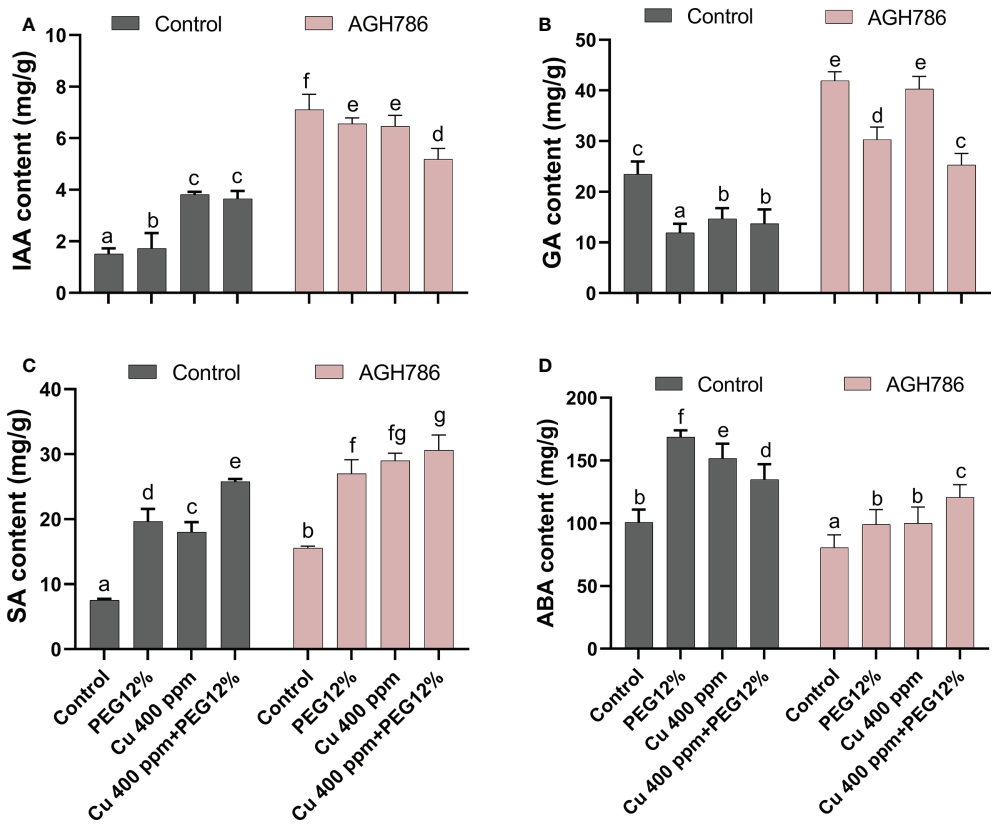

FIGURE 5
Effect of *P. spadiceum* AGH786 on hormonal content of *S. lycopersicum* L. under heavy metal (Cu) toxicity and drought stress (12% PEG). **(A)** Indole-3-acetic acid (IAA), **(B)** gibberellic acid (GA), **(C)** salicylic acid (SA), and **(D)** abscisic acid (ABA). Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

inoculated with *P. spadiceum* AGH786 also showed significant induction in the enzymatic (CAT, POX, and AAO) and non-enzymatic antioxidants (AsA), compared with non-inoculated plants under stress (Figures 7D–I).

3.8 Effects of *P. spadiceum* AGH786 on heavy metal Cu uptake in *S. lycopersicum* L. under normal and drought stress

P. spadiceum AGH786 decreased the toxicity of Cu through limited uptake, translocation, and accumulation in the upper parts of the *S. lycopersicum* plants (Figure 8). A significant reduction in Cu was found in the leaves of *S. lycopersicum* associated with *P. spadiceum* AGH786 as compared with the stem and root tissues of inoculated and non-inoculated plants under combined stress of heavy metal (Cu) and drought stress (12% PEG). Significantly higher Cu content was retained in the soil pots with *S. lycopersicum* L. plants associated with the *P. spadiceum* AGH786 fungal endophyte compared with the non-inoculated control indicating a reduction in the uptake of the Cu content from the soil by root tissue of plants under Cu stress, as shown in Figures 8C, D.

However, an opposite trend was recorded in the plants treated with Cu, which showed an abrupt increase in the uptake of Cu by root tissues and overaccumulation in the shoot tissue, whereas the highest Cu content was quantified in the leaf tissues of the non-inoculated *S. lycopersicum* plants under Cu stress compared with the control. Significantly low Cu content was retained in the soil pots with non-inoculated *S. lycopersicum* L. plants compared with the inoculated control, indicating a sufficient uptake of Cu to the root tissues of the plants under Cu and drought stress.

3.9. Effects of AGH786 on Cu^{2+} *transporters* and *metallothioneins* gene expressions in *S. lycopersicum* L. under stress

The RT-qPCR analysis was carried out to evaluate the effect of AGH786 inoculation on the expression level of selected Cu^{2+}

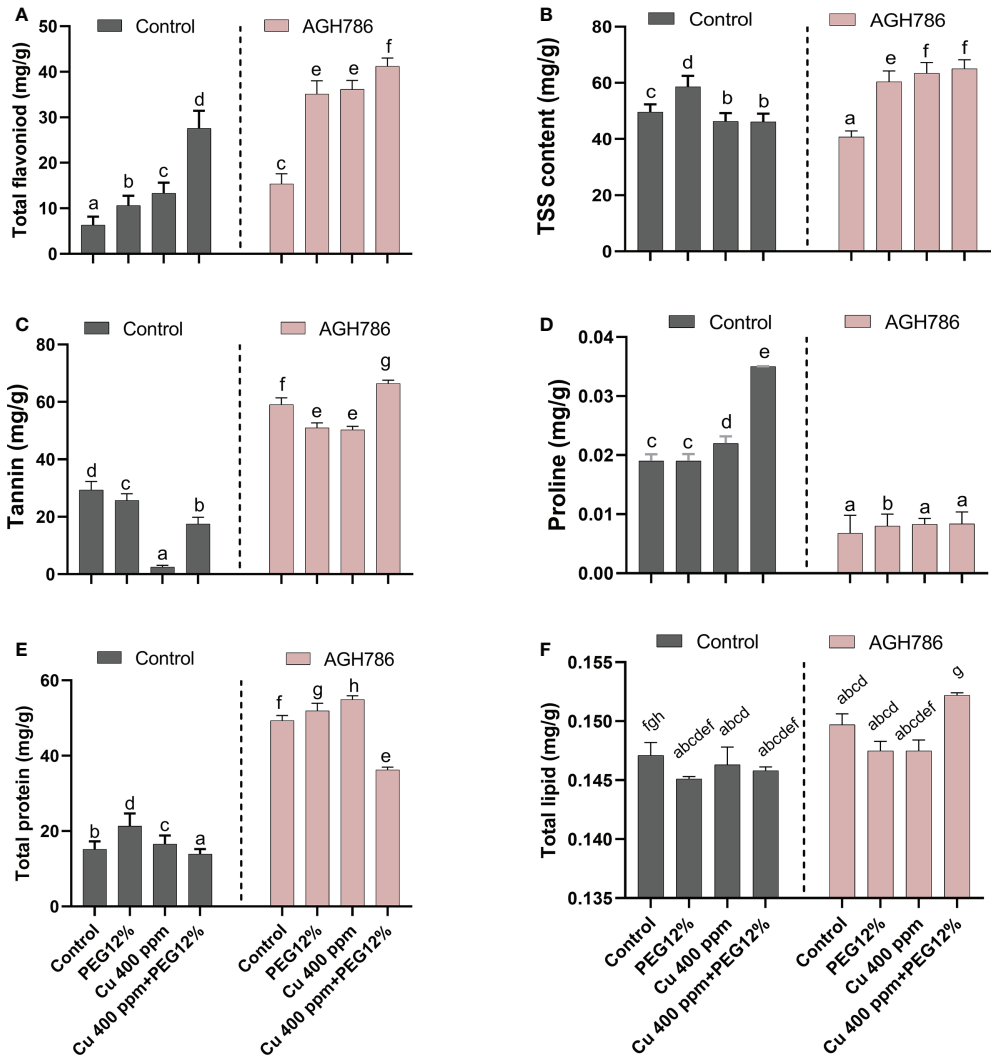

FIGURE 6
Effect of *P. spadiceum* AGH786 on metabolic attributes of *S. lycopersicum* L. under heavy metal (Cu) toxicity and drought stress (12% PEG). **(A)** Total flavonoids, **(B)** total soluble sugar, **(C)** tannins, **(D)** proline, **(E)** total protein, and **(F)** total lipids. Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

transporters (COPTs) and *MTs* genes in the leaf and root tissues of *S. lycopersicum* L. plants grown under single and combined Cu and drought stress.

The expression levels of the *SlCOPT1*, *SlCOPT2*, *SlCOPT3*, *SlCOPT4*, *SlCOPT5*, and *SlCOPT6* and *SlMT1, SlMT2, SlMT3*, and *SlMT4* in the roots and leaf tissues of *S. lycopersicum* L. are shown in Figure 9. The analysis revealed that *SlCOPT3* and *SlCOPT6* genes exhibited a significantly higher expression (>4-fold), both in root and shoot tissue, in response to Cu stress. However, AGH786 inoculation significantly decreased the expression of *SlCOPT3* and *SlCOPT6* genes up to the basal level, both in root and shoot tissues of *S. lycopersicum* L. under single and combined stress of Cu and drought (Figure 9).

The expression levels of *SlMT1, SlMT2, SlMT3*, and *SlMT4* in the roots and leaf tissues of *S. lycopersicum* L. are shown in Figure 9. The analysis revealed that *SlMT1, SlMT2*, and *SlMT3* genes exhibited a significantly higher expression in leaf and root tissues (>4-fold change) in response to Cu stress, whereas expression was downregulated (>0.5 fold) in response to drought stress in root and shoot tissues. AGH786 inoculation induced the expression of *SlMT1, SlMT2*, and *SlMT3* genes (>5-fold) both in root and shoot tissues of *S. lycopersicum* L. Moreover, the combined stress of Cu and drought was induced (>3-fold), which was further increased (>6-fold) by AGH786 inoculation in *S. lycopersicum* L. plants (Figure 9).

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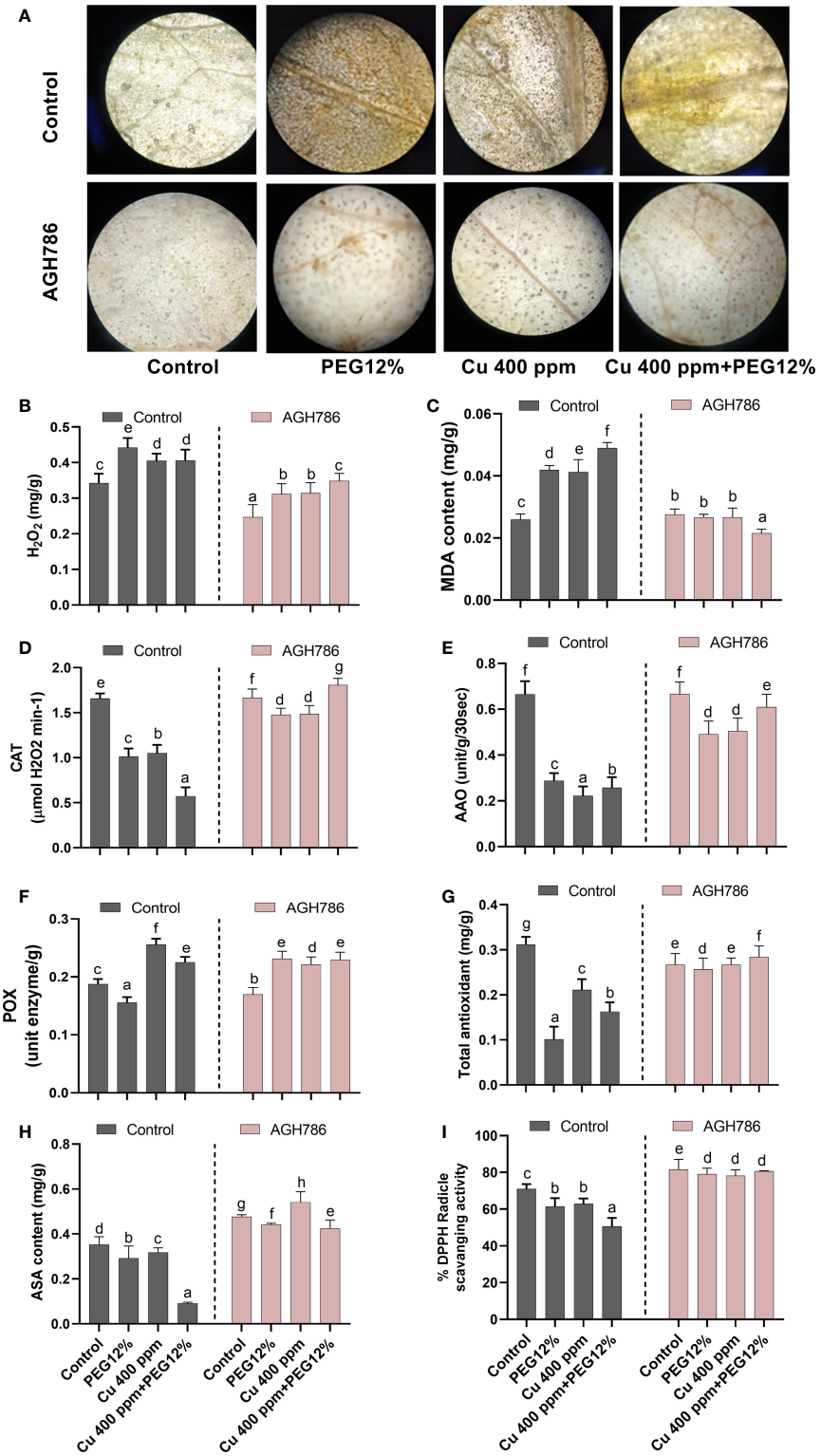

FIGURE 7
Effect of *P. spadiceum* AGH786 on the antioxidant potential of *S. lycopersicum* L. under heavy metal (Cu) toxicity and drought stress (12% PEG). **(A)** Effects of AGH786 on endogenous ROS accumulation. **(B)** H_2O_2 level. **(C)** MDA content. **(D)** Catalase activity. **(E)** Ascorbate oxidase activity. **(F)** Peroxidase activity. **(G)** Total antioxidants. **(H)** Ascorbic acid. **(I)** DPPH activity. Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

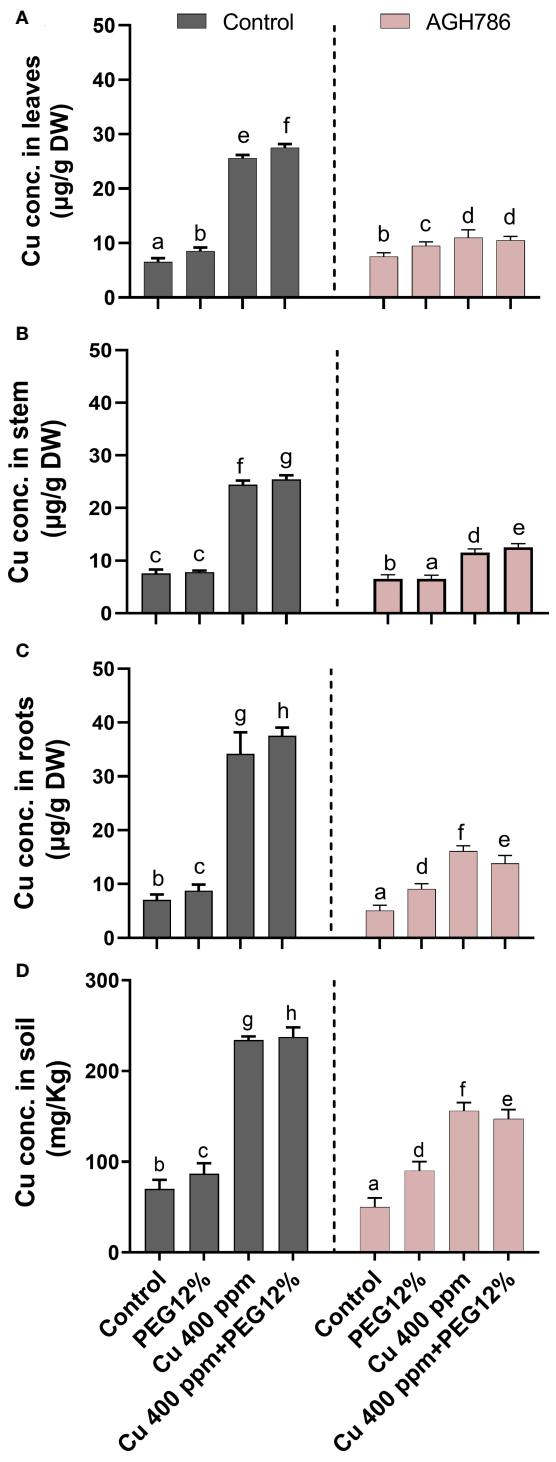

FIGURE 8
Effect of *P. spadiceum* AGH786 on the endogenous copper accumulation in various tissues of *S. lycopersicum* L. under heavy metal (Cu) toxicity and drought stress (12% PEG). **(A)** Cu accumulation in leaves. **(B)** Cu concentration in the stem. **(C)** Cu concentration in roots. **(D)** Concentration of Cu in soil. Data represent the mean with standard error, and letters represent the significant difference ($p < 0.05$).

4 Discussion

In general, microorganisms exhibit high tolerance to multiple stresses (drought and heavy metals), acquired likely through an evolutionary adaptation to a contaminated, harsh environment. Fungi are more tolerant to environmental heavy metals (HMs) than other microorganisms, for instance, bacteria, because of differences in cellular metabolism (Rajapaksha et al., 2004; Agustinho et al., 2018). Higher osmotic pressure in the cell structure of fungi allows them to survive adverse conditions (Agustinho et al., 2018). Moreover, fungi can survive in the soil as sclerotia, chlamydospores, or other structures that allow the microorganisms to survive under unfavorable conditions (Golubović-Ćurguz 2010).

High tolerance of fungi has been observed when the tolerance thresholds to Cu of pure cultures of systematically distant soil microorganisms were compared. At high Cu concentrations (128 mmol kg^{-1}) applied to growing media, fungal activity (acetate-in-ergosterol incorporation rate) increased by seven times as compared with the control (Rajapaksha et al., 2004).

Most of the plant growth-promoting endophytic fungi belong to the group of sac fungi known as *Ascomycota*. However, members of club fungi (*Basidiomycota*) have also been shown to exist as endophytes in plant tissues and promote growth by different mechanisms (Waller et al., 2005; Khan et al., 2009).

Several *Ascomycota* filamentous fungi have been known to be heavy metal stress resistant. For example, the *Rhizopus microsporus* was found highly tolerant to a wide range of Cu concentrations (400–1,000 mg kg^{-1}); however, its high tolerance capacity was apparent only at 25 mg kg^{-1} of Cd and 125 mg kg^{-1} of arsenic (As) (Oladipo et al., 2018). Organic acids induced tolerance to copper-exposed filamentous fungi (*A. niger* and *P. citrinum*) (Sazanova et al., 2015).

However, among *Basidiomycota*, only a few members have been reported to be heavy metal stress tolerant. For example, white rot basidiomycetes *Abortiporus biennis* and *C. unicolor* showed a species-specific response to Cd stress. Cd biosorption onto the mycelial surface was the predominant Cd sequestration mechanism in *C. unicolor* that induced the Cd stress tolerance of *C. unicolor* in comparison to *A. biennis* (Cd-sensitive). These species-specific responses toward Cd suggest that *C. unicolor* possesses a more efficient system than *A. biennis* to keep intracellular Cd concentrations low. *A. biennis* showed higher content of thiol compounds (cysteine, γ-glutamylcysteine, and glutathione in both its reduced and oxidized form) by Cd application, whereas *C. unicolor* showed higher production of oxalate and laccase by Cd application, which is corroborated by the Cd stress tolerance response of *C. unicolor* (Jarosz-Wilkołazka, 2006). Oxalic acid overproduction also triggered Cu toxicity tolerance in brown rot basidiomycete fungi *(P.*

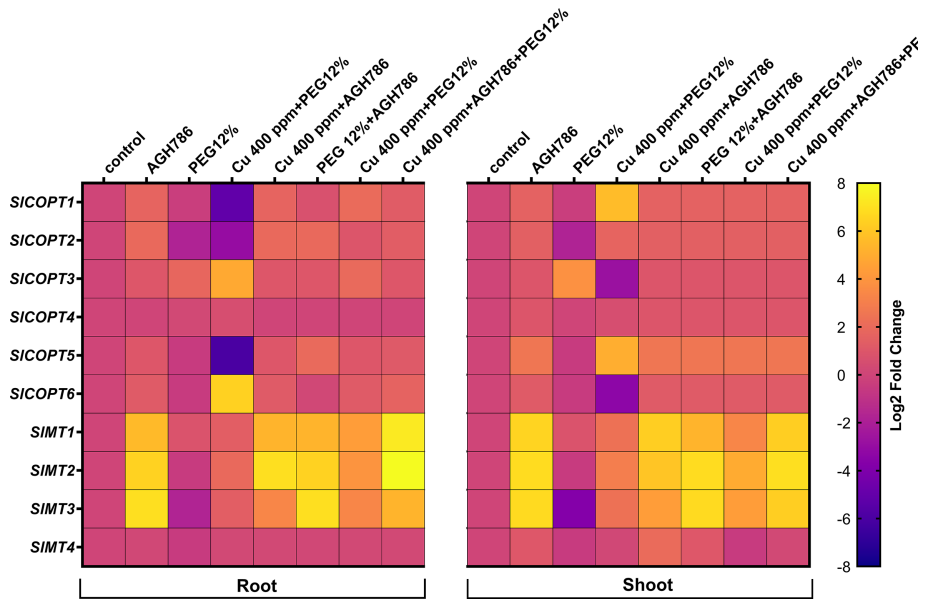

FIGURE 9
Differential expression profile of *SlCOPT* and *SlMT* genes by Reverse transcription–quantitative PCR in the root and shoot tissues of *S. lycopersicum* L. subjected to the single and combined stress of Cu and drought inoculated with AGH786 compared with the control. Quantitative data represent the means ± standard deviation of three independent experiments and at least three technical replicates each.

placenta, M. incrassate, W. cocos, and *A. vaillantii*) (Clausen and Green (2003). Reddy et al. (2014) found differential expression of MTs in response to heavy metals and their involvement in metal tolerance in the symbiotic basidiomycete *L. bicolor*. Combining plants and their associated microorganisms to eliminate contaminants and provide environmental stress alleviation provides a cost-effective, *in situ*, and promising technology (Tiodar et al., 2021).

Moreover, root-associated microorganisms, such as mycorrhizal fungi and endophytic fungi, can remove, inactivate, or degrade harmful environmental contaminants (Aziz et al., 2021a; Aziz et al., 2021b; Wang et al., 2022). Endophytic fungi are the essential components of root microflora in the metal-contaminated ecosystem. They possess various degradation pathways by which they increase host heavy metal tolerance and assist the host's survival in contaminated soils, for example, extracellular metal sequestration (by secreting organic acids and compounds), metal binding to cell walls (hydroxyl, amide, carboxyl, and phosphate-rich cell walls of the lignin-degrading fungus), intracellular metal immobilization (through metal transporters and efflux pumps), and chemical transformations and compartmentalization (through metal chelators) (Gajewska et al., 2022).

During symbiosis, endophytic fungi either directly induce resistance of the host plants to deal with heavy metal toxicity as "phytoremediators" or indirectly improve tolerance by improving water and mineral nutrient uptake in plants, increasing shoot biomass and causing modification in the root morphology. In addition to their ability to promote plant growth, endophytes can chelate and/or sequester heavy metals in polluted soil (Zahoor et al., 2017). Therefore, these are called "mycoremediators." In addition, endophytic fungi–assisted phytoremediation is a cost-effective and environmentally friendly strategy (Wani et al., 2015).

Only a few reports of endophytic fungal members of *Basidiomycota* are there, such as *P. indica*, that could improve the tolerance of host plants to heavy metals that immobilized the heavy metals in host plant roots, which can be very promising in phytoremediation (Shahabivand et al., 2017; Ghorbani et al., 2020).

Endophytic fungus *P. spadiceum* AGH786 (a member of *Basidiomycota*) isolated from the roots of soybean (cv. Hwangkeumkong) by Hamayun et al. (2017) demonstrated resistance to drought and Cu stress and induced combined stress tolerance against drought and Cu in *S. lycopersicum* L. by colonizing the roots of host plants under stress.

Sessile plants are permanently confined to their germination place. Some plant species have adapted growth responses (morphological, physiological, biochemical, and molecular adaptations) to deal with the profuse and quick variations in environmental stress, such as drought, through diversity in the context of stress adaptation, higher plants develop sophisticated abiotic stress responses too, such as resistance to drought, to optimize growth under stress (Takahashi et al., 2020). ABA is

known as a stress hormone that responds to stress conditions like drought by closing its stomata and expressing stress-related genes (Cutler et al., 2010). In the scarcity of water, ABA accumulates in leaf vasculature because of the response to drought stress. ABA biosynthesis occurs in leaf vasculature tissues (Takahashi et al., 2018).

Unfortunately, not all plant species have the capacity to adapt to the changing environment for their survival and growth under stress. Researchers have found that endophytic fungi directly or indirectly induce the resistance of the host plants against various biotic and abiotic stresses (Rauf et al., 2021; Javed et al., 2022; Rauf et al., 2022) to deal with water stresses by improving water and mineral nutrient uptake, modulating antioxidant capacity to cope with ROS-prone destruction upon stress in plants. Endophytic fungi–assisted drought stress alleviation is a cost-effective and environmentally friendly strategy.

Endophytic fungi have also been found to secrete large amounts of secondary metabolites such as terpenoids, alkaloids, phenalenones, cytochalasins, terphenyls, xanthones, diphenyl ether, sterols, squalene, gliotoxins, and their derivatives with varied biological functions (El-Hawary et al., 2020; Rauf et al., 2022).

In this study, *P. spadiceum* AGH786 ably tolerated with a normal growth response on media supplemented with different concentrations of Cu from 100 to 1,000 ppm. Moreover, the growth response of *P. spadiceum* AGH786 was equally normal upon induced drought 12% PEG compared with the control. These findings support the hypothesis that *P. spadiceum* AGH786 is a multistress-tolerant endophytic fungus that can be exploited for growth promotion and induction of multistress resistance in *S. lycopersicum* L.

Current research also shows that *P. spadiceum* AGH786 has a strong potential for producing and secreting primary and secondary metabolites and growth hormones such as IAA, GA, SA, ABA, flavonoids, phenolics, sugar, and proline. The sufficiently produced growth-related metabolites and hormones consistently supported the positive role of *P. spadiceum* AGH786 as a growth-promoting endophytic fungus. Moreover, *P. spadiceum* AGH786 also produced enough enzymatic antioxidants (CAT and AAO), both under PEG-induced drought and Cu stress (in a dose-dependent differential manner).

It is known that ROS are the metabolic byproduct of photosynthesis and respiration that upon overproduction have the potential to cause oxidative damage to cells during environmental stresses. However, ROS play a key role in plants as signal transduction molecules involved in mediating responses to environmental stresses and different stimuli for growth and development. The basal antioxidant system of the cell helps to mediate the ROS overaccumulation by scavenging activities (Tudzynski et al., 2012). Consistent with previous reports, higher H_2O_2 accumulation in the culture filtrate of *P. spadiceum* AGH786 grown in media supplemented with 12% PEG and Cu (100–1,000 ppm) compared with the control can be explained by the activation of signaling mechanisms to support fungus growth and stress responses. The scenario may be suitable for stress alleviation in associated host plants with H_2O_2 produced by endophytic fungus tended to induce the antioxidant machinery of not only fungal cells but also for plant tissues resided by endophytic fungus.

In the current situation, *P. spadiceum* AGH786 inoculation to *S. lycopersicum* L. and heavy metal and induced drought stress proline content were positively regulated in non-inoculated plants, whereas in the same condition, lipid content was negatively regulated under copper and 12% PEG stressed environments. This is the reason that proline acts as an osmolyte, direct free radical (ROS) scavenger, as well as normalizes intracellular redox homeostasis.

In addition, plants can respond rapidly to water imbalance (drought) by accumulating various osmolytes like proline Many plants have been shown to accumulate proline in large quantities when exposed to heavy metal stress. However, despite its beneficial effects, proline may be toxic if overaccumulated or applied in excessive concentrations (Mostofa et al., 2015). In this work, we found that Cu stress induced a high increase in the proline level, whereas AGH786 inoculation to *S. lycopersicum* moderately reduced the accumulated proline content to a moderate level and induced the tolerance in the seedlings under single and combined Cu and drought stress.

Several reports have shown that ROS has the potential to cause oxidative damage to cells during environmental stresses. However, ROS plays a key role in plants as signal transduction molecules involved in mediating responses to environmental stresses, programmed cell death, and different developmental stimuli (Mittler et al., 2004; Torres and Dangl 2005). The rapid increase in ROS production is referred to as "the oxidative burst."

In our study, it was found that *S. lycopersicum* L. exposed to abiotic stresses such as copper and induced drought 12% PEG reduced host growth by slowing down their metabolic activities. *P. spadiceum* AGH786 inoculation enhanced and stimulated the growth of the host plant under combined stress of drought and Cu, helped to detoxify copper metal by restricting the Cu uptake by roots and sequestrating the excessive amount in roots by metal chelators, and adapted to induced drought conditions by strengthening osmolyte production and enhancing the antioxidant potential of *S lycopersicum* L.

Consistent with the previous studies, the *P. spadiceum* AGH786 endophytic fungus also modulated the hyperactivity of various antioxidants (CAT, AAO, POD, and DPPH) in *S. lycopersicum* L., which primarily helped to scavenge the overproduced ROS under the combined stress of Cu toxicity and drought (Evelin et al., 2019; El-Esawi et al., 2020). Among the antioxidant enzymes' catalytic activity by converting the molecules of H_2O_2 into simple molecules of water and oxygen,

ascorbate peroxidases (AAO) convert H_2O_2 into H_2O and use it as an electron donor. POD oxidizes aromatic electron donors such as guaiacol and pyrogallol at the expense of H_2O_2 (Engwa, 2018). The present research also demonstrated the ability of *P. spadiceum* AGH786 to associate with *S. lycopersicum* L. seedlings and the potential of quenching DPPH to reduce the accumulation of free radical ROS.

Previous reports have shown that MDA is overproduced upon stress in plants because of the cellular destructive activities of ROS (Hasanuzzaman et al., 2020). As a result, endophytic fungi (*P. spadiceum*, AGH786) in the current study help *S. lycopersicum* produce enough antioxidant enzymes to stop MDA production and detoxify the cells from ROS by scavenging the overproduced free radicals in the stressed host.

Although the Earth crust is made up of natural heavy metal elements, their proportion has been altered by anthropogenic activities such as rapid industrialization, extensive irrigation systems, and agricultural practices. Involuntarily, these heavy metals enter the food chain through overabsorption or accumulation by growing crop plants in contaminated soils. The overaccumulation of these heavy metals in plants decreases plant growth. In such conditions, bioremediation techniques (including mycoremediation and phytoremediation) are useful as compared with other approaches (Aziz et al., 2021a). Our results showed that *P. spadiceum* AGH786 is a growth-promoting endophytic fungus. Inoculation of *S. lycopersicum* L., along with copper stress and induced drought stress, relieved copper toxicity and reduced induced drought effects on host plants through biochemical, physiological, and molecular strategies.

Our results also revealed the positive role of *P. spadiceum* AGH786 in helping in restricting Cu uptake by roots and translocation of Cu from root to shoot. Thereby, copper accumulation in roots, stems, and leaf tissues was predominantly less than the toxic level for host plants, compared with non-inoculated *S. lycopersicum* L.

Root-to-shoot translocation is a crucial activity for plants that is an important limiting factor for the transportation of the soil resources up to the fruits. A current study consistently showed that *P. spadiceum* AGH786 association helped the plant to prevent copper metal transport to leaves and other upper parts like stems and leaves of the host plant during the vegetative stage of plant growth. The roots of plants have direct contact with soil, and all types of toxic metal ions affect the roots directly (Shahabivand et al., 2016). Increased accumulation of heavy metals in roots and their translocation to the upper aerial part are observed in *S. lycopersicum* L. without the *P. spadiceum* AGH786 association. These findings indicated the potential of *P. spadiceum* AGH786 to remediate the excessive Cu ions in the soil, as well as the roots of the host plant, by restricting the uptake through plant root–localized Cu transporter channels. Moreover, since *P. spadiceum* AGH786 can take up and accumulate Cu content in its biomass, most of the Cu content from the soil is probably eliminated by the mycoremidiation activity of the *P. spadiceum* AGH786 fungus. Previously, the role of fungal endophytes has also been identified to restrict these heavy metals outside the roots through extracellular absorption mechanisms, and the huge accumulation of these metals in the root endodermis in casparin strips blocks the translocation of metal to the leaves (Li et al., 2014).

Fungal endophytes have also evolved various ways to eliminate the heavy metal contents from soil and the host plants directly or indirectly, such as *Lindgomycetaceae* P87 and *Curvularia geniculata* P1, which were found to reduce mercury ion Hg (II), and the reaction led to the formation of volatile forms of Hg enabling its evaporation (Pietro-Souza et al., 2020). However, *A. flavus–associated* tomato plants developed tolerance against Cd and Cr toxicity *via* the expression of *SlGSH1* and *SlPCS1* genes. Both genes helped in metal chelation and mitigated Cd and Cr toxicity. Previously, the overexpressions of *GSH1, GSH2, PCS1*, and *PCS2* (Gasic and Korban, 2007; Kühnlenz et al., 2014) were also shown to increase heavy metal tolerance by raising glutathione (GSH) and phytochelatin (PCs) levels. In addition, metal-tolerant proteins (MTPs) are divalent cation transporters and play fundamental roles in plant metal tolerance and ion homeostasis. The expression patterns of cucumber MTP genes under Zn^{2+}, Cu^{2+}, Mn^{2+}, and Cd^{2+} stress have been studied where these MTPs were induced by a metal ion, suggesting their involvement in metal tolerance or transportation (Jiang et al., 2022).

Several genes have also been reported to be upregulated by Cu excess, including laccase-like multicopper oxidases (Berni et al., 2019). They oxidize Cu (I) to a less toxic Cu (II). The genes upregulated by Cu excess also include *Cu2+ transporters (COPT), a Cu2+ transporting P-type ATPase (HMA5)*, or two Cu chaperones (*antioxidant protein1; ATX1* and *ATX1-like Cu chaperone*) and *copper-modified resistance1 (cmr1)* protein (Puig and Thiele, 2002; Sancenón et al., 2004; Andrés-Colás et al., 2006; Juraniec et al., 2012; Shin et al., 2012). The Cu chaperones, *antioxidant protein1 (ATX1)* family of Cu chaperones specifically deliver Cu to heavy metal P-type ATPases. The *Arabidopsis thaliana* expresses the *ATX1-like Cu chaperone CCH*, which exhibits a plant-specific *carboxy-terminal domain* with unique structural properties (Andrés-Colás et al., 2006).

It is also known that non-Cu accumulator plants store excess Cu in S-rich MTs, as suggested by Mijovilovich et al. (2009) that control heavy metal homeostasis and attenuate heavy metal–induced cytotoxicity by chelation, thus lowering their intracellular concentrations. Therefore, MTs have been used as bimolecular markers for evaluating metal toxicity response indicators within plants and environmental pollution in the soil.

In this study, to reveal the uptake, transport, and accumulation of mineral elements in *S. lycopersicum* L., it was inevitable to quantify the endogenous mineral concentration and biomarker gene expression analysis induced or fluctuated by the perturbance. A six-member family of *COPT (SlCOPT1-6)* was identified and characterized in *S. lycopersicum* L. that are

known to play important roles in Cu homeostasis, including absorption, transportation, and growth in plants. Furthermore, all the *SlCOPTs* contained several Cu-responsive elements (*CuRE, GTAC motif*) and different types of *cis*-elements related to hormone response, in which those related to ABA predominated. The responsive elements associated with ABA, cytokinins, GA, and auxin were found in all the *SlCOPT* members (Romero et al., 2021), indicating the induction of *SlCOPT under the control of Cu and hormonal signaling.*

It is also known that non-Cu accumulator plants store excess Cu in S-rich MT-type structures, as suggested by Mijovilovich et al. (2009). Plant MTs are thought to have a functional role in heavy metal homeostasis, and they are used as biomarkers for evaluating environmental pollution. MTs have low molecular weight (7–9 kDa), are cysteine-rich, and possess high affinity for heavy-metal, stress-responsive proteins. Different expressions of *MTs* may be linked to their biochemical and physiological functions. Additionally, MTs act as chelators of heavy metals. They are essential for metal homeostasis and detoxification, and they have important functions in the elimination of intracellular free radicals. In addition, the thiol groups in MTs can act as powerful antioxidants, so MTs may have a role in protecting against oxidative damage. *MT* expression is tissue specific and under developmental control, and several key plant hormones can play a prominent role in the regulation of the *MT* gene expression.

Previously, it was also reported that the *SlMT* genes showed a differential expression pattern when exposed to some heavy metals such as Cu, Zn, and Fe (Ryan et al., 2013). The expression of *SlMT3* was induced in roots, leaves, and fruits exposed to Cu compared with untreated groups, and *SlMT4* was significantly increased in fruits of *S. lycopersicum* L. exposed to Cu and 12% PEG. Although Cu and applications have increased *SlMT1* and *SlMT2* gene expressions compared with the control in all tissues of *S. lycopersicum* L. subjected to different concentrations of heavy metals, the highest levels of *SlMT1* and *SlMT2* transcripts were found in roots and leaves, respectively (Ryan et al., 2013). We also aimed to evaluate the expression of biomolecular marker *SlMT* genes (*SlMT1*, *SlMT2*, and *SlMT3*) in plants exposed to single and combined copper and drought stress. Consistently, this study also revealed the differential expression of *SlMT1, SlMT2*, and *SlMT3* induced in *S. lycopersicum* L. plants under single and combined Cu and drought stress inoculated with the *P. spadiceum* AGH786 endophytic fungus. From current findings, we concluded that AGH786 appeared as an efficient *S.*

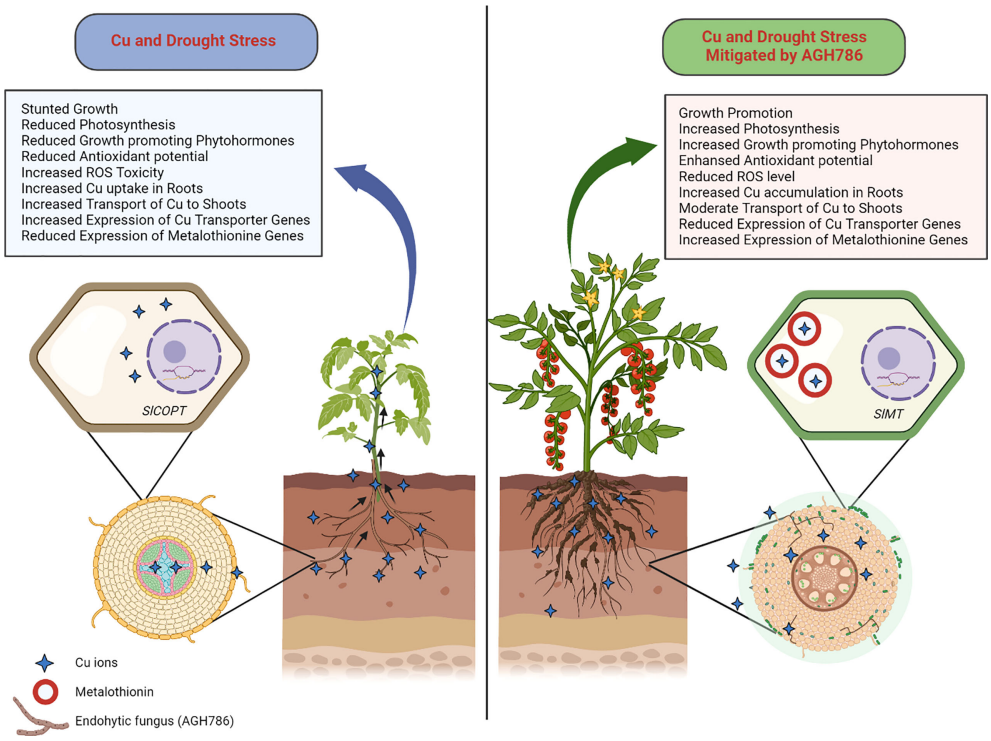

FIGURE 10
Role of *P. spadiceum* AGH786 under combined stress of Cu and drought in *S. lycopersicum* L. Cu and drought stress inhibited plant growth, while the association of AGH786 ameliorated *S lycopersicum* L. growth under Cu and drought stresses, through secreting phytohormones and essential secondary metabolites under stress conditions and modulating the plant gene expression of Cu transporters, metal chelators, and stress-related biomarker genes such as the *SlCOPT* and *SlMT* genes in the *S. lycopersicum* L. for restricting and sequestering the heavy metal ions in the root tissue.

lycopersicum L. growth-promoting and multistress-alleviating endophytic fungus, and hence, it can be used as a biofertilizer in heavy metal–contaminated fields to rescue the crops under combined stress of Cu toxicity and drought.

5 Conclusion

Based on the outcomes of this study, it can be stated that the plant growth-promoting endophytic fungus *P. spadiceum* AGH786 is a multistress-resistant isolate that not only eliminated the Cu contamination from the soil through mycoremediation but also triggered the plants' defense mechanism to cope with Cu toxicity. Moreover, the *P. spadiceum* AGH786 fungal association also boosted the signaling mechanism of host plants to modulate and optimally suppress the Cu uptake and translocation machinery and enhance the toxic metal chelation mechanism in roots, thus hindering the Cu uptake from roots and transport to upper vegetative parts and converting the host plants into efficient phytoremediators for Cu-contaminated soils (Figure 10).

Moreover, being drought resistant, the *P. spadiceum AGH786* isolate efficiently induced the resistance of host plants against PEG-induced drought stress. In addition to this, the *P. spadiceum* AGH786 isolate efficiently induced soil-related multistress tolerance in host plants against drought as well as Cu contamination.

Data availability statement

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

Author contributions

MH conceived the idea and designed the experiments. FN performed the main experiments. FN, MR, and MH prepared the manuscript. FN, MR, and MA analyzed the data. FN and SAK performed heavy metal content analysis. JU, MA, and MR performed the qRT-PCR analysis. HG, AH, AI, and I-JL reviewed the manuscript critically. MH, MA, H-YK, and I-JL provided financial support. All authors contributed to the article and approved the submitted version.

Funding

The authors declare that the current research was supported by a National Research Foundation of Korea grant funded by the Korean government (MSIT) (No. 2022R1A2C1008993).

Acknowledgments

Resources have been provided by Abdul Wali Khan University Mardan (34°12′4.4″N and 72°01′33″E), Pakistan. The present research experiments were mainly carried out at the Department of Botany (PMI Lab, Molecular Biology Lab, Botanical Garden) and the Department of Biotechnology (Plant Biotechnology Lab).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 17 November 2022
DOI 10.3389/fpls.2022.1026575

OPEN ACCESS

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

RECEIVED 24 August 2022
ACCEPTED 13 October 2022
PUBLISHED 17 November 2022

CITATION
Kumari M, Qureshi KA, Jaremko M, White J, Singh SK, Sharma VK, Singh KK, Santoyo G, Puopolo G and Kumar A (2022) Deciphering the role of endophytic microbiome in postharvest diseases management of fruits: Opportunity areas in commercial up-scale production. *Front. Plant Sci.* 13:1026575. doi: 10.3389/fpls.2022.1026575

Deciphering the role of endophytic microbiome in postharvest diseases management of fruits: Opportunity areas in commercial up-scale production

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As endophytes are widely distributed in the plant's internal compartments and despite having enormous potential as a biocontrol agent against postharvest diseases of fruits, the fruit–endophyte–pathogen interactions have not been studied detail. Therefore, this review aims to briefly discuss the colonization patterns of endophytes and pathogens in the host tissue, the diversity and distribution patterns of endophytes in the carposphere of fruits, and host–endophyte–pathogen interactions and the molecular mechanism of the endophytic microbiome in postharvest disease management in fruits. Postharvest loss management is one of the major concerns of the current century. It is considered a critical challenge to food security for the rising global population. However, to manage the postharvest loss, still, a large population relies on chemical fungicides, which affect food quality and are hazardous to health and the surrounding environment. However, the scientific community has searched for alternatives for the last two decades. In this context, endophytic microorganisms have emerged as an economical, sustainable, and viable option to manage postharvest pathogens with integral

colonization properties and eliciting a defense response against pathogens. This review extensively summarizes recent developments in endophytic interactions with harvested fruits and pathogens—the multiple biocontrol traits of endophytes and colonization and diversity patterns of endophytes. In addition, the upscale commercial production of endophytes for postharvest disease treatment is discussed.

KEYWORDS

endophytes, molecular interactions, biocontrol screening, commercial hurdles, postharvest management, fruits

Introduction

In the recent era of climate change and the rising global population, food security is one of the most critical issues worldwide. At the same time, postharvest losses of fresh products, including fruits, vegetables, or horticultural crops, accelerate food security challenges. Currently, it has been estimated that approximately 50%–60% of the total agricultural production (Kumar and Kalita, 2017) and 30%–50% of the total fruit production are lost after harvesting due to improper storage, attack of pathogens, or the incidence of diseases (Zhang et al., 2017). However, on the broad industrial scale or even a laboratory scale, various chemical pesticides or fungicides have been broadly employed to prevent postharvest loss caused by phytopathogens or diseases. Nevertheless, the undistributed use of chemical pesticides adversely affects the nutrient constituents, texture, flavor, and quality of the fruits and negatively impacts consumer health. Furthermore, the emergence of resistant pathogen varieties against existing pesticides is a severe problem (Hahn, 2014; Nicolopoulou-Stamati et al., 2016). Therefore, the negative consequences of chemical pesticides on fruit quality, human health, and the environment urgently need the development of a reliable and sustainable approach to replace toxic agrochemicals with suitable microbial antagonists.

Utilizing the endophytic microbiome as a biocontrol agent (BCA) during preharvest or postharvest storage conditions has emerged as a suitable alternative to chemical pesticides in the last few years (Singh et al., 2019; Kumar et al., 2021; Ahmad et al., 2022). Endophytes are the microbes that colonize intercellular/intracellular spaces of plants without causing any apparent sign of infection (Bacon and White, 2016; Pathak et al., 2022). Endophytes are well known for inducing plant growth-promoting traits and ameliorating biotic and abiotic stresses (Glassner et al., 2015). In addition, it synthesizes a plethora of bioactive compounds that enhance the host's immune response and protect the plant from pathogen attacks or disease incidence (Nair and Padmavathy, 2014; Singh et al., 2017). For practical biocontrol efficacy, the most challenging task is the administration and establishment of microorganisms inside the host plant. An endophytic microbiome is a suitable option in this context due to better colonization and proliferation efficacy (Busby et al., 2016; O'Brien, 2017). Nevertheless, there is still a need to explore the endophytic microbiome for its practical application as microbial antagonistic agents against various phytopathogens or plant diseases during postharvest storage conditions.

Furthermore, the diversity of endophytic microbiome in the fruits, its role in biotic stress amelioration, and an insight into the mechanistic aspects are still under investigation (Aiello et al., 2019; Chaouachi et al., 2021). Therefore, research on the endophytic microbiome and its role in minimizing postharvest loss of horticultural crops, including fruits, needs special attention with an in-depth discussion regarding their prospects and their transition from lab to field or industry. This review summarizes the molecular interaction of plant endophytes, the diversity of endophytic microbiome, the screening of BCAs, and the technological aspect of endophytic microbiome postharvest management. This review also focuses on the literature and discussion on the modes of application, the future aspects, and the hurdles to be overcome for converting endophytes into the success stories of postharvest management of fruits in a sustainable manner.

An overview of microbial endophytes

Plants host diverse communities of microorganisms as epiphytes (on the surface) or endophytes (inside the plant tissue) and share a complex relationship. These host–microbe interactions play significant roles in maintaining the plant normal physiology under biotic and abiotic stress conditions (Khalaf and Raizada, 2018; Verma et al., 2021). The term endophyte was firstly introduced by De Bary (1866) as the fungal species living inside the host tissue. However, Petrini (1991) considered endophytes, of either fungal or bacterial

strains, as those that reside in the host tissue or plant for at least some part of their life cycle without causing any disease or apparent sign of infection. With technological advancement or next-generation sequencing (NGS), it has been estimated that each plant species harbors multiple endophytic microbes during its life cycle (Senthilkumar et al., 2011; Verma et al., 2021). The latest NGS revealed that *Proteobacteria* is the most prominent endophytic bacterial phylum, followed by *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. In contrast, Glomeromycota is the major fungal phylum followed by *Ascomycota* and *Basidiomycota*; however, *Pseudomonas*, *Pantoea*, *Acinetobacter*, and *Enterobacter* members of Gamma-Proteobacteria are the commonly found bacterial genera. Arbuscular mycorrhizal fungi (AMF) are the most prominent fungal taxa among endophytic fungi in plant tissues (Hardoim et al., 2015; Kumar et al., 2020; Verma et al., 2021).

The endophytic microbes within plant tissue interact with plants and modulate the plant's growth, fitness, and physiology. The mutualistic endophytes live inside the host and mutually benefit each other; for example, endophytes produce phytohormones, solubilize nutrients, and modulate bioactive compounds of the host, all resulting in the growth and development of the plant, and in return, the plant provides shelter and nutrients to the endophytes (Papik et al., 2020; Khalaf and Raizada, 2020).

Colonization by microbial endophytes

The host–endophyte share a complex relationship that is driven by various intrinsic and extrinsic factors (White et al., 2019; White et al., 2021). However, the entry or establishment of microorganisms in the host tissue is the primary step for any strain to be an endophyte (White et al., 2019; Micci et al., 2022). According to Kandel et al. (2017), endophytic colonization refers to the entry, growth, and multiplication of endophytes within the internal compartments of the plant host. However, colonization is a complex process regulated by different signaling molecules in several consecutive steps (Kumar et al., 2020). Firstly, the plant species attract the microbes by the specific components of their exudates, which are generally composed of sugars, organic acids, amino acids, lipopolysaccharides (LPSs), flavonoids, and proteins and may be specific for each microbial strain (White et al., 2019). The microbes showed a chemotactic response toward the specific components of the exudates and facilitated effective colonization (Oku et al., 2012). The motility of the microbial strain/s toward the host surface is facilitated by appendages that protrude from the cell surface, such as flagella, or through type IV pili (Knights et al., 2021). Several reports reinforce the importance of lateral appendages during this movement (Sauer and Camper, 2001; Zheng et al., 2015). For instance, flagella were reported to have direct involvement in adhering to *Azospirillum brasilense* with wheat roots (Pinski et al., 2019). Böhm et al. (2007) reported type IV pili and their direct role in the colonization of *Azoarcus* sp. BH72 to the surface and root interior of rice. However, attachment of the endophyte on the host surface is facilitated through secretory products such as exopolysaccharides (EPSs), LPSs, cell surface saccharides, and cellulase of the microbial strain. For example, Meneses et al. (2011) reported that the inactivation of gene *gumD*, which is responsible for EPS synthesis, decreased the colonization rate of the endophytic strain *Gluconacetobacter diazotrophicus* in rice roots.

Similarly, Monteiro et al. (2012) observed that inactivation of gene wssD, bcsZ, which are responsible for the synthesis of beta-1,4, glucanase (cellulose), decreased the colonization rate of *Herbaspirillum rubrisubalbicans* M1 in *Zea mays*. The endophytic microorganism, before its entry or colonization, confronts the challenges of oxidative environments of the host tissue. This situation is similar to the one the pathogens face during infection of the host. The host plant provides a barrier to oxidative burst, resulting in only a few microorganisms that can enter plant cells (White et al., 2019; White et al., 2021). Experiments have shown that this initial oxidative burst can be reduced by treating seedlings with low concentrations of humic substances, resulting in increased entry of bacteria into root cells at root tips (White et al., 2021). To be an endophyte, microbial strains must be able to survive in the oxidative environment within plant cells (Di Pietro and Talbot, 2017; White et al., 2019). In this context, several authors reported the successful acclimation potential of endophytic strains; for example, *Enterobacter* spp. encodes antioxidant enzymes during the colonization of poplar plants (Balsanelli et al., 2016).

Additionally, Malfanova et al. (2013) reported genes responsible for antioxidative enzymes used by *Klebsiella* to protect the host plant from reactive oxygen species (ROS). Similarly, strain *G. diazotrophicus* showed the expression of antioxidant enzyme genes during the early stage of colonization in rice plants (Meneses et al., 2017). In addition, the colonization efficacy of the endophyte depends upon several factors; host genotype, nutrient status, and specificity of microbial strain are the prime factors (Hardoim et al., 2015).

Colonization patterns of endophytes and pathogens in the host tissue

The colonization patterns of the pathogens and endophytes are similar to some extent. However, the response of plant defense systems differs and depends upon the nature of the microorganisms. Similarly, the expression patterns against oxidative stress are also different. Chen et al. (2020a) reported the colonization patterns of endophytic strain *Azoarcus olearius* and the pathogen *Xanthomonas oryzae* in rice plants and observed differential expression patterns of genes. The pathogen followed the salicylate pathway; however, the

Azoarcus used the jasmonate signaling pathway during colonization. The colonization patterns of symbiotic endophytes and pathogenic strains are also dissimilar regarding secretions. Pathogenic strains secrete comparatively higher amounts of cell wall-degrading enzymes at the infection sites. In contrast, a lower amount of cell wall-degrading enzymes was reported during endophyte colonization, which could not elicit the plant immune system and make easy access to endophytes inside the host tissue (Elbeltagy et al., 2000; Reinhold-Hurek et al., 2006; Naveed et al., 2014). The overview of endophytic dynamics, entry, colonization, transmission, and interacted factors is presented in Figure 1.

Diversity of endophytic microbiota in the fruit

The physiology and biochemistry of the plant depend upon the surrounding biotic and abiotic factors, which ultimately affect the diversity and composition of the microbiota, either epiphytes or endophytes. For instance, seasonal variations affect the number of plant exudates, which are a determining factor in rhizospheric microbial population and endophytic colonization (Wang et al., 2009; Kuffner et al., 2012). The genotype (Mocali et al., 2003), cultivars (Pettersson and Bååth, 2003), and host plant's age influence endophytic microbial compositions.

Recently published reports reinforce the variation in the endophytic populations among the plant organs. For example, Ren et al. (2019a) reported variations in the endophytic bacterial microbiome among the different organs of the same Jingbai pear (*Pyrus ussuriensi* Maxim.) plant. Maximum richness and diversity were observed in the root tissue, followed by flower, stem, and fruit, and the lowest were in the leaf tissue. This report illustrates that each plant organ has a specific richness or diversity.

Furthermore, in another study, Ren et al. (2019b) reported variations in fungal richness or diversity in the different plant organs of the Jingbai pear forest. They observed that the root tissue had maximum fungal richness and diversity, followed by stem, fruit, and leaf, and the lowest were observed in the flower tissue. Thus, the diversity patterns of both bacteria and fungi are different in the same plants. Finally, Dong et al. (2019) reported a similar observation of bacterial distribution patterns among the root zone, rhizosphere, phyllosphere, and endosphere of roots, stems, leaves, fruits, and seeds of tomatoes under greenhouse conditions. They observed that the root zone and rhizospheric soil had the highest diversity and richness, followed by stem, flowers, and fruits; however, the lowest diversity and richness were observed in the phyllosphere tissue.

Abdelfattah et al. (2015) also reported that leaves contain higher diversity than flowers or olive fruits (*Olea europaea*), and the fungal diversity consequentially decreased from fruitlets to mature stages of the olive. However, the trends of the fungal community were very similar from fruitlets to the flowering stage, which later changed. However, the microbial diversity in the flower or fruit section is similar to the diversity of some other parts. Therefore, the uniqueness and diversity of endophytic microbiota may vary among the different compartments of the fruits (Ottesen et al., 2013). The uniqueness may be due to the ovaries, which turn into flesh and create a new environment that harbors specific microbiota or microbial strains (Tadych et al., 2012; Aleklett et al., 2014).

Host–endophyte interaction in terms of biocontrol agents

It is well known that during plant–microbe interactions, microbial strains showed neutral, commensalism, mutualistic, or pathogenic interaction with the host plants. The establishment depends upon several factors, including the genotype of microorganisms or host plants and the surrounding environment (Brader et al., 2017). Plants rely on their sophisticated defense systems to counteract attacks of phytopathogens (Jones and Dangl, 2006), as the pathogenic strains secrete numerous biomolecules inside the host during infection. The host plant responds accordingly after recognizing conserved structure and elicits its immune behavior as the first line of defense to control the pathogen by the present pattern recognition receptors (PRRs). The PRRs sense the nature of microbes through the perception of microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) (Plett and Martin, 2018). Bacterial flagellin, elongation factor Tu (EF-Tu), fungal chitin, and yeast mannans are the most commonly reported PAMPs/MAMPs (Newman et al., 2013).

During co-evolution with the host plant, pathogenic strains improved the strategies to suppress the MAMP/PAMP-triggered immunity. In response, the host plant developed a second line of defense known as effector-triggered immunity. The plant system develops receptors that sense or recognize the pathogen's constituents. For instance, for the pathogenic microbes (biotrophic) that depend upon the nutrient uptake of living cells, a hypersensitive response may be activated, which leads to the programmed cell death of plants under attack (de Wit, 2007). However, this response must be suppressed in the case of necrotrophic pathogens or endophytes or symbiotic microorganisms (Liu et al., 2017). However, to cope with the plant immune system, the endophytic microorganisms produce their MAMPs, which do not significantly elicit the host immune or defense system. However, there is significant variation between the cell surface components (flagellin proteins in the endophytic microbes) of endophytic/symbiotic or pathogenic microbial strains (Trdá et al., 2014), which show differential patterns at the time of recognition by the receptors (Figure 2).

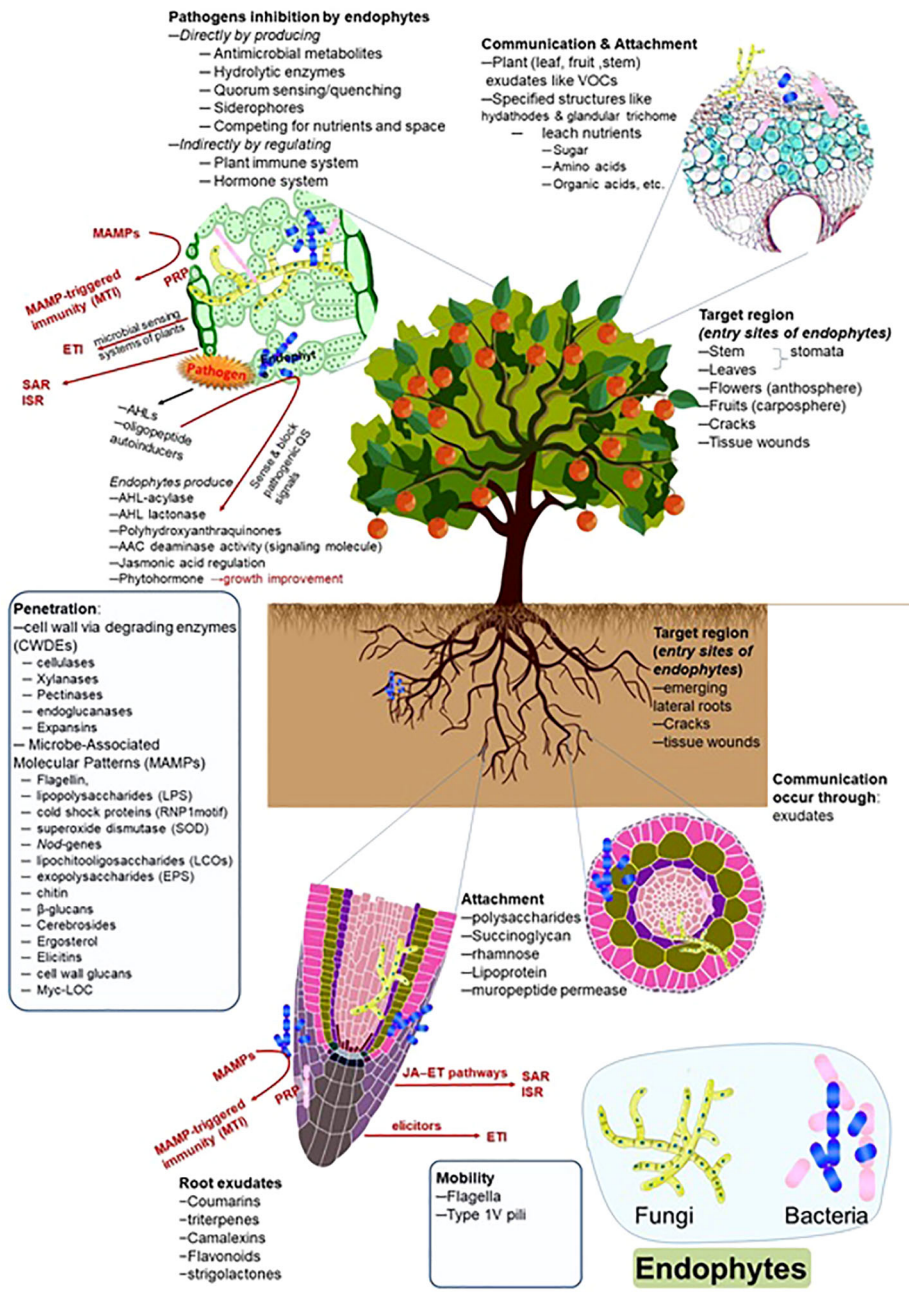

FIGURE 1
Endophytes and their interaction with the host plants. The figure describes the detailed role and approach of root exudates, communication, mobility, attachment, penetration, and target region (entry site) during endophyte colonization.

Endophytes as biocontrol agents

To explore endophytes as biological control agents, several factors have been considered relevant, including survival, stability, storage, application, and marketability. Despite the massive exploration of various microbial strains as BCAs *in vivo* or *in vitro*, only a limited number of strain/s, bacteria, fungi, or yeast, have been commercialized, and the possible reason is the survivability or stability of BCAs. The endospore formation of *Bacillus subtilis* or chlamydospore structure of *Trichoderma* makes them most suitable compared to other microbial strains because of stability or survivability under unfavorable conditions to fulfill the requirement of commercial exploitation. However, the endophytic microbiome can easily be administered,

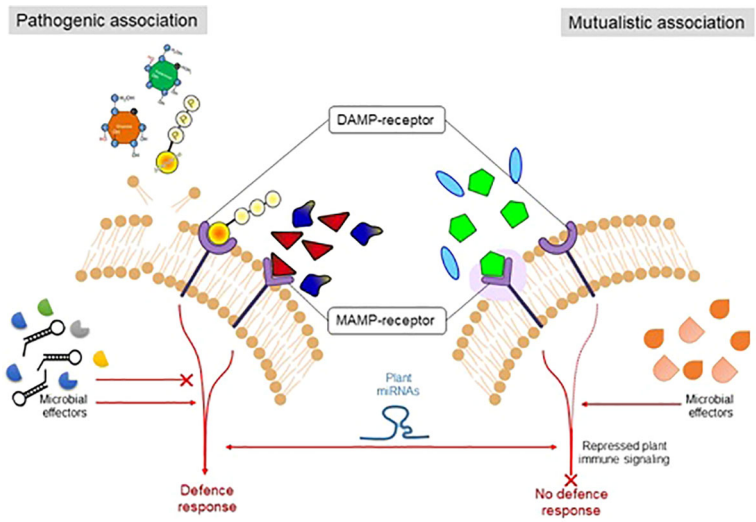

FIGURE 2
The figure illustrates the mechanism by which plants sense to differentiate symbiotic and pathogenic microorganisms.

penetrating and colonizing the host tissue, unlike other microorganisms where colonization is a complex process. However, the effectiveness of BCAs against the pathogen may also depend upon various factors, including the growth or physiological state of the plant, genotype, colonization pattern, population dynamics, and the surrounding environmental conditions (Card et al., 2016; Bolívar-Anillo et al., 2020).

Recent studies have reported the antagonistic activities of a diverse range of endophytes, which is present on the fruit surface. A number of bacterial, actinomycetes, and fungal species are present on the fruit surface that can impact the growth of postharvest pathogens (Huang et al., 2021). Similar to field conditions, *Pseudomonas*, *Citrobacter*, *Paenibacillus*, *Burkholderia*, and *Bacillus* sp. are some of the most prevalent biocontrol bacteria found on fruit surfaces (Shi et al., 2013; Huang et al., 2021). The use of endophytic yeast *Metschnikowia pulcherrima* along with chitosan prevented the growth of *Alternaria alternata* in table grapes (Stocco et al., 2019). *Aureobasidium pullulans* prevented the growth of *Botrytis cinerea* and *Monilinia laxa* in sweet cherries and table grapes, decreasing the decomposition rate of fruits between 10% and 100% (Schena et al., 2003). *Pantoea dispersa* controlled the black rot of sweet potato by exhibiting antibiosis (Jiang et al., 2019). *Trichoderma* and *Nodulisporium* are some of the most found fungal BCAs on the carposphere. Recently, mycofumigation with the fungal volatile organic compounds (VOCs) has also gained attention to inhibit the growth of postharvest pathogens (Zhi-Lin et al., 2012). Suwannarach et al. (2013) reported on biofumigation with the *Nodulisporium* spp. CMU-UPE34, an endophytic fungus, to prevent the postharvest decay of citrus fruits. The endophytic fungal stain *Nodulisporium* sp. strain GS4d2II1 produced six different VOCs, which inhibited *Fusarium oxysporum* growth in cherry tomato fruits after their harvest (Medina-Romero et al., 2017). Details of endophytic microbial strains and their utilization in postharvest disease or pathogen control of fruits have been discussed in Table 1.

Screening of endophytic biocontrol agents

The search for endophyte agents with biocontrol capacities is imperative in detecting those agents with excellent antagonistic capacities against potential pathogens. Detecting these characteristics depends on having better chances of generating microbial endophyte-based biocontrols with good chances of being successful in open field application and not just showing good actions in the laboratory. Next, we detail some tools for detecting and selecting endophytic BCAs. Screening microbial antagonists against various phytopathogens is one of the most crucial steps. The BCAs are generally screened on the basis of some specific characteristics such as parasitism, in which BCAs live together with the host plant, resulting in antagonistic effects (Mukherjee et al., 2012). Furthermore, strains having the capability to synthesize antimicrobial or volatiles compounds and enzymes such as pectinases and cutinases, which can interfere with pathogenicity factors or reduce the virulence of pathogens, are preferred for BCA screening (Zimand et al., 1996; Kapat et al., 1998).

However, other direct or indirect mechanisms have been employed to screen suitable BCAs for particular or broad-scale phytopathogens causing plant diseases. Dual-culture assay is one

TABLE 1 Endophytic microbial strains used for the postharvest disease or pathogen management in fruits.

Endophytic strains	Domain	Disease/Pathogens	Plants/Fruits	References
***Bacillus velezensis* QSE-21**	Bacteria	Postharvest gray mold of fruit	Tomato	Xu et al., 2021
Paenibacillus polymyxa	Bacteria	*Penicillium digitatum*	Citrus	Lai et al., 2012
***Bacillus subtilis* L1-21**	Bacteria	*Penicillium digitatum*	Citrus Fruits	Li et al., 2022
Endophytic bacteria	Bacteria	*Monilinia laxa* and *Rhizopus stolonifer*	Stone fruits	Pratella et al., 1993
Bacillus amyloliquefacies	Bacteria	*Botryosphaeria dothidea*	Kiwi fruit	Pang et al., 2021
Pseudomonas synxantha	Bacteria	Monilinia fructicola and *Monilinia fructigena*,	Stone fruit	Aiello et al., 2019
Lactobacillus plantarum CM-3	Bacteria	*Botrytis cinerea*	Strawberry fruit	Chen et al., 2020b
***Bacillus subtilis* L1-21**	Bacteria	Botrytis cinerea	Tomato	Bu et al., 2021
***Penicillium* sp.**	Fungi	*Botrytis cinerea*	Grapes fruits	Noumeur et al., 2015
Daldinia eschscholtzii	Fungi	*Colletotrichum acutatum*	Strawberry fruits	Khruengsai et al., 2021
Saccharomycopsis fibuligera	Yeast	Botrytis cinerea	Guava fruits	Abdel-Rahim and Abo-Elyousr, 2017
***Muscodor suthepensis* CMU-Cib462**	Fungi	*Penicillium digitatum*	Tangerine fruit	Suwannarach et al., 2016
***Fusarium* sp.**	Fungi	*Fusarium oxysporum*, *Aspergillus niger* and *Rhizopus stolonife*	Postharvest pathogens of vegetables	Tayung et al., 2010

of the standard phenotype-based direct screening methods for identifying microbial antagonists during *in vitro* identification. In this assay, BCAs and pathogens were cocultivated on semisolid media. The pathogen's antagonistic behavior toward BCAs and pathogenicity are evaluated by measuring the lesion diameter (Shi et al., 2014). During the evaluation, both the BCAs and the pathogen were grown together on the plates at different locations, and a significant decrease in mycelium growth and fungal spores was observed (Comby et al., 2017). In another case, the pathogen has been evenly spread over the plate, and BCA was spotted over the medium. The clear zone around the spotted BCA was measured to evaluate biocontrol activity. The larger the clear zone, the higher the biocontrol potential (Shehata et al., 2016).

Synthesis of antimicrobial compunds, either diffusible or volatile, by the microbial endophytic strain is also one of the parameters for biocontrol screening. During *in vitro* volatile analysis, the BCA and the pathogen grow on an agar base plate, which is grown under physically separated conditions and sealed with parafilm or tape to avoid VOC escape (Stinson et al., 2003). However, screening of BCAs in liquid media has also been done under which both the BCAs and pathogen were grown either simultaneously or consecutively, and their impact has been evaluated either by measuring the optical density or by the microscopic evaluation of pathogen spore or germination tube of mycelia tube (Omar and Abd-Alla, 1998).

However, *in vivo* screening is the standard method for evaluating potential BCAs under natural or greenhouse conditions through several parameters such as measuring lesion diameter, disease severity, or defined disease index (Lecomte et al., 2016). *In vivo* screening not only is based on antagonistic activity but also includes the physiological status of the plant by measuring water status (e.g., transpiration, stomatal conductance), variation in antioxidant activity (e.g., enzymatic activity levels), production of plant defense molecules (e.g., phytoalexins), morphological growth parameters such as plant height, the dry or fresh weight of certain plant parts, or the flowering date (Lecomte et al., 2016). The antagonistic potential of the BCAs varies with plant genotype or species; differences in host genotypes differentially regulate the physiological functions that may modulate the rate of infections and response of host immune systems. Similarly, the colonization potential of the endophytes, which depends upon the various physiochemical nature of plant exudates, also impacts the biocontrol potential against the pathogen more efficiently and effectively (Martin et al., 2015).

Postharvest factors that affect the quality of food and disease incidence

Postharvest diseases can result from incorrect postharvest practices and faulty preharvest management. The significant postharvest factors that affect the storage of food are as follows.

Fruit storage conditions

Fruits are generally transported to supermarkets and cold chains before reaching customers' hands. Temperature, pH, and humidity conditions in cold chains significantly affect the growth of pathogens and endophytes (Carmona-Hernandez et al., 2019).

Low pH due to fruit metabolism and high humidity support the growth of fungal pathogens (Arah et al., 2015). In addition, temperature and pH conditions also influence the production of volatile secondary metabolites (VOCs) from the microbes (Lazazzara et al., 2017; Fadiji and Babalola, 2020). In a study, a lower pH condition of the fermentation medium significantly influenced the production of phloroglucinol and gallic acid from isolated endophytic fungus *Colletotrichum gloeosporioides* (Gasong and Tjandrawinata, 2016).

Physical handling and gaseous treatments

The rough handling of already ripened fruits invites the attack of pathogens on soft and brushed surfaces. In addition, mechanical injuries to the fruits due to improper handling can increase the metabolism and ethylene production, which can cause adverse biotic stresses on the stored fruits (Miller, 2003). The stored fruit's carbon monoxide (CO) treatment increases ripening and decreases pathogen infestation. The *Alternaria* rot in jujube fruits was effectively controlled by CO application in fruit storage conditions (Zhang et al., 2020). High carbon dioxide concentration around fruits also reduced the respiratory activities and consumption of soluble solids, which results in a reduction in pathogen infection (Huyskens-Keil and Herppich 2013). Apart from the growth of pathogens, physical handling and food storage conditions can also play a significant role in the growth and secondary metabolite production of endophytes.

Postharvest management strategies by endophytes: Action mechanisms

Endophytes are known to show a myriad of mechanisms against pathogens ranging from direct competition to change in the molecular architecture of the host plants. Endophytes against postharvest pathogens, being a relatively new field, require an in-depth literature review to understand the possible mechanisms employed against postharvest pathogens. Following are the possible mechanisms that endophytes employ to combat pathogenic attacks on the harvested fruits.

Direct competition for space and nutrients

In the tripartite system of fruit–pathogen–endophyte interaction, the nutrition and space of the host are limited. Nitrogen, carbon, macronutrients, and micronutrients are essential for the survival of both endophytes and pathogens (Kumari et al., 2020a, b). Endophytes, being fast in growth and colonization, quickly occupy the exposed fruit surface and outnumber pathogens in the space competition and utilization of nutritional resources (Adame-Álvarez et al., 2014; Spadaro and Droby, 2016). Different studies have demonstrated the utilization of carbon resources by endophytic *Bacillus* spp., inhibiting spore germination of the pathogens; however, bacterial dosage needs to be optimized according to the fruit (Carmona-Hernandez et al., 2019). A phenotypic and gene transcription study revealed the increased expression of genes involved in nutrition uptake by the bacterium *Lactobacillus plantarum* when cocultivated with the pathogen *Aspergillus carbonarius* isolated from grape berries (Lappa et al., 2018). The *L. plantarum* culture effectively inhibited the growth of four fungal pathogens isolated from the grape berries. A 32%–90% inhibition in mycotoxin produced by *A. carbonarius* was also observed after coculturing with *L. plantarum*. Successful *in vivo* application of this bacterium not only may help in controlling postharvest pathogens but also will act as a source of probiotics for modulating gut microflora.

Production of siderophores (iron-chelating compounds)

Iron is one of the essential minerals required for the growth, survival, and virulence of pathogens. Siderophores are the secondary microbial metabolites produced by many endophytes, which can form a tight and stable octahedral Fe (H2O6)3+ complex with available iron (Miethke and Marahiel, 2007). The exposed fruit surface is an adverse niche, where the bioavailability of nutrients, especially iron, is relatively low. In the competition for survival, endophytes are known to colonize faster than pathogens, chelating the available iron by producing several types of siderophores and thus depriving the postharvest pathogen of any iron source (Chowdappa et al., 2020). Genome mining of the endophytic *Pseudomonas fluorescens* BRZ63 has revealed siderophore production by the bacterium, protecting against several postharvest pathogens, including *Colletotrichum dematium* K, *Sclerotinia sclerotiorum* K2291, and *Fusarium avenaceum* (Chlebek et al., 2020). Many endophytic *Bacillus* sp. produce bacilibactin type of siderophore-protecting bacterial wilt in banana (Carmona-Hernandez et al., 2019). *Trichoderma* spp. has been known to produce hydroxamate siderophore, which can deplete iron and inhibit the growth of postharvest pathogens in apples and citrus fruits (Sood et al., 2020). Though the endophytic *Trichoderma* spp. is still in the nascent stage for controlling postharvest diseases of fruits, it can pave a new and sustainable path for the disease control of fruits after harvest. However, optimizing the concentration of endophytes and factors affecting siderophore production should not be neglected to increase endophytic efficiency against postharvest pathogens.

Production of bioactive antimicrobial compounds and antibiosis

Endophytic microbiomes have recently emerged as potent and novel sources of secondary metabolites, many of which are antimicrobial. They are known to produce alkaloids, flavonoids, phenolics, terpenoids, steroids, non-ribosomal peptides, and VOCs (Kumari et al., 2018). For example, endophytic *Trichoderma* sp. produced antifungal epipolythiodioxopiperazines, peptaibols, koninginins, and pyrenes, which combat postharvest diseases in kiwi fruit, apple, and banana (Khan et al., 2020). The recently published review article by Huang et al. (2021) briefly covered the bioactive compounds produced by endophytes and how they enhance the resistance against postharvest diseases of fruit and vegetables. Similarly, Carmona-Hernandez et al. (2019) also covered the bioactive compounds, volatiles produced by the endophytic strains, and their role in postharvest disease management. The details of bioactive metabolites produced by endophytes, which can potentially be used against postharvest pathogens of fruits, are described in Table 2.

Though the potential of bioactive secondary metabolites is enormous in postharvest disease control of fruits, the low quantity produced, *in planta* pressure, and influence of the culture conditions are some of the factors that need optimization.

Mycoparasitism and production of lytic enzymes

One of the essential mechanisms employed by endophytic fungi against pathogenic fungi is mycoparasitism by the production of cell wall-degrading enzymes and direct parasitism. The lytic enzymes, including glucanase, chitinase, and cellulose produced by endophytes, can degrade the pathogenic cell wall. For example, *Talaromyces acidophilus* a fungal strain AUN-1 emerged as a novel mycoparasite of postharvest pathogen *B. cinerea* by producing lytic enzyme chitinase, lipase, and protease (Abdel-Rahim and Abo-Elyousr, 2018). Endophytic fungus *Choiromyces aboriginum* inhibited postharvest pathogen *Pythium* sp. by producing β-1,3-glucanases and degraded the pathogenic cytoplasm coiling around the hyphae (Cao et al., 2009). In the same sense, plant beneficial fungus *Trichoderma* spp. can inhibit the growth of several pathogens through parasitism, for example, a *Trichoderma* sp. strain inhibited the fungal pathogen *F. oxysporum* by producing a lytic enzyme and coiling around the pathogenic fungal hyphae (Rajani et al., 2021).

Some bacterial strains are also prolific producers of lytic enzymes, making them suitable candidates for postharvest disease management, though endophytes specifically have not been explored much. For example, endophytic *Bacillus* sp. are known to produce β-1,3-glucanase, chitinase, and protease, which can disrupt fungal cell walls (Carmona-Hernandez et al., 2019). The hydrolytic enzymes produced by *B. subtilis* 739 caused the lysis of phytopathogenic fungi *A. alternata*, *B. sorokiniana*, *F. culmorum*, and *R. solani*. The cocktail of cold-adapted lytic enzymes produced by archaea and cold-adapted bacteria has also shown their potential against antagonistic fungal pathogens (de Oliveira et al., 2020), which provides an excellent opportunity to explore endophytes from extreme conditions.

Production of endotoxins and lipopolysaccharides

Endophytes are being developed as prolific producers of LPSs of several lengths of fatty acids. For example, phengicines and iturins produced by *B. subtilis* GA1 inhibited the growth of *B. cinerea* in apple fruits (Toure et al., 2004). Thus, the optimized media conditions for synthesizing LPSs from endophytes can pave a sustainable path for the biological control of postharvest fruit diseases. The toxin Leu7-surfactin was produced from the endophytic bacterium *Bacillus mojavensis* RRC 101 against antagonistic fungus *Fusarium verticillioides* (Snook et al., 2009). Several mycotoxins produced by endophytic fungi can also be explored for their efficacy against the antagonistic pathogens to control postharvest disease, though their safety also needs to be analyzed thoroughly (Lacava and Azevedo, 2013).

Modulating the redox homeostasis of harvested fruits and pathogens

Many postharvest pathogens overcome the fruit defense system by manipulating their redox potential. For example, *Penicillium digitatum*, the causative agent of green mold in citrus fruits, produces catalase that decomposes hydrogen peroxide to establish an infection (Macarisin et al., 2007). Endophytes provide oxidative stress protection to plants (Hamilton et al., 2012; White et al., 2019). However, their role in modulating stress in postharvest disease management is not much explored. Endophytes help plants combat biotic stress by lowering lipid peroxidation and accumulation of proline (Spadaro and Droby, 2016). As an example, endophytic fungus *Paraburkholderia phytofirmans* strain PsJN increased the expression of genes involved in reactive oxygen species (ROS)-scavenging pathways, resulting in detoxification of ROS and modulating the signaling pathways (Pacifico et al., 2019). The plant–pathogen and endophytic relation has been documented well in literature, but the research on the role of endophytes in modulating redox homeostasis of stored fruits needs special attention.

TABLE 2 Bioactive compounds produced by endophytic microbes used in the management of postharvest diseases of fruits.

Endophytic microbes	Production of bioactive compound	Putative role against postharvest pathogens	References
Bacillus subtilis	Iturin A, lipopolysaccharide	Antifungal activity against *F. oxysporum*, *Pythium ultimum*, and *Phytophthora* sp.	Ek-Ramos et al., 2019
***Bacillus* sp.**	Surfactin, fengycin	Used against bacterial diseases	Jasim et al., 2016
Pseudomonas aeruginosa	Phenyltetradeca-2,5-dienoate	Used against bacterial diseases	Pratiwi et al., 2017
***Bacillus amyloliquefaciens* CEIZ-11**	lipopolysaccharide	Antifungal activity against *Botrytis cinerea* and Alternaria alternata	Zouari et al., 2016
***Pseudomonas putida* BP25**	VOCs	Antifungal activities against *Phytophthora capsici* and *Radopholus similis*	Sheoran et al., 2015
Chaetomium globosum	Chaetomugilin A and D	Antifungal activity against *Fusarium* sp. and *Verticillium* sp.	Pimentel et al., 2011
Trichoderma lixii (IIIM-B4)	Peptaibol	Shows antibacterial activities	Katoch et al., 2019
***Trichoderma* sp.**	VOCs	Antifungal activities against *Sclerotium rolfsii* and *Fusarium oxysporum*	Rajani et al., 2021
Aspergillus fumigatus	Alkaloids	Shows antifungal activities against postharvest pathogens	Li et al., 2012
Trichoderma polyalthiae	Violaceol I and Violaceol II	Showed antimicrobial activities	Nuankeaw et al., 2020
***Streptomyces* sp.**	Enduspeptide B, neomaclafungins A-I	Strong antifungal activities	Jakubiec-Krzesniak et al., 2018
Streptosporangium oxazolinicum K07-0460	Polyketides	Antibacterial activities against *Xanthomonas* sp.	Matsumoto and Takahashi, 2017
***Xylariales* sp.**	α-pyrone derivatives	Antifungal activities against *Botrytis cinerea, Fusarium oxysporum* and *Alternaria* sp.	Rustamova et al., 2020
***Alternaria* sp.**	Alternarilactone-A	Antifungal activities against *Verticillium cinnabarium* and *Gaeumannomyces graminis*	Rustamova et al., 2020

Quorum sensing and biofilm formation and disruption by endophytes

Bacterial endophytes, including *Bacillus* spp. and *Pseudomonas* spp., are known to colonize exposed fruit areas by quorum sensing (QS) and biofilm formation. The ability of endophytic bacteria to secrete small molecules such as tyrosol, farnesol, and phenethyl alcohol to regulate colonization helps them outnumber the pathogenic microbes in the competition for space and nutrients (Carmona-Hernandez et al., 2019). Recently, endophytes were also found to produce anti-QS molecules, which can help combat the biofilm established by pathogenic bacteria on fruit surfaces. For example, endophytic fungi *Fusarium graminearum* and *Lasiodiplodia* sp. isolated from the plant *Ventilago madraspatana* produced secondary metabolites with anti-QS potential (Mookherjee et al., 2017). Furthermore, the isolated fungi produced QS inhibitors that were quantified spectrophotometrically by their ability to inhibit the production of violacein in wild and mutants of *Chromobacterim violaceum* (Rajesh and Rai, 2013). Whether it is biofilm formation or the production of anti-QS molecules by endophytes, both properties can be exploited in postharvest disease management in fruits, as this field of research remains unexplored.

Modulation and synthesis of phytohormones

Endophytic microbes can synthesize phytohormones, including auxin, gibberellins, cytokines, ethylene, nitric oxide, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which provide additional immunity to postharvested plants to cope up with biotic and abiotic stresses (Ali et al., 2017). The increased phytohormone synthesis helps to overcome the stress-induced wilting. Not only are the endophytes capable of synthesizing plant hormones themselves, but they can also modulate the plant–hormone metabolic pathways for enhanced stress tolerance. For example, the interaction of endophytic fungus *Piriformospora indica* in the synthesis of auxins, cytokinin, gibberellins, abscisic acid, ethylene, salicylic acid (SA), jasmonates, and brassinosteroids resulted in better efficiency of stress tolerance in higher plants (Xu et al., 2018).

Induction of disease resistance in fruits

In response to a pathogenic attack, plants develop two kinds of disease resistance mechanisms: 1) systemic acquired response (SAR) and 2) induced systemic resistance (ISR). Many endophytic microbes have been known to elicit ISR, thereby providing solid immunity against biotic stress (Pacifico et al., 2019). Endophytes activate ISR pathways by synthesizing pathogen-related proteins, enhancing the synthesis of phenolic compounds, and activating signaling pathways by jasmonate/SA and ethylene (Jacob et al., 2020) (Figure 3).

The endophytic bacterial strain *Pseudomonas putida* MGY2 was able to control anthracnose caused by *C. gloeosporioides* in harvested papaya fruit (Shi et al., 2011). It was found that the endophyte induced ISR by increasing the gene expression of

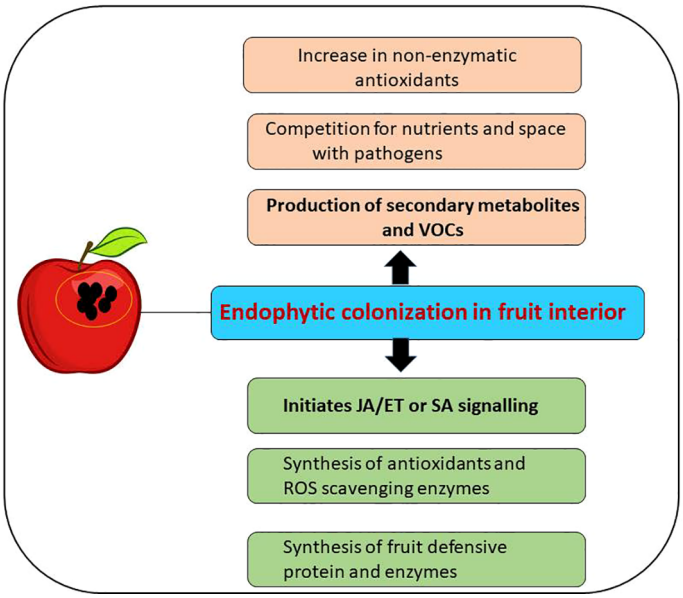

FIGURE 3
Activation of induced systemic resistance (ISR) signaling pathway and production of bioactive secondary metabolites after colonization of endophytes in the host (postharvested fruits).

phenylalanine ammonia-lyase (PAL), catalase (CAT), and peroxidase (POD), increasing the phenolic content and decreasing the production of ethylene. The same group demonstrated the control of *Phytophthora nicotianae* disease in papaya fruits by induction of the pathogenesis-related protein 1 gene (PR1) and non-expression of PR1 gene (NPR1) after inoculation of *P. putida* MGP1 strain (Shi et al., 2013). Louarn et al. (2013) demonstrated a significant change in the endophytic community in organically and conventionally grown carrots. Endophytic *Bacillus amyloliquefaciens* YTB1407 strain elicited ISR by activating the expression of SA-responsive PR1 gene, thus inhibiting pathogenic fungus *Fusarium solani*. The literature is insufficient regarding the elicitation of molecular responses of fruits in postharvest conditions. Furthermore, in-depth mechanistic studies are required to understand the disease resistance of fruits after endophytic microbe application.

Modulating the native microbiota and ecological effects

The endophytic microbial population modulates the native microbiota of fruits, roots, leaves, and soil, promoting a sustainable crop production system. Therefore, it is of great economic relevance (Sturz et al., 2010; Baghel et al., 2020). However, its interference with the native population of harvested fruit microbiota is still waiting to be explored. Endophytes bear the potential to shift the native bacterial population toward favorable conditions for plant growth and stress amelioration (Baghel et al., 2020). It has been found that healthy fruits tend to have a diverse microbial community, whereas diseased fruits have a limited microbial growth dominated by pathogen microorganisms (Huang et al., 2021). In their study, Diskin et al. (2017) found that colonization of endophytic communities was much less prevalent in mango fruits suffering from stem-end rot disease than that in their healthier counterpart. By utilizing multiple mechanisms, including parasitism, production of bioactive compounds, lytic enzymes, and siderophores against postharvest pathogens, endophytes can modulate the native microbiota of the harvested fruits to increase their resistance against biotic stresses.

Controlling mycotoxins

Mycotoxins are a major cause of qualitative and quantitative loss in stored fruits. Deoxynivalenol, alternariol, aflatoxin, and patulin, produced by antagonistic fungi, can impact fruit and human health negatively (Bartholomew et al., 2021). Many endophytes and their secondary metabolites have shown the effectiveness of controlling mycotoxins *in vitro* and *in planta* (Abdallah et al., 2018) in maize and other crops, although studies on their impact on postharvested fruits are limited. Sarrocco and Vannacci (2018) emphasized preharvest application of

endophytes for controlling postharvest damage caused by mycotoxins. The VOCs produced by endophytic fungi can be incorporated in edible biofilms or can be an ingredient during packaging to effectively control mycotoxins in store fruits (Mari et al., 2016).

As biocontrol strategies usually rely on a single or mixture of antagonists, endophytic microbial strains have been suggested as antagonistic microorganisms against various diseases in various crops. The additional effect of endophytic microbiota as BCAs is the phytohormone synthesis, metabolites, and nutrients utilized for growth promotion and stress management in host plants (Lodewyckx et al., 2002; Suhandono et al., 2016).

In the recent past, various BCAs, including bacteria, yeast, and fungi, have been frequently applied for effective management of postharvest pathogens, while practices with endophytes are very limited. Endophytes' properties appear superior to those of epiphytic microorganisms due to their better colonization and tolerance potential against various biotic and abiotic stresses (Shi et al., 2010). In recent years, several pieces of literature regarding utilizing the endophytic microbiome for screening BCAs against postharvest pathogens have been reported. Shimizu et al. (2009) reported on the endophytic actinomycete *Streptomyces* sp., which showed effective biocontrol potential against the pathogen *Colletotrichum orbiculare*, the causal agent of anthracnose disease in cucumber. Similarly, Shi et al. (2010) reported on *P. putida* biovar isolated from the pericarp of papaya with strong colonization potential and showed potent inhibition against several pathogens.

Additionally, the strain effectively inhibits the growth of *P. nicotianae* just after a short period of treatment. Lai et al. (2012) screened the endophytic strain *Paenibacillus polymyxa* isolated from the root tissue of *Sophora tonkinensis* and showed antagonistic potential against *P. digitatum*, one of the most devastating pathogens causing postharvest diseases in citrus fruit. The application of endophytic strains effectively reduces postharvest decay by inhibiting conidia germination in a fungal cell suspension. Additionally, the unwashed cell suspension of the strain was found to be more effective than the washed cell suspension and culture filtrate in the *in vivo* trials.

Ji et al. (2008) isolated 45 endophytic bacterial strains from the mulberry leaves (*Morus alba* L.) and reported the strong inhibitory potential of *B. subtilis* Lu144 against *Ralstonia solanacearum*, the causal agent of bacterial wilt of mulberry fruits. Furthermore, Furuya et al. (2011) utilized the strain *B. subtilis* KS1 isolated from the skin part of grape berry and applied it as a potential antagonistic agent against fungal grapevine diseases. *In vitro* screening showed that the strain effectively suppressed the growth of *B. cinerea* and *C. gloeosporioides*. Furthermore, after applications in the vineyards, the strains significantly reduce the incidence of downy mildew from the leaves and skin of the berry. Chen et al. (2016) screened the *B. amyloliquefaciens* PG12 strain isolated from apple fruits as a potential BCA against apple ring rot disease. The strain significantly suppressed the *Botryosphaeria dothidea* growth during *in vivo* and *in vitro* screening and showed a potent antagonistic effect against different fungal pathogens. Madbouly et al. (2020) evaluated the biocontrol potential of endophytic yeast strains *Schwanniomyces vanrijiae*, *Galactomyces geotrichum*, *Pichia kudriavzevii*, isolated from apple fruits, against the pathogen *Monilinia fructigena*, the causal agent of apple fruit brown rot of golden delicious apples. During *in vitro* test analysis, all three endophytic yeast strains showed inhibitory potential against *M. fructigena* and significantly inhibited conidial germination by 67.6%–89.2%. In the last few years, rapid enhancement can be seen in the use of endophytic microorganisms in postharvest disease management in fruits. However, still, most of the experiments are limited to the laboratory scale. Furthermore, we need to study how the fruit microbiome affects the fruit's physiology and disease resistance and how the fruit-associated microbial communities shifted during the postharvest stages and after applying BCAs.

Commercial upscale production and hurdles ahead

Antagonistic endophytic application against postharvest diseases, especially in fruits, has emerged as a new generation of pesticides. Though the mechanisms are still to be deciphered completely, many endophytes have paved their path to commercial applications. *B. subtilis* strain B-3 has been patented, and pilot experiments have been conducted against the peach brown rot disease. It was observed that after the application of the endophyte in either powder or paste form, it was as effective as traditional pesticide benomyl in Clemson, SC, USA (Pusey et al., 1988). Products based on *B. subtilis* QST713 with the trade name Serenade™ are produced commercially by AgraQuest Inc., USA, against powdery mildew, brown rot, and late blight of apple, pear, and grapes (Punjia et al., 2016). Multiple formulations in many countries with trade names, including Candifruit™, Shemer™, and Boni-protect™, have been successfully used against postharvest pathogens (Fenta et al., 2019). The endophytes, a new concept, have to face many hurdles for their successful commercialization. In addition to the agricultural giants such as Dupont, Monsanto, and Bayer, many small startup companies such as Indigo and NewLeaf Symbiotics have entered the microbial domain with promising contributions. The following hurdles need to be overcome to achieve economically and sustained commercial-scale production of antagonistic endophytes or their products.

Increased shelf life and multiple stress-tolerant endophytic microbes

In the niche of postharvest fruits, endophytes have to overcome several biotic and abiotic stresses (Diskin et al., 2017). For the successful application and upscale production of antagonistic endophytes against postharvest diseases of fruits, the endophytes must be stress-tolerant to prolong their shelf life and sustain antipathogenic activities. Many stress-tolerant endophytic microbes are already studied for plant growth promotion in adverse conditions (Giauque et al., 2019; Singh et al., 2022). Furthermore, the synergistic application of endophytes can also help increase the shelf life of endophytes in their battle against postharvest pathogens (Huang et al., 2021). Therefore, exhaustive screening of stress-tolerant endophytes and their *in vitro* and *in vivo* stress amelioration potential should be conducted for the endophytes to go from lab to field.

Some endophytes are deeply associated with their host for stress tolerance and the production of the desired natural products (Khare et al., 2018). Therefore, their ability to cope up with the stress condition in the absence of their host plants and the niche of postharvest fruits should also be assessed before their commercialization.

Optimizing the modes of endophyte application

The modes of application of endophytes to the surface of postharvest fruits also play a crucial role in plant disease management and increasing the shelf life of the endophytes. Therefore, the application of endophytes on fruit surfaces should be optimized on a case-by-case basis. Generally, the formulations are applied as liquid or powder/paste formulations. Though the dry form provides a longer shelf life, it can cause a loss of viability of microbes through repeated rehydration-dehydration processes (Kumari et al., 2020a, b). Many rehydration agents, including whey proteins and maltodextrins, have been suggested to coat dry formulations (Martin et al., 2017). For sustained release of endophytes, their secondary metabolites, and VOCs, nanoencapsulation of the products and nanoemulsions can also be studied (Pandey et al., 2020). Recently, Ghazy et al. (2021) studied the role of anise extract oil nanoemulsion against different postharvest antagonistic bacteria for their sustained release. A combination of SA with endophytic *B. subtilis* was used to treat postharvest diseases by *F. oxysporum* and *P. infestans* (Lastochkina et al., 2020). Preharvest and postharvest modes of endophytic application should also be considered for their antagonistic application. For the upscale production of endophytes as postharvest disease management in fruits, the mode of application is an important parameter, whose optimization should be carried out in detail.

Sustained release and cost-effective production of microbial metabolites

The commercialization of secondary metabolites and VOCs derived from endophytes faces hurdles in sustainable release and economic upscale production. Media optimization, selection of potent microbial strains, and metabolic engineering are some of the parameters that can be employed (Sah et al., 2020; Kamat et al., 2020; Taritla et al., 2021) for the sustained production of desired antimicrobial secondary metabolites from endophytes. The addition of some of the precursors from the host system has also been studied during media optimization for continuous upscale production of the antimicrobial metabolites from endophytes during the fermentation process.

The second hurdle faced during their commercialization includes the hydrophobicity of natural products. To overcome the solubility issue, several solutions, including their encapsulation in non-toxic and biodegradable polymers, have been proposed (Soh and Lee, 2019), which provide solubility and the slow release of the active ingredient. Chitosan, carrageenan, starch, and alginate nanopolymers have been used to encapsulate natural products, including polyphenols, alkaloids, and terpenoids with increased water solubility and bioactivity (Detsi et al., 2020).

Overcoming the *in planta* pressure for survival and stress amelioration

The biggest hurdle in successfully applying endophytic microbes in the fruit microbiome is overcoming their host pressure. Endophytes have always lived as symbionts with their host, sharing many physical and chemical attributes with their host plants (Spadaro and Droby, 2016). Several hypotheses, including the defensive mutualism hypothesis, xenohormesis hypothesis, and trait-specific endophytic infallibility (TSEI) hypothesis, have been shared among the research community to describe the co-evolution of the host and the endophytes (Kusari et al., 2015; Pathak et al., 2022). Their isolation and survival without their hosts may alter their growth cycle and physiological performance in the competition of the new fruit microbiome. The question of replacement dynamics with the preexisting microbiome of fruits is always relevant while introducing a new endophytic strain. The mode of application and the growth and production of secondary metabolites *in vitro* should be monitored before their *in vivo* application in postharvested fruit microbiomes.

Genome mining and metagenomics

Getting the superior strains of endophytes required digging deep into the unexplored wealth of endophytes and exploring the biosynthetic pathways to synthesize beneficial secondary metabolites, siderophores, and phytohormones. To bypass the tedious process of endophyte isolation and screening for postharvest disease management, genome mining and metagenomic studies can be performed to select the right strain economically (Kusari et al., 2015). For example, genome mining of the endophytic fungus *Penicillium dangeardii* revealed a cluster of 43 biosynthetic genes demonstrating their strong ability to synthesize secondary metabolites (Wei et al., 2021) exploited in postharvest disease management. Thus, genome mining and metagenomics can provide better endophytic strains that can be commercially produced for the desired secondary metabolites.

Change in policymaking and awareness regarding the use of antagonistic endophytes

The most critical parameter for introducing endophytes as substitutes for conventional pesticides in postharvest disease management is to increase the awareness of the end-users and people involved in the distribution chain. Therefore, outreach programs and workshops related to these new ideas should constantly be organized to bring awareness and benefits of using endophyte-based biopesticides.

Any effort is not fruitful without governments, policymaking, and funding agencies to implement new technologies in agri-business sectors. Earlier, the Department of Biotechnology (DBT), India, launched the National Biocontrol Network Programme (NBNP) to popularize and commercialize more than 30 biopesticides (Kumari et al., 2020a, b). Similar programs should be launched and funded to popularize financial, most effective, and eco-friendly products for managing postharvest diseases of fruits.

Safety of endophytes and their secondary metabolites for consumers and the environment

Endophytes, a new aspect of BCAs in postharvest disease management in fruits, need thorough scrutiny regarding their safety for consumers and the environment. Endophytes themselves or their products should not be opportunistic pathogens or should not pose any harm to the environment. Unfortunately, many of the earlier studied rhizobacteria or their secondary metabolites have acted as opportunistic human pathogens or environmental contaminants in certain conditions (Keswani et al., 2019). To avoid similar conditions with the endophytes, their safety in animal models and their effect on the environment due to higher dosage should also be assessed.

Conclusion

Endophytic microorganisms can colonize different organ tissues of the host plant and interact in multiple ways to regulate physiological and metabolic pathways, which can further be utilized in the effective management of postharvest diseases. Endophytic bacterial, actinomycetes, and fungal strains have been broadly utilized as BCAs against various plant pathogens during preharvest and postharvest stages. Currently, it is estimated that approximately 30% of the total fruit production is lost annually due to various diseases. Therefore, the potential colonization efficacy of endophytes is a crucial characteristic for disease management.

In addition, next-generation omics may be applied to identify the gene(s) responsible for disease management. Thus, during the application, consortia of mixed microbial agents (bacteria-bacteria; bacteria-fungus; fungus-fungus) showed a practical approach in disease management, but the survival and better adaptability of both strains together are reasons for further investigation, particularly under diverse environmental conditions. Endophytes have reported multiple mechanisms that are used to inhibit pathogenic growth and increase fruit health. Though there are numerous examples of successful bioformulations of microbial endophytic strains capable of controlling the pathogenicity of the pest or pathogens during preharvest conditions, their application in postharvest pathogen control is in the nascent stage. Further application of endophytic microbiome can further reduce, or at some point will eliminate, the harmful dependence on chemical pesticides and fungicides in postharvest disease management.

Author contributions

MK and AK designed the study. MK, KQ ,SS, VS, KS, and AK wrote the manuscript. KQ and MJ acquired funding. KQ, MJ, JW, GS, and GP reviewed and provided valuable feedback to this study. All the authors contributed to the article and agreed to the published version of the manuscript.

Funding

The research is financially supported by King Abdullah University of Science and Technology, Thuwal, Jeddah, Saudi Arabia.

Acknowledgments

The authors are thankful to the Agriculture Research Organization for providing lab facilities.

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.

The reviewer SP declared a shared affiliation with the author SS to the handling editor at the time of review.

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frontiers | Frontiers in Plant Science

TYPE Mini Review
PUBLISHED 18 January 2023
DOI 10.3389/fpls.2022.1092105

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Plant Symbiotic Interactions, a section of the journal Frontiers in Plant Science

RECEIVED 07 November 2022
ACCEPTED 22 December 2022
PUBLISHED 18 January 2023

CITATION
Mushtaq S, Shafiq M, Tariq MR, Sami A, Nawaz-ul-Rehman MS, Bhatti MHT, Haider MS, Sadiq S, Abbas MT, Hussain M and Shahid MA (2023) Interaction between bacterial endophytes and host plants. *Front. Plant Sci.* 13:1092105. doi: 10.3389/fpls.2022.1092105

Interaction between bacterial endophytes and host plants

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Endophytic bacteria are mainly present in the plant's root systems. Endophytic bacteria improve plant health and are sometimes necessary to fight against adverse conditions. There is an increasing trend for the use of bacterial endophytes as bio-fertilizers. However, new challenges are also arising regarding the management of these newly discovered bacterial endophytes. Plant growth-promoting bacterial endophytes exist in a wide host range as part of their microbiome, and are proven to exhibit positive effects on plant growth. Endophytic bacterial communities within plant hosts are dynamic and affected by abiotic/biotic factors such as soil conditions, geographical distribution, climate, plant species, and plant-microbe interaction at a large scale. Therefore, there is a need to evaluate the mechanism of bacterial endophytes' interaction with plants under field conditions before their application. Bacterial endophytes have both beneficial and harmful impacts on plants but the exact mechanism of interaction is poorly understood. A basic approach to exploit the potential genetic elements involved in an endophytic lifestyle is to compare the genomes of rhizospheric plant growth-promoting bacteria with endophytic bacteria. In this mini-review, we will be focused to characterize the genetic diversity and dynamics of endophyte interaction in different host plants.

KEYWORDS

host endosymbiont interactions, mechanism of interaction, bacterial endophytes, plants, endophytic

1 Introduction

Plants interact with diverse microbial populations in the ecosystem (Delaux et al., 2015). Microorganisms can colonize on plants' surfaces or internal parts depending on the host genotype and the molecular signals released by plant roots. Microorganisms can colonize on plants' surfaces or internal parts depending on the host genotype and the molecular signals released by plant roots. Endophytes are prokaryotic bacteria found within the healthy host tissue (Brader et al., 2014). Bacterial endophytes can benefit the host in several ways, such as biotic and abiotic stress resistance, increased availability of nutrients, degradation of toxic molecules, and production of phytohormones (Kandel et al., 2015).

Plant population dynamics have soil microbial intermediation. The plant has a microbial population in the phyllosphere, endophytes, or rhizospheric microbes. The ecology and phenotype of the plants can be affected by the influence of symbiotic microbes on the atmosphere and competition for soil resources.

The plant genotype affects the microbial make-up of the phyllosphere, rhizosphere, and endophytic microorganisms (Lynch et al., 2001). Although the precise method involves the plant-associated microorganisms and ecosystem function, the other specific mechanism is still unknown. Because they are co-evolved with bacteria, plants are immobile and need to control the results of their intricate interactions (Schnitzer and Klironomos, 2011). Different sorts of chemicals are continuously produced by plant roots, gathered, and secreted into the soil (Wood et al., 2012) known as the root exudates which contain enzymes, water, mucilage, H^+ ions, and primary, secondary compounds made up of carbon (Singh, 2015). Every plant species' rhizosphere is known to have a microorganism population that is 100 times higher than soil and is mostly controlled by compounds generated by roots (Jonkers et al., 2003; Bever, 2003). The favorable plant-soil microbial response enhances the microbial populations' spatial spread (Schimel et al., 2007), while negative reaction results in plant replacement, which demands recolonization of locally specific roots (Bever et al., 2010; Pedrotti et al., 2013).

It has been proposed that endophytic bacteria vary from rhizobacteria in their genetic architecture, which may account for their capacity to colonise plant tissues internally. However, no specific gene or gene family has been found to explain the endophytic regime. In a 2014 study, the whole genomes of nine Proteobacteria were compared to identify a list of genes that may play a role in the endophytic activity. So yet, only a few of those genes have undergone experimental testing to determine whether they are involved in endophytic colonisation (Shen et al., 2013; Ouyabe et al., 2019). In this study, we have documented some mechanisms involved in plant endophyte interaction at the molecular level.

2 Plant growth promotion by endophytes

PGPEs enhance plant development through three interconnected mechanisms: phytostimulation, biofertilization, and biocontrol. Phytostimulation is the production of phytohormones for direct plant development (Vishwakarma et al., 2021). The amount of the plant hormone ethylene frequently declines as a result of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Cruz Barrera et al., 2020). According to numerous studies, the pea plant and the pepper plant (*Pseudomonas putida* and *Piper nigrum*, respectively) both have bacterial endophytes that release ACC deaminase to aid plant growth (Ruduś et al, 2013). By controlling ethylene levels in plants, ACC deaminize production may minimize abiotic stress because an increase in ethylene can obstruct DNA synthesis, root and shoot growth, and cell division. However, the specific method for enhanced plant development is still unknown (González Candia, 2021). Bacterial strains also produced other hormones which include abscisic acid, indole-3-acetic acid, and jasmonic acid, to stimulate plant growth (Forchetti et al., 2007).The endophytes can enhance plant growth by increasing the availability of important nutrients known as bio-fertilization.

Nitrogen fixation is the most studied phenomenon of bio-fertilization which is the conversion of atmospheric nitrogen into ammonia (Mishra and Arora, 2016). Bacterial species like *Azospirillum* spp., *Pantoea agglomerans*, and *Azoarcus* spp. all are known to be involved in a substantial amount of nitrogen fixation in plant roots (Indiragandhi et al., 2008). Nonetheless, only 21 PGPEs can increase plant phosphorus availability by solubilizing phosphate. The metal cation linked to phosphorous is chelated as a result of the release of low molecular weight acids, making it more available to plants. The researchers have isolated, identified, and assessed the ability of *Achromobacter xiloxidans* and *Bacillus pumilus* to solubilize phosphate in sunflowers (Barrera et al., 2020). PGPEs were utilized to treat corn, lowering the quantity of artificial phosphorus fertilizer required while increasing yields by up to 50% (Cruz Barrera et al., 2019).

The protection of plants from phytopathogens and their growth promotion is known as biological control. Antibiotic and siderophores production are involved in biological control mechanisms. Siderophores like pyochelin and alicyclic acid and chelate iron are not directly involved in disease control due to their competition with pathogens for trace metals (Leopold, 1964). The disease can be suppressed in plants by antimicrobial metabolites secreted by bacterial endophytes such as 2,4-diacetylphloroglucinol (DAPG). Seed treatment of eggplant (*Solanum melongena*) with DAPG-producing bacterial endophytes reduced 70% of eggplant wilt caused by *Ralstonia solanacearum* (Rana et al., 2020a).

Burkholderia, Bacillus, Pseudomonas, Enterobacter, and *Serratia* are just a few of the bacterial endophyte strains that are successful at preventing the growth of pathogenic germs in

both *in vitro* and *in vivo* settings (Khan and Doty, 2009). Aside from that, bacteria from the genera *Bacillus, Enterobacter, Arthrobacter, Azotobacter, Isolptericola, Streptomyces*, and *Pseudomonas* improved the crop's stress resistance from heat, drought, and salt (Rana et al., 2020b; Khalil et al., 2021). The most important interaction between these endophytes and symbiotic plants allowed the plants to significantly increase their biomass and height while lowering stress. Although, it is not yet clear how bacterial endophytes lessen abiotic stress (Liu et al., 2014).

2.1 Rhizobium and process of nodule formation

Rhizobium is a member of the family *Rhizobiaceae* and the class *Alphaproteobacteria*. Rhizobium, was the name given to this genus for the first time by Frank in 1889. There are 11 non-rhizobial species and 49 rhizobial species in the family *Rhizobiaceae* at the moment (Ledermann et al., 2021). The rhizobial species induce the nodules on the roots of plants (*Fabaceae* family) and are linked to symbiotic nitrogen-fixing bacteria. The nodule's nitrogen fixation activity is extremely oxygen sensitive. The host plant receives continual supplies of reduced nitrogen from the bacterial enzyme system in this symbiotic connection, and the bacteria in exchange receive nutrients and energy from the plant (Van Rhijn and Vanderleyden, 1995). Nodules can occur in about 10% of legumes. The majority of the rhizobacteria in soil are oxygen sensitive and feed on the decomposing remains of other organisms.

In roots, nitrogen-fixing bacteria occur as irregular cells known as bacteroids, which are frequently Y, club-shaped and appear as straight rods with a regular structure (Figure 1). Bacteroidsencode genes that determine the rhizobium's host specificity (Lodwig and Poole, 2003). Rhizobia that generate nodules but are unable to fix nitrogen are sometimes referred to as ineffective strains, whereas effective strains cause nitrogen fixation in nodules. Nodule development is controlled by certain genes known as nod genes i.e. nodF, nodE, nodL, nodP, nodQ,

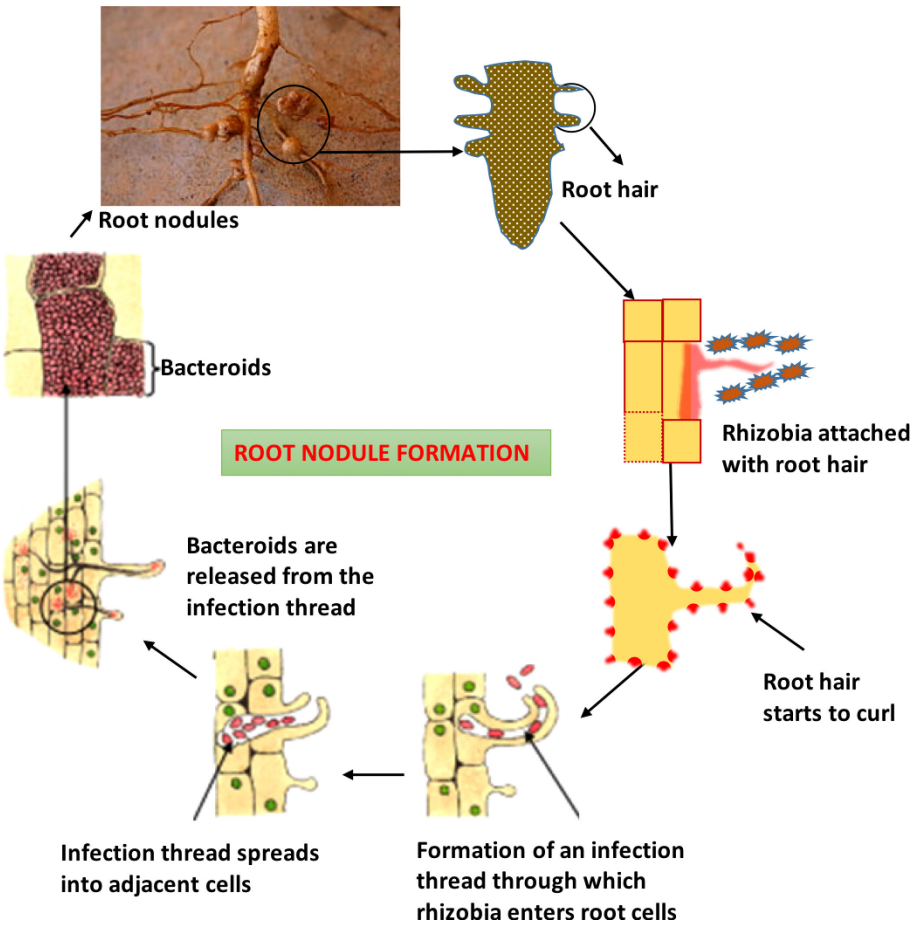

FIGURE 1
Diagrammatic representation of the whole process of nodule formation through rhizobia.

and nodH (Basile and Lepek, 2021). Some substances, such as flavonoids, are released by the root cells and trigger the production of nodules in bacteria by activating the nod gene. In essence, these chemicals are in charge of identifying the proper host and attaching to the root hairs.

The nod factors, which are secreted by bacteria, cause the root hairs to curl (Moran, 1997). The root hair tip is damaged by rhizobia, which also causes the infection thread to arise. The thread then extends to neighboring cells by thread branching, and the bacteria continue to grow within the growing network of tubes, continuing to create nod factors that encourage the growth of the root cells and ultimately result in the formation of root nodules (Oldroyd et al., 2011). Following a week of infection, nodules are visible with the unaided eye and each nodule contains thousands of living rhizobium bacteria, the majority of which are malformed and are referred to as bacteroids. Small sections of the plant cell membranes called symbiosomes, which may or may not include multiple bacteroids, are located next to bacteroids and are active sites for nitrogen fixation (Ratu et al., 2021). Through the *Nitrogenase enzyme*, also known as Nitrogenize catalysis, nitrogen gas from the atmosphere is converted inside legume nodules into ammonia, which is then assimilated into amino acids, DNA, and RNA as well as significant energy molecules like ATP or other chemicals like vitamins, flavones, and hormones (Bergersen, 1961). The Nitrogenize complex is protected by a variety of mechanisms used by aerobic free-living bacteria, including physical barriers and fast metabolic rates. Azotobacter, for instance, circumvents this issue by maintaining the lowest oxygen concentration in its cells and the greatest rate of respiration of any organism. In the instance of Rhizobium, the nodule's red iron-containing protein, similar to hemoglobin in function to bond with oxygen, maintains control over the oxygen level (Lindström and Mousavi, 2020). However, this avoids the accumulation of free oxygen to prevent the loss of Nitrogenize activity while still providing enough oxygen for the metabolic functioning of bacteriods. Rhizobia and plants work together to make leghemoglobin, something neither of them could ever do on their own. Even in poor soil with few nutrients and insufficient nitrogen to support the growth of other plants, these nodules increase crop output (Lodwig and Poole, 2003).

2.2 Spread and variation of microbes from seed to plants

Plants and their microbial diversity vary throughout their life span of plants. These factors, prompt the structure and variety of the microbial community (Honma and Shimomura, 1978). Seed-born microbes gain entry into the germinating plant and take advantage of other colonizing microbes as well as opportunistic pathogens from the surrounding soil (Glick et al., 1999; Oteino et al., 2015). Hence the overall microbial biota and population changed dramatically throughout the life cycle of plants. The important ways of entry into host plants are through root hair cells, root cracks, and wounds whereas other sources include stomata particularly of young stems and leaves; lenticels, and germinating radicles (Figure 2). Vertical seed transmission is another possible way to receive endophytic bacteria through plant host generations (Bergersen, 1961).

2.3 Presence of plant microbes in different parts of plants

Microorganisms associated with plants formed a complex network. Different studies suggested that plant-associated microbes live inside plant tissues or on the surface of plant parts such as leaves, stems, fruit, and roots (Clarholm, 1985). The microbiome studies of *A. thaliana* leaves showed that plant genotype, surrounding plants, and abiotic features affected the microbial population structure (Teixeira et al., 2013). These interactions are responsible for expediting the defense signals between plants and the efficacy of natural biological control agents (Morgan et al., 2005). Microbial populations might indirectly affect the other taxa of microbes by altering the host growth response or metabolites without direct interaction with microbes.

3 Beneficial effects of microbes on plant growth and development

Plants usually take nutrients from the soil which constitutes a pool for microscopic life forms including bacteria, fungi, actinomycetes, algae, and protozoa. So, among them, the bacteria are the most common ones and have the maximum proportion in soil. The maximum number of bacteria present in the rhizosphere near the roots of plants is different from bulk soil (Luu et al., 2020). As these bacteria are present in more concentration in the soil so the bacteria may affect a plant through three different pathways (Edwards and Harding, 2004). PGPEs can promote plant growth directly by expediting the procurement of compounds or modifying levels of plant hormones and reducing the inhibitory effect of plant growth and pathogenicity by acting as biocontrol agents (Yan et al., 2019). The benefits provided by the endophytes to the host plants and their mechanisms are described in (Table 1).

4 Role of PGPEs against biotic stress

Throughout their lives, plants are exposed to harmful abiotic and biotic stresses. The damage that bacteria, fungi, viruses, nematodes, viroids, and insects do to plants is referred to as "biotic stress." Rhizobacteria that promote plant growth by generating phytohormones or facilitating the uptake of particular nutrients might affect plant growth through biotic stress (Tiwari et al., 2020). However, PGPR reduces or even

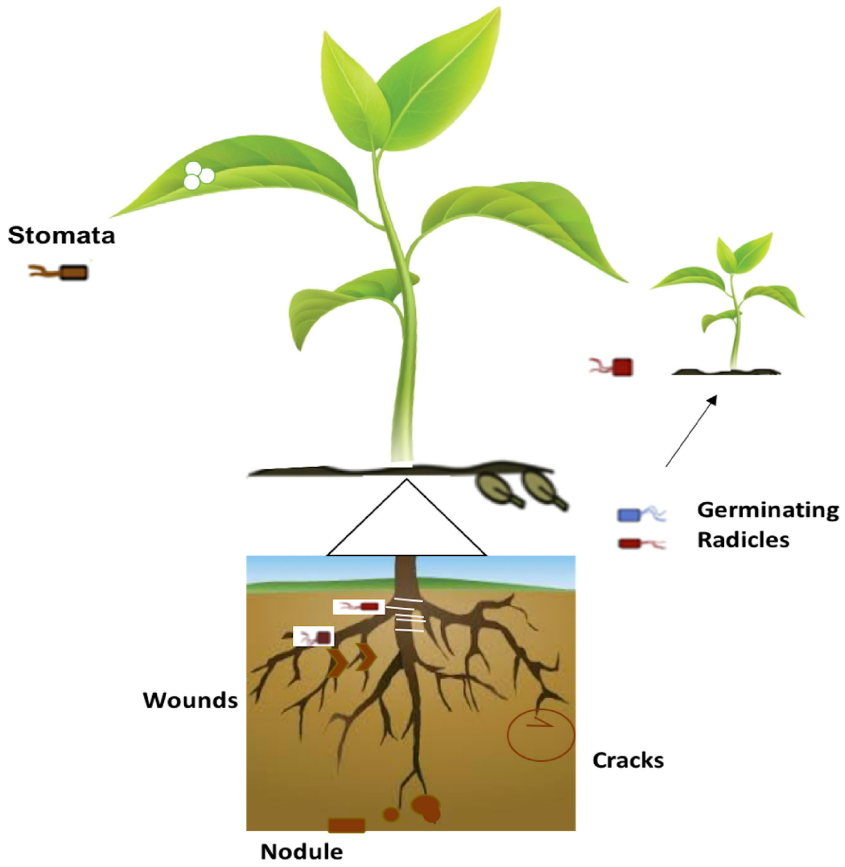

FIGURE 2
Overview of the endophytic bacterial mode of entry into different plant tissues.

eliminate the negative impacts of plant pathogens. For example, *Pseudomonas fluorescens* produces 2,4-Diacetyl Phloroglucinol, which inhibits the development of pathogenic fungi in plants (Suslow and Schroth, 1982). Chitinase and laminarinase, two extracellular enzymes generated by *P. stutzeri*, caused the lysis of *Fusarium solani* mycelia and root rot (Cano-Salazar et al., 2011). During a seven-month field trial, the endophytic *B. cenocepacia* reduced the prevalence of fusarium wilt disease in banana plants by 3.4%, compared to 24.5% in untreated infected plants (Sapak et al., 2008). The antibiotic Pyrrolnitrin, which helps to reduce cotton damping off losses brought on by *Rhizoctonia solani*, was developed by several endophytic *Pseudomonas fluorescens* strains (Timper et al., 2009). *Fusarium oxysporum*, which was used as a bio-agent to create resistance in tomato plants, was successfully protected against *P. fluorescens* in flowering plants (Dudai, 2011). A bacteria that inhabit plant roots called *Bacillus amyloliquefaciens* has the power to control plant diseases and promote plant growth (Vardi et al., 2021).

In a study, it was discovered that bacterial endophytes shield cucumber plants from the cucumber anthracnose produced by *Pseudomonas fluorescents* (Akköprü et al., 2021). It was once believed that *Achromobacter* sp., *Streptomyces* sp., and *Bacillus licheniformis* were responsible for the foliar disease known as downy mildew. The downy mildew disease infestation level was lowered by *Pseudoperonospora cubensis* (Basu et al., 2022), which ultimately resulted in an increased yield.

The management of pests, which has become a challenge for most crops since pests have evolved a tolerance to pesticides, is another use for these endophytic bacteria (Deng et al., 2014). Entomopathogenic bacteria have been used to combat pests that are immune to insecticides (Figure 3). A few fungi from the genera *Podonectria, Verticillium, Hirsutella, Sphaerostilbe, Agerata, Metarhizium Aschersonia*, and *Myriangium* are used for the biological management of pests. *Brevibacillus laterosporus* is effective against nematodes, Lepidoptera, Coleoptera, and toxic fungi in plants in addition to insects (Skinner et al., 2014).

5 Identification of endophytic bacteria interaction with Host

In recent years, next-generation sequencing (NGS) techniques have been utilized to study the whole population of

TABLE 1 Examples of plant growth-promoting rhizobacteria tested for various crop types.

PGPR	Plant	Benefits to plant growth	References
Pseudomonas sp.	Green gram	Increased plant dry weight, number of nodules, total chlorophyll content, root/shoot N, P seed protein, and yield.	(Del Carmen Orozco-Mosqueda et al, 2020)
	Soybean Wheat	Increased soil enzyme activity, nutrient absorption, and yield	(Kalyani et al., 2008)
	Chickpea	An enhanced fresh and dry weight of plants	(Berendsen et al., 2012)
	Rice	More ability to control fungal and bacterial pathogens	(Bulgarelli et al., 2012)
	Canola	Encouraged growth and cadmium accumulation in plants	(Agler et al., 2016)
	Mustard	Improved growth and reduced Cr contents among plants	(Foster, 1988)
	Soybean, mung bean, wheat	Promotes growth of plants	(Bertin et al., 2003)
Pseudomonas putida	Mung bean	The ethylene production repressed in treated plant Increase the growth and decreases Pb and Cd uptake	(Glick, 2012) (Ahemad and Khan, 2012)
	Lectuca	Enhancement of shoot/root length attained through concentrated inoculants	(Sharma et al., 2011)
	Artichoke	PSB along with N fixers increase in shoot length/weight, germination percentage seedling vigor, and reduction in germination time	(Tank and Saraf, 2010)
Pseudomonas aeruginosa	Maize	Endorsed plant growth and helped soil metal utilization, increase Pb and Cr uptake	(Lawongsa et al., 2008)
	Black gram	Reduced Cd deposition in tissues, widespread rooting, and increased plant growth	(Wu et al., 2015)
	Indian mustard and pumpkin	Increased in plant growth, decrease in Cd uptake	(Rajkumar et al., 2006)
	Tomato, Okra, African spinach	Increase in Dry weight of tomato, okra, and spinach	(Gupta et al., 2002)
Pseudomonas fluorescens	Alfalfa	Enhanced Fe and Cu movement from root/shoot	(Mayak et al., 1999)
	Peanut	Increase in pod yield and nodule dry weight	(Lobo et al., 2019)
	Soybean	Increased plant growth	(Rekha et al., 2007)
	Canola	Protect plants against the inhibitory effects of Cd	(Jahanian et al., 2012)
	Maize	Increase of plant growth, height, seed weight, no. of seed/ear, leaf area, shoot dry weight	(Curá et al., 2017)
Azospirillum amazonense	Rice	Grain dry matter deposition, panicle count, and nitrogen buildup at the grain maturity stage all increase	(Sant'anna et al, 2011)
Azospirillum brasilense	Common bean	Increase of Root growth in plants	(Adesemoye et al, 2008)
Azospirillum lipoferum	Cotton	An increase in soil microorganisms, plant height, and seed production was observed, but no changes in boll weight or staple length.	(Fayez and Daw, 1987)
Azotobacter chroococcum	Chinese mustard	Increased plant development and metal toxicity protection for the plant	(Jha, 2017)
Azospirillum brasilense	Rice	Increased grain yield	(Gupta et al., 2005)
Kluyvera ascorbate	Mustard, Tomato Canola,	Heavy metals reduce plant growth but do not boost metal uptake.	(Safronova et al., 2006)
Bradyrhizobium	Green gram	The development traits at all of the studied pesticide dosages (quizalafop-p-ethyl and clodinafop)	(Wani et al., 2007)
	Soybean and yellow Lupin	Increased biomass, nitrogen content, deposition of metals	(Dell'amico et al, 2008)

(Continued)

TABLE 1 Continued

PGPR	Plant	Benefits to plant growth	References
	Green gram	Increase of nodule number, seed yield, grain protein, root/shoot N at 290 mg Ni/kg soil	(Burd et al., 2000)
Brevundimonas	Canola	Isolated cadmium directly from the solution	(Gholami et al., 2009)
Enterobacter cloacae	Canola	Significant increases in root and shoot length were observed.	(Bashan and González, 1999)
Klebsiella oxytoca	Maize	Increase of plant growth parameters	(Remans et al., 2008)
Enterobacter sakazakii			
Brevibacillus	White clover	Increased plant growth and nutrition and decreased zinc conc.	Anjum et al., 2007)
Methylobacterium oryzae, Berkholderia sp.	Tomato	Significant increase in shoot/root length attained through bacterial cells inoculation	(Wu et al., 2006)
Sinorhizobium sp.	Brown mustard	Increased the efficacy of Pb	(Thakuria et al., 2004)
Bacillus spp	Barley	Increased root/shoot weight	(Dary et al., 2010)
Rhizobium sp.	Pea	Increase of the dry matter, nodule numbers, root/shoot nitrogen	(Lugtenberg and Kamilova, 2009)
Mycobacterium sp.	Canola	Prevent plant against the inhibitory effects of cadmium	(Wani et al., 2008)
Bacillus sp. *Paenibacillus* sp.	Rice	Considerably encouraged the root/shoot growth.	(Robinson et al., 2001)

cultivable or non-cultivable bacteria inside plants, as well as their genomes. The interaction of host and bacterial endophytes has insightful concerns for the biological functioning of plants. As a result of interactions, rapid changes in host phenotype occurs also it is assumed as a driving force for the speciation and co-evolution of both the genetic system of host and bacteria (Fawcett, 1944). Though old genetic techniques to study plant-microbe interaction are less efficient, time-consuming, costly, and labor-intensive required a wide range of experiments and are usually limited to certain known genes (De Oliveira et al., 2004) in comparison to investigating the host-microbe interactions in molecular levels, it is needed to understand the phenotypic phenomena and genomics in depth. So the development of NGS technologies or metagenomic studies has provided the best way to understand the host-pathogen system. Through this technology, we can construct genome models of different organisms, which includes strains, their natural populations over time and their evolutionary histories (Navas et al., 2017; Sharma et al., 2021).

These complicated interactions can be analyzed and integrated by viewing plant microbiomes as a system. To better understand endophytism, contemporary genomic investigations incorporating metaomics and comparative studies can be quite beneficial (Dubey et al., 2020). A better understanding of endophyte interactions could be used to improve agricultural management by increasing plant development, biocontrol, and bioremediation (Alaimo et al., 2018). Some of the tools being utilized or that could be used to understand the link between plants and endophytes include genome sequencing, comparative genomics, microarray, next-generation sequencing, metagenomics, and metatranscriptomics (Dixit et al., 2022). To study endophytes and their apparent function in host plant ecology, contemporary methods and approaches need to be investigated (Gaiero et al., 2013).

Another way to identify the endophytic bacteria interact with the plant is to isolate the endophytic bacteria culture and then classify based on its phenotypic traits, and a few isolates from each category are identified further through partial sequencing of the 16S rRNA gene (Khare et al., 2018). The results of partial sequencing show that the isolates belonged to the genera *Pseudomonas*, *Stenotrophomonas, Bacillus, Pantoea*, and *Serratia* of bacteria (Liu et al, 2017>2552). These isolates are tested for their ability to produce siderophores, phosphate solubilization, atmospheric nitrogen fixation, protease, and hydrogen cyanide, as well as phytohormones like auxin and gibberellin (Eid et al., 2019). Auxin and gibberellin, two plant growth hormones, can be produced by all strains, though to varying degrees. Almost all strains could solubilize phosphate (Lata et al., 2019). The outcomes of protease, siderophore, and atmospheric nitrogen-fixing ability vary between strains. These findings provide information on the relationship between endophytic bacteria and their host plant (Vandana et al., 2021).

Furthermost genomic methods require recognition of variations among sequences within species or populations, like point mutations, Addition/deletions, and structural variations in structures (Bulgarelli et al., 2013).

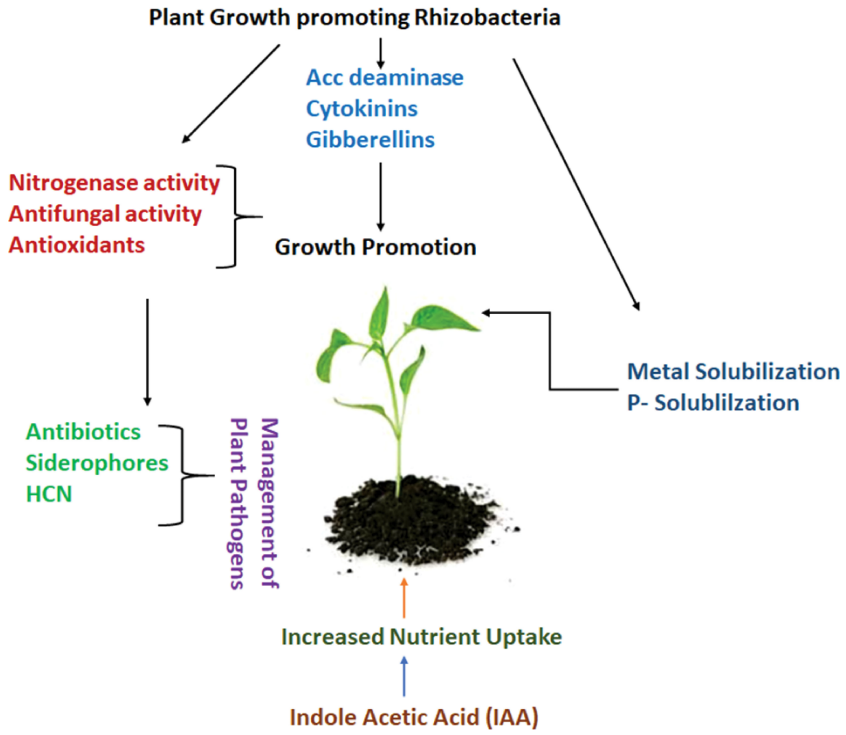

FIGURE 3
Mechanism of plant growth promotion by rhizobacteria (PGPR).

5.1 Evolution of new pathogenic strains of microbes

One of the great evolutionary changes in life is the development of advantageous symbioses between eukaryotic (plants) and prokaryotic creatures (Chebotar et al., 2015). According to certain theories, the relationship between endophytic bacteria and plants frequently depends on two fundamental elements: currency and a system for exchanging currency. The currency could be, for instance, a root exudate that bacteria can take up in the context of interactions between plants and endophytic bacteria (Mercado-Blanco and JJ Lugtenberg, 2014). Similarly, bacteria may release hormones that encourage plant growth, such as auxin and gibberellins, which may be favorable for plant growth (Maksimov et al., 2018). It is anticipated that selection will favor the evolution of mutualism when the exchange of currencies between the two parties is balanced. Therefore, it is hypothesized that increased mutualistic dependency develops through reciprocal co-evolution or adaptation by one of the partners through the selection of features directly related to the mutualistic interaction (Chen et al., 2021).

Competition for scarce shared resources like iron may also lead to asymmetrical currency exchange, which could help to explain why some plant-microbe interactions are hostile (Hong and Park, 2016). Furthermore, because the rhizosphere is open, the free diffusion of resources derived from plants may promote higher levels of cheating in which mutant bacterial genotypes take benefit of "public goods" without producing substances that aid plant growth (Pandey et al., 2017). Because of this, mutualistic plant-microbe interactions may need additional enforcement from the plant, such as penalizing dishonest bacterial genotypes or positively identifying genotypes that promote plant growth (Ryan et al., 2008).Intriguing research would also be done to see whether endophytic bacteria and plants may coevolve from first neutral interaction and whether plants can coevolve in response to rhizosphere bacteria (Santos et al., 2018). In conclusion, by showing that plant-associated bacteria can quickly evolve along the symbiotic connection within a few growth cycles, our results urge eco-evolutionary management of endophytic bacteria and plants interactions in agriculture (Aswani et al., 2020).

5.2 Endophytic bacteria in disease management

Crop productivity is impacted by a number of common plant diseases that are present worldwide. Some of the serious ones are wilt disease, root rot, powdery mildew, leaf spot, leaf curl, and blight. To counter these phytopathogens, endophytic bacteria are crucial (Latha et al., 2019).

By producing proteins associated with pathogenesis (PRPs) and defense enzymes that stop the growth of phytopathogens that cause disease, endophytic bacteria can produce siderophores, antimicrobial compounds, and systemic resistance (Pandey et al., 2019). Bacterial endophytes are also potentially useful biocontrol agents. Plant diseases degrade plant performance and crop quality, which reduces crop output (Muthukumar et al., 2017). It has been shown that the nitrogen-fixing bacteria *Azotobacter chrococcum*, the phosphate-solubilizing bacteria PSB (*Pseudomonas cepacia*), the endophytic bacterial strains *Lysinibacillus* sp. and *Bacillus subtilis*, and their combination as bio-fertilizers can reduce the incidence of bacterial wilt disease in chili plants by up to 80% (Tewari et al., 2019).

The endophytic bacterial strain *B. subtilis* showed the strongest (80%) illness suppression (Jacob et al., 2020). This endophyte could also considerably aid the growth of the chili. Chemical pesticides are typically used to manage such phytopathogens, but this tactic has raised concerns about environmental contamination and contributed to the emergence of resistance to specific chemicals over time (Prasad et al., 2020). New insecticides must always be developed to address this. Chemical pesticides are thought to be ineffective when compared to endophytic bacteria acting as biocontrol agents or bioinsecticides. A broad array of mechanisms, including direct antagonism *via* the generation of antibiotics, siderophores, hydrogen cyanide, hydrolytic enzymes (chitinases, proteases, and lipases), etc., are involved in the biocontrol of plant diseases (Puri et al., 2017).

6 Conclusion

Some of the bacterial endophytes or PGPR are commonly used to control different diseases and as biological control agents so nowadays most of the focus is the understanding of complex interactions and their mechanisms and outcome either beneficial or harmful. It is hard to find the exact mechanism of interaction among complex microbial populations residing in the soil and environment near to host. So that proper characterization and management strategies can be devised according to the current need of time. In recent time peoples are preferring organic food and disliked the use of fertilizers and chemicals in agriculture. As the world population is increasing and food shortage issues are raised, in the current situation food security is an important topic for debate. Hence bacterial endophytes can be used as an alternative to chemical fertilizers, nutrient sources, and biological control agents for various plant pathogens. Scientists are focusing on the use of these endophytes in the form of biopesticides, and biofertilizers with different trade names for the control of different diseases and sustainable agricultural systems. Although the application of these endophytes in combination may lead to the development of optimum PGPEs inoculants that robust, and slight variation of environmental factors will not affect the plant growth promotion.

Author contributions

SM, MN, MH, MS, and MA conceived and conceptualized the study. MAS, AS, MB provided materials and technical assistance. SM, MS wrote original draft. SS, MSH, MS and MT technically reviewed and finalized the draft. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors are thankful to the University of the Punjab Lahore, Pakistan for providing the facilities for study.

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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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 01 February 2023
DOI 10.3389/fpls.2023.1118698

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to
Plant Symbiotic Interactions,
a section of the journal
Frontiers in Plant Science

RECEIVED 07 December 2022
ACCEPTED 20 January 2023
PUBLISHED 01 February 2023

CITATION
Pereira EC, Zabalgogeazcoa I, Arellano JB,
Ugalde U and Vázquez de Aldana BR
(2023) *Diaporthe atlantica* enhances
tomato drought tolerance by improving
photosynthesis, nutrient uptake and
enzymatic antioxidant response.
Front. Plant Sci. 14:1118698.
doi: 10.3389/fpls.2023.1118698

Diaporthe atlantica enhances tomato drought tolerance by improving photosynthesis, nutrient uptake and enzymatic antioxidant response

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Functional symbiosis with fungal endophytes can help plants adapt to environmental stress. *Diaporthe atlantica* is one of the most abundant fungal taxa associated with roots of *Festuca rubra* subsp. *pruinosa*, a grass growing in sea cliffs. This study aimed to investigate the ability of a strain of this fungus to ameliorate the impact of drought stress on tomato plants. In a greenhouse experiment, tomato plants were inoculated with *Diaporthe atlantica* strain EB4 and exposed to two alternative water regimes: well-watered and drought stress. Several physiological and biochemical plant parameters were evaluated. Inoculation with *Diaporthe* promoted plant growth in both water treatments. A significant interactive effect of *Diaporthe*-inoculation and water-regime showed that symbiotic plants had higher photosynthetic capacity, water-use efficiency, nutrient uptake (N, P, K, Fe and Zn), and proline content under drought stress, but not under well-watered conditions. In addition, *Diaporthe* improved the enzymatic antioxidant response of plants under drought, through an induced mechanism, in which catalase activity was modulated and conferred protection against reactive oxygen species generation during stress. The results support that *Diaporthe atlantica* plays a positive role in the modulation of tomato plant responses to drought stress by combining various processes such as improving photosynthetic capacity, nutrient uptake, enzymatic antioxidant response and osmo-protectant accumulation. Thus, drought stress in tomato can be enhanced with symbiotic fungi.

KEYWORDS

symbiosis, *diaporthe*, drought stress, fungi, antioxidant defense, nutrient uptake, proline, photosynthetic capacity

1 Introduction

Drought is a multidimensional stress that causes a wide range of morphophysiological, biochemical and molecular modifications on plants, affecting their growth and development (Farooq et al., 2009; Chaves et al., 2011). At a cellular scale, a series of harmful perturbations in some central processes occur, including disorders in water homeostasis, perturbations in metabolic functions and hormonal imbalance. In addition, changes in chlorophyll synthesis, root differentiation, foliage development, stomatal movement, and water and mineral nutrition occur, leading to a decrease in plant yield and water use efficiency (Kapoor et al., 2020; Kaur et al., 2021). Drought also induces the generation of reactive oxygen species (ROS), which cause oxidative damage and disturb the cell redox regulatory functioning (Cruz de Carvalho, 2008; Impa et al., 2012).

To cope with water deficit, plants have developed mechanisms to capture more water from the soil or to minimize water loss *via* transpiration (Osakabe et al., 2014; Takahashi et al., 2020). Morphological changes such as an increase in root size for better exploring the soil and increasing surface absorption can occur (Hund et al., 2009). In response to drought stress, the stomatal closure reduces transpirational water loss, but also causes a decrease in both CO_2 diffusion and photosynthetic carbon assimilation rate (Shahzad et al., 2016). The production of compatible organic solutes, such as proline, is another important mechanism to adapt to water deficit, contributing to osmotic adjustment, ROS detoxification, and stabilization of membrane, enzyme and protein structures (Farooq et al., 2009; Takahashi et al., 2020). In order to cope with oxidative stress under drought, plants also use antioxidant defense systems (Shahzad et al., 2016). The antioxidant apparatus helps to scavenge reactive oxygen species (ROS) and to regenerate ascorbate (AsA) using enzymatic antioxidants like catalase (CAT), ascorbate peroxidase (APX) or dehydroascorbate reductase (DHAR) (Koffler et al., 2014; Noctor et al., 2014; Laxa et al., 2019).

Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural crops in the world. Its high sensitivity to water deficit has prompted different approaches for obtaining drought-resistant cultivars. The plant microbiome can have an important role in plant growth and stress tolerance, having applications related to crop production (Ray et al., 2020; Pozo et al., 2021).

Diaporthe is one of the most abundant fungal taxa associated with roots of *Festuca rubra* subsp. *pruinosa*, a grass growing in sea cliffs (Pereira et al., 2019). In this habitat, *F. rubra* grows in rock fissures where nutrient availability is scarce, and exposure to salinity is intense (Castroviejo, 2021). When inoculated in agricultural grasses, a *Diaporthe* strain ameliorated salt stress, increasing proline, nutrient uptake, and phytohormones, resulting in plant growth improvement (Toghueo et al., 2022). That fungal strain belongs to *Diaporthe atlantica*, a dominant species of the genus in *Festuca* roots (Toghueo et al., 2023). Symbiotic microorganisms from saline environments might benefit plants in their adaptation to drought stress (Rodriguez et al., 2008; Hosseyni Moghaddam et al., 2021). Plant responses to drought and salinity have much in common because both conditions induce osmotic stress and oxidative damage in an early stage, which leads to a decrease in growth, stomatal aperture, and a deficit in nutrients (Forni et al., 2017; Ma et al., 2020). Therefore, plant adaptation to both stresses could be mediated by similar mechanisms involving plant responses such as growth attenuation, accumulation of compatible solutes as proline, increased levels of antioxidants and protective proteins, suppression of energy-consuming pathways and gene expression regulation (Bartels and Sunkar, 2005; Munns, 2011).

Thus, the main objective of this work was to evaluate the ability of a *Diaporthe atlantica* strain isolated from *Festuca rubra* subsp. *pruinosa* to improve the growth and drought tolerance of tomato plants. For this purpose, the changes of tomato plants in physiological and biochemical parameters such as chlorophyll, gas exchange, mineral elements, proline, antioxidant enzyme activities and antioxidant capacity were evaluated.

2 Materials and methods

2.1 Fungal material

The *Diaporthe* strain EB4 was originally isolated from surface-disinfected roots of an asymptomatic plant of *Festuca rubra* subsp. *pruinosa*, collected in a natural population on the northern coast of Galicia, Spain (Pereira et al., 2019). This strain belongs to *Diaporthe atlantica*, a newly described species (Toghueo et al., 2023).

Most *Diaporthe atlantica* strains, including EB4, do not sporulate on laboratory media (Toghueo et al., 2023), for this reason, fungal mycelium was used as inoculum. To produce *Diaporthe* EB4 mycelial inoculum, 30 g of sugar beet pulp pellet mixed with 9.0 g $CaCO_3$, 4.5 g $CaSO_4$ and hydrated with 60 ml of water were autoclaved in wide-mouth glass bottles for 30 minutes at 121°C (Vázquez de Aldana et al., 2020). Each bottle of sugar beet pulp substrate was inoculated with four plugs of mycelium from a potato dextrose agar (PDA) culture and incubated at room temperature (20-22°C) for four weeks.

2.2 Experimental design

To determine the effect of *Diaporthe* inoculation on tomato plants under drought stress, a bioassay with two variables was designed: *Diaporthe* inoculation (inoculated or uninoculated plants) and water treatment (well-watered and drought stress). For each of the four treatments, ten replicates were considered. To inoculate plants, seeds of tomato cv. Marmande were sown in a plastic tray containing a substrate composed of seven parts of peat and perlite (1:1 v/v), previously treated at 80°C for 24 h, and one part of *Diaporthe* EB4 inoculum. Uninoculated plants were obtained from seeds sown in a tray containing only the peat and perlite mixture. Ten-day-old seedlings were individually transplanted to 300-ml plastic plots containing the heat-treated substrate with or without inoculum for the inoculated and uninoculated seedlings, respectively.

During the first week, all plants were exposed to a well-watered regime. After this period of adaptation, two watering treatments were applied for five weeks: a well-watered, and a drought stress regime. In the well-watered regime, plants were watered three times per week at 100% of the water holding capacity. In the drought stress treatment, plants were watered three times per week at 10% of the water holding

capacity of the soil. To avoid plant death under drought stress, these plants were watered once at 100% of the water holding capacity three weeks after the drought treatment was initiated.

Five weeks after the start of the watering treatment, all plants were harvested. Three leaves from the same branch were collected from each plant and immediately immersed in liquid nitrogen and kept at −80°C for antioxidant enzyme analysis. Then, each plant was separated into leaves, stems, and roots and lyophilized to measure dry weight and for chemical analyses.

2.3 Detection of *Diaporthe* in inoculated plants

The presence of *Diaporthe* in inoculated plants was diagnosed by light microscopy in root samples collected at harvest time. Fresh root fragments were cleared in 5% KOH at 90°C for 15 min, neutralized with approximately three volumes of 1% HCl at 20°C overnight, stained with trypan blue (Berthelot et al., 2016), and visualized.

2.4 Measurements of plant physiological and biochemical parameters

2.4.1 Photosynthetic parameters

The chlorophyll content was determined 24 h before plant harvesting by means of a leaf-clip sensor (Dualex Force, Orsay, France). In each plant, three leaves of the third branch from the top were selected, and the average chlorophyll content was obtained from three measurements taken at the central position of each leaf.

The gas exchange measurements at 400 ppm CO_2, including stomatal conductance, CO_2 assimilation rate, and water use efficiency (WUE) were obtained from leaves of the third branch from the top of four randomly replicate plants per treatment, making use of a CIRAS-3 portable gas exchange system (PP-Systems, Amesbury, MA, USA) 24 h before plant harvesting. The leaves were pressed between the upper and lower gaskets of the leaf cuvette head of CIRAS-3 and pre-acclimated for 15–20 min.

2.4.2 Analysis of mineral element content

The concentration of mineral elements (N, P, K, Ca, Fe, S and Zn) was analyzed in five replicates of leaf samples. For that purpose, freeze-dried and ground samples were calcined at 450°C for 8 h, and ashes were dissolved in HCl : HNO_3:H_2O (1:1:8). Then, P, K, Ca, Fe, S and Zn contents were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES) in a Varian 720-ES spectrometer (Agilent, USA). Carbon and Nitrogen contents were analyzed by the Dumas combustion method in a C-N analyzer (Leco CHN-628, USA).

2.4.3 Antioxidant enzyme determination

At harvest time, the third leaf from three different branches of the same plant were pooled for antioxidant enzyme activity assays. Samples of fresh leaves previously stored at −80°C were ground with liquid nitrogen and kept at −80°C until the measurement of the antioxidant enzyme activities. The antioxidant activities of catalase (CAT), ascorbate peroxidase (APX), and dehydroascorbate reductase (DHAR) were measured in leaf samples of four plant replicates per treatment following the methods described below by Bendou et al. (2022) and Pérez-López et al. (2009). APX was selected as a representative peroxidase activity enzyme because it belongs to the ascorbate-glutathione cycle, it is very sensitive to stress conditions, and it is well established that APX also regulates redox signaling pathways in normal plant development (Caverzan et al., 2012). A 96-well microplate reader FLUOstart® Omega (BMG Labtech, Ostenberg, Germany) was used for all the spectrophotometric methods.

For CAT activity, 40 mg of the ground samples were mixed with 0.5 ml of 50 mM Tris-HCl (pH= 7.8), 0.1 mM EDTA, 0.2% (v/v) Triton X–100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol and beaten with glass beads for 1 min. The homogenates were filtered through a layer of muslin and gel-filtered over MicroSpin G25 columns (Amersham Biosciences, Sweden) equilibrated with 50 mM Tris–HCl (pH= 7.8), 0.1 mM EDTA and 0.2% (v/v) Triton X–100. CAT activity was measured spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm in a reaction mixture of a final volume of 300 µl containing 50 mM potassium phosphate buffer (pH= 7.0), 25 mM H_2O_2 and 5 µl of the filtered supernatant.

The homogenizing medium for DHAR analysis consisted of 50 mM potassium phosphate (pH= 7.8), 0.1 mM EDTA, 0.2% (v/v) Triton X–100, 2 mM AsA, 5 mM cysteine, 0.1 mM PMSF and 1% (w/v) poly(vinylpolypyrrolidone). An amount of 40 mg of ground samples were incubated with 0.5 ml of the homogenizing buffer for 10 min at 6–8°C, filtered through a layer of muslin and centrifuged at 16,100 *g* for 15 min. DHAR activity was determined by monitoring AsA formation *via* dehydroascorbate (DHA) reduction at 265 nm. Briefly, the final volume of the assay mixture was 300 µl, and contained 2.5 mM glutathione (GSH), 0.1 mM EDTA, 50 mM potassium phosphate (pH= 6.6) and 10 µl of supernatant. The reaction was initiated by adding 10 µl of 0.2 mM DHA to the reaction mixture. The reaction rate was corrected for the non-enzymatic reduction of DHA by GSH.

For the APX activity, the ground samples were homogenized as in the previous paragraph. APX activity was analyzed by measuring the oxidation of AsA at 290 nm. Briefly, a volume of 290 µl of reaction mixture containing 0.8 mM AsA and 50 mM HEPES (pH= 7.6) was mixed with 10 µl of the supernatant. The oxidation rate of AsA measured as the decline in absorbance at 290 nm was estimated 1–6 min after starting the reaction with the addition of H_2O_2 at a final concentration of 1.2 mM. Corrections were made for the non-enzymatic oxidation of ascorbate by H_2O_2 and for the oxidation of ascorbate in the absence of H_2O_2.

The measurement of the CAT, APX and DHAR activities were carried out 25°C and protein content in the supernatant was measured according to the Bradford method (Bradford, 1976).

2.4.4 Ferric reducing antioxidant potential assay

The total antioxidant capacity was determined in leaves of five replicates of each treatment using the ferric ion reducing antioxidant power (FRAP) method (Benzie and Strain, 1996). This method is based on the reduction of the colorless [Fe(III)–,4,6-tri(2-pyridyl)-*s*-triazine)$_2$]$^{3+}$ complex, abbreviated as Fe(III)-TPTZ, to the blue-

colored Fe(II)-TPTZ complex, formed by the action of electron donating antioxidants at low pH. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH=3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20.35 mM $FeCl_3$ at a ratio of 10:1:1 (v/v/v). Five mg of each plant sample were extracted in 700 µl of 50% aqueous acetone for 30 min in an ultrasound bath at 8°C. The mixture was centrifuged and transferred to a 96-well plate where 8 µl of the sample, 8 µl of phosphate buffer saline, and 200 µl of FRAP reagent were added to each well. The absorbance was measured at 593 nm after 30-min incubation in a microplate reader FLUOStar Omega (BMG Labtech, Ostenberg, Germany). A standard curve was prepared using different concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The results were expressed as µmol trolox equivalent/g dry weight.

2.4.5 Total phenolic compounds content

The content of total phenolic compounds in leaf samples (five replicates of each treatment) was determined spectrophotometrically according to the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007). An aliquot of 100 µl of 50% aqueous acetone extract of each sample, prepared as previously described for the FRAP assay was mixed with 500 µl of Folin-Ciocalteu reagent (Scharlab Chemie S.A.). After 5 min, a volume of 400 µl of a 700 mM Na_2CO_3 solution was added. The mixture was incubated for 60 min and the absorbance at 765 nm was measured in a 96-well plate in a microplate reader FLUOStar Omega (BMG Labtech, Ostenberg, Germany). Gallic acid was used as a reference standard, and the results were expressed as µmol gallic acid equivalent/g dry weight.

2.4.6 Proline content

Proline content was quantified in leaves of five plant replicates per treatment using the spectrophotometric method described by Shabnam et al. (2016), adapted to 96-well plates in our laboratory. Approximately 15 mg of freeze-dried and ground plant material were homogenized in 500 µl of 3% aqueous sulfosalicylic acid and kept for 10 min in ice. The mixture was centrifuged at 10°C and 16,000 *g* for 10 min and the supernatant was mixed with 250 µl of glacial acetic and 500 µl of ninhydrin reagent. Then, the mixture was heated at 99°C for 40 min and immediately cooled with ice. The mixture was centrifuged and an aliquot of 200 µl was transferred to a 96-well plate where the absorbance was measured at 513 nm in a microplate reader FLUOStar Omega (BMG Labtech, Ostenberg, Germany). L-proline (Acrós Organics) was used as a standard for quantification.

2.5 Statistical analyses

The data were evaluated for statistical assumptions of the ANOVA using the Shapiro-Wilk normality test and Levene´s equal variance test. The effect of *Diaporthe* inoculation and water treatment on plant parameters were analyzed with a two-way ANOVA. Differences between treatment means were evaluated by Tukey's test. All the statistical analyses were performed by means of SigmaPlot 14.5.

3 Results

3.1 Detection of *Diaporthe* in inoculated plants

Fungal structures were not observed by light microscopy in the roots of inoculated plants. Therefore, it appears that the association of *Diaporthe* EB4 with tomato plants may be rhizospheric and not endophytic.

No visual disease symptoms were observed on roots or leaves of plants inoculated with *Diaporthe*, regardless of the water regime. This indicates that this *Diaporthe* strain is not pathogenic to tomato plants.

3.2 Effect of *Diaporthe* and water regime on plant biomass production

In terms of dry weight, both inoculation and water treatment significantly affected the shoot growth of tomato plants. However, the interaction of both factors was not significant (Figure 1; Table 1). The shoot biomass increased in inoculated plants regardless of drought stress. Compared to uninoculated plants, *Diaporthe* increased the shoot biomass by 45% in well-watered plants, and by 80% under drought. Compared to the well-watered treatment, drought significantly reduced the shoot biomass by 58% (Figures 1A, B).

For the root biomass, a significant effect of inoculation, water treatment, and their interaction was detected (Figure 1C; Table 1). The root biomass increased in inoculated compared to uninoculated plants in the well-watered treatment (33%), but this difference was not significant under drought stress (Figure 1C). The root:shoot ratio increased in uninoculated repect to inoculated plants under drought, but the difference in the well-watered treatment was not significant (Figure 1D).

3.3 Effect of *Diaporthe* and water regime on photosynthesis activity and WUE

A significant effect of *Diaporthe*, water treatment, and their interaction was detected on the chlorophyll content (Table 1). Compared to uninoculated plants, the chlorophyll content increased significantly with *Diaporthe* inoculation, and this increase was larger under drought stress than in well-watered plants (Figure 2A). The inoculation with *Diaporthe* significantly increased the stomatal conductance regardless of the water regime (Figure 2B; Table 1).

A significant effect of *Diaporthe* and its interaction with water treatment was detected on the CO_2 assimilation rate (Table 1). Compared to uninoculated, this parameter increased in inoculated plants under drought stress, but the difference in well-watered plants was not significant (Figure 2C). In parallel to these results, the WUE increased in inoculated plants compared to uninoculated under drought stress, but such a difference was not significant in well-watered plants (Figure 2D).

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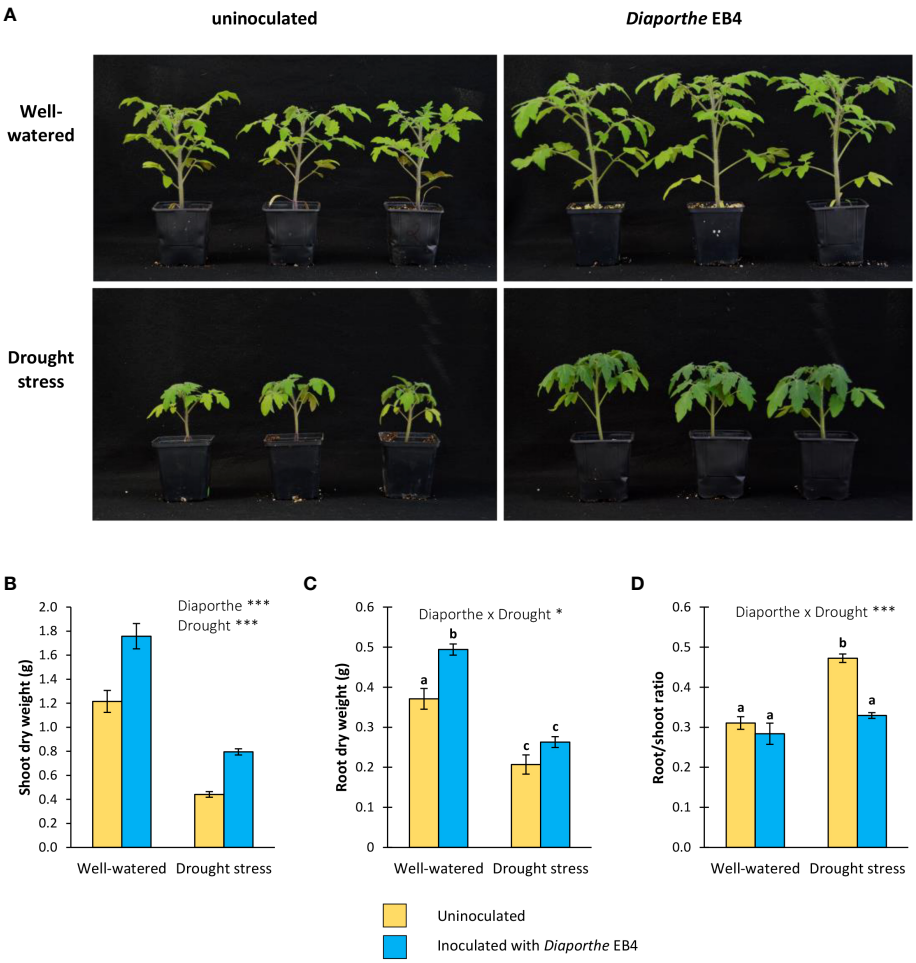

FIGURE 1
(A) Six-week-old tomato plants uninoculated (yellow) or inoculated with *Diaporthe* strain EB4 (blue), with two different water treatments (well-watered or drought stress), **(B)** shoot biomass, **(C)** root biomass, and **(D)** root/shoot ratio. Different letters indicate different means (Tukey $p<0.05$) for the [*Diaporthe* inoculation × Drought] interaction. Values are means +SE (n=10). Level of significance: $*p < 0.05$; $***p < 0.001$.

3.4 Effect of *Diaporthe* and water regime on mineral elements content

The N, P, K, Fe, S and Zn content was significantly affected by the *Diaporthe* × water treatment interaction (Table 1). Compared to uninoculated, the concentration of N, P, K, Fe and Zn increased significantly in inoculated plants under drought stress, but differences in the well-watered treatment were not significant (Figure 3). The S content increased due to *Diaporthe* in both well-watered and drought treatments (Figure 3F). The Ca concentration was only significantly affected by *Diaporthe* inoculation, increasing in inoculated plants regardless of water regime (Figure 3D). The total C content was not significantly affected by any factor or their interaction (Figure 3H, Table 1).

3.5 Effect of *Diaporthe* and water regime on biochemical plant parameters

3.5.1 Antioxidant enzyme activity

A significant effect of *Diaporthe*-inoculation, water treatment, and their interaction was detected on the activity of catalase (CAT) (Table 1). The CAT activity increased with *Diaporthe* inoculation, but only when plants were subjected to drought stress (Figure 4A). DHAR activity was affected by *Diaporthe* inoculation and drought stress, but not by their interaction (Table 1). The DHAR activity increased under drought stress regardless of inoculation, and in inoculated plants regardless of water treatment (Figure 4B). The APX activity was significantly lower in plants under drought stress regardless of inoculation (Figure 4C).

3.5.2 Antioxidant capacity and phenolic compounds content

A significant effect of the inoculation × water treatment interaction was detected on the antioxidant capacity (Table 1). Compared to uninoculated, this parameter decreased in *Diaporthe*-inoculated plants, but only under drought stress (Figure 5A). The phenolic compound content was not significantly affected by any factor (Figure 5B).

3.5.3 Proline content

A significant effect of *Diaporthe* inoculation, drought stress, and their interaction was detected on the proline content (Table 1). Compared to uninoculated plants, this osmolyte increased significantly in inoculated plants under drought stress; however, *Diaporthe* did not change the proline content in well-watered plants (Figure 6).

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TABLE 1 Results of two-way analysis of variance showing the effect of inoculation with *Diaporthe* EB4, water treatment and their interaction on tomato.

	Diaporthe inoculation		Water treatment		*Diaporthe* × watering	
	F	P	F	P	F	P
Shoot dry weight	39.60	**<0.001**	146.1	**<0.001**	1.738	0.196
Root dry weight	14.47	**<0.001**	113.6	**<0.001**	5.724	**0.022**
root/shoot ratio	25.91	**<0.001**	38.58	**<0.001**	12.12	**0.001**
Chlorophyll content	122.9	**<0.001**	18.67	**<0.001**	33.92	**<0.001**
Stomatal conductance	16.21	**0.002**	0.574	0.463	1.007	0.335
CO_2 assimilation	26.69	**<0.001**	0.462	0.510	7.023	**0.021**
WUE	8.332	**0.014**	24.97	**<0.001**	23.94	**<0.001**
N	26.47	**<0.001**	81.15	**<0.001**	9.942	**0.006**
P	16.51	**<0.001**	45.90	**<0.001**	6.105	**0.025**
K	0.704	**0.414**	156.0	**<0.001**	20.46	**<0.001**
Ca	13.99	**0.002**	0.439	0.517	1.125	0.305
Fe	1.172	0.295	56.13	**<0.001**	18.24	**<0.001**
S	240.4	**<0.001**	84.97	**<0.001**	43.72	**<0.001**
Zn	37.43	**<0.001**	76.33	**<0.001**	26.96	**<0.001**
C	1.403	0.253	0.352	0.561	0.622	0.442
CAT	9.126	**0.011**	48.80	**<0.001**	8.816	**0.012**
DHAR	4.880	**0.047**	9.241	**0.002**	2.207	0.163
APX	0.095	0.763	40.10	**<0.001**	3.366	0.091
Antioxidant capacity	1.472	0.243	25.27	**<0.001**	16.59	**<0.001**
Phenolic compounds	2.107	0.166	1.168	0.296	4.555	0.057
Proline	6.297	**0.023**	11.97	**0.030**	4.662	**0.046**

Numbers in bold indicate that the factor significantly affects the variable.

4 Discussion

Diaporthe species are one of the most abundant components of the culturable fungal microbiome of *Festuca rubra* subsp. *pruinosa* roots (Pereira et al., 2019). These plants grow in an habitat where exposure to salinity and limited soil nutrients are characteristic. *Diaporthe atlantica* strain EB4, isolated from roots of *Festuca rubra* subsp. *pruinosa*, was recently shown to improve plant growth and alleviate salt stress in two agricultural grasses: tritordeum and perennial ryegrass (Toghueo et al., 2022). This finding prompted us to analyze new symbiotic systems in which we could investigate the potential benefits of *Diaporthe* EB4 with non-gramineous agricultural plants of economic relevance such as tomato.

The genus *Diaporthe* includes pathogenic and endophytic species (Gomes et al., 2013). Tomato plants inoculated with *Diaporthe* EB4 exhibited an apparently healthy phenotype with no obvious disease symptoms. In addition, we did not observe by light microscopy any fungal structures inside the plant root tissues. This led us to conclude that *Diaporthe* EB4 should hold a non-pathogenic, epiphytic association with tomato plants, and moved forward to run experiments in which tomato plants were challenged with drought stress.

Although there was no experimental evidence for an endophytic association between *Diaporthe* EB4 and tomato, inoculated plants performed better than uninoculated plants, showing more biomass under both water regimes. Plants under drought stress showed evident changes in morphology, including lower plant biomass, smaller height, lower number of branches and reduced leaf area, all detrimental characteristics usually associated with slower plant cell expansion and division rates (Jaleel et al., 2009). This proved that a beneficial symbiotic association between *Diaporthe* EB4 and tomato plants occurred. Some plant-fungal symbiotic associations are known to enhance water retention and nutrient absorption, which, in turn, increase photosynthesis and production of stored material resulting in better root and shoot biomass (Li et al., 2019; Sarkar et al., 2021).

Previously it was observed that *Diaporthe* EB4 caused an enhancement of the content of abscisic (ABA) and indole-acetic acid (IAA) in leaves of tritordeum under salt stress, accompanied by an increase in the root and shoot biomass (Toghueo et al., 2022). In addition, *Diaporthe* EB4 cultures produced extracellular IAA (Toghueo et al., 2022). ABA and IAA are well known for their roles in maintaining water retention capacity and hydraulic properties in plants under drought, and modulating changes in root morphology (Tiwari et al., 2017; Saleem et al., 2018). Thus, *Diaporthe* EB4 could induce the formation of fine roots under drought stress, increasing the root-soil contact, and improving nutrient and water uptake. Recently, *Diaporthe masirevici* was demonstrated to have a positive effect on

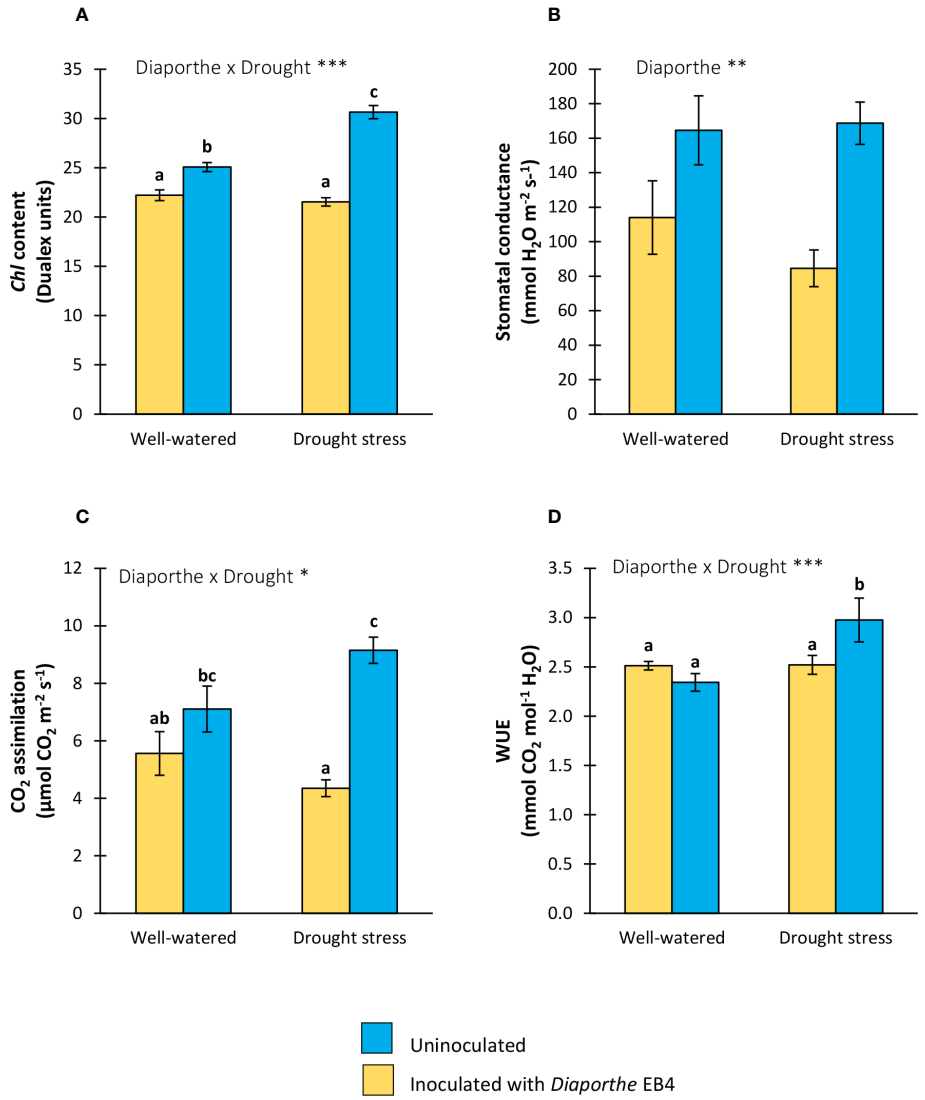

FIGURE 2
(A) Chlorophyll content, **(B)** stomatal conductance, **(C)** CO_2 assimilation rate, and **(D)** water use efficiency (WUE), of tomato plants uninoculated (yellow) or inoculated with *Diaporthe* EB4 (blue), with two different water treatments (well-watered or drought stress). Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* inoculation × Drought] interaction. Values are means +SE (n=5). Level of significance:*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

plant development by enhancing IAA production and phosphate solubilization (da Silva Santos et al., 2022).

In this study, *Diaporthe* EB4 stimulated soil uptake and mobilization to the plant shoot of several macro- and micronutrients (N, P, K, Ca and S, and Fe and Zn) with essential roles in plant development, biosynthesis of photosynthetic pigments and proteins, photosynthesis and hormonal water regulation (Ahmad and Abdin, 2000; Peng et al., 2007; Hänsch and Mendel, 2009). The increase in the content of the above mineral nutrients, related to an increase in shoot biomass, was particularly significant in *Diaporthe*–inoculated plants under drought conditions. *Diaporthe* EB4 could suppress, at least in part, the negative effect of drought stress on plant biomass through a more efficient system of absorption of nutrients (Figure 7). The fact that the inoculated plants under drought had an unexpectedly higher mineral content than those under well-watered conditions was attributed to a dilution effect on the mineral nutrient content in inoculated plants under well-watered (and more favorable growth) conditions, in which the C metabolism was not downregulated and the partitioning of C towards structural components was not restricted as observed under drought stress (Ghaffari et al., 2019). In our study, the increase in biomass of *Diaporthe*-inoculated tomato plants seems to be conveyed by hormone mediated root structural changes leading to improved mineral uptake and water retention.

The decrease in plant growth caused by drought is also associated with the downregulation of photosynthesis (Parkash and Singh, 2020). In the present study, drought stress caused an evident reduction in the stomatal conductance and the CO_2 assimilation rate of leaves in uninoculated plants, thereby limiting the synthesis and sink distribution of photosynthates. However, no significant changes in chlorophyll content were observed in uninoculated plants between drought and well-watered conditions, suggesting that, although there was a prominent decline in shoot biomass, the photosynthetic apparatus did not sustain severe photodamage.

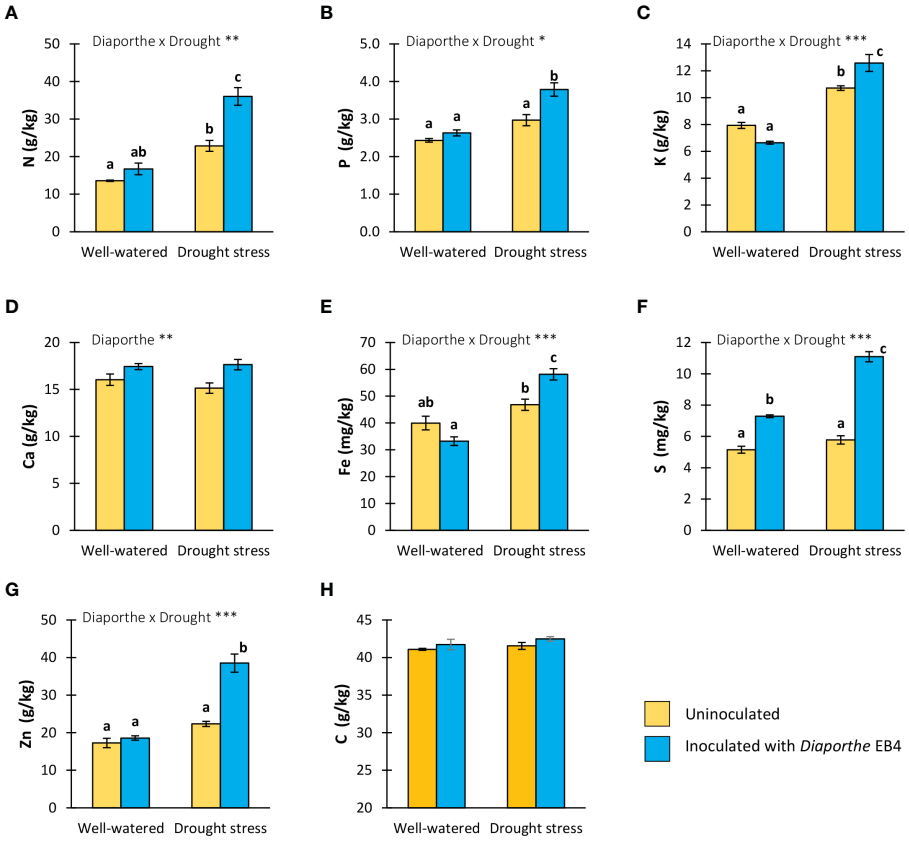

FIGURE 3
(A) Total nitrogen, **(B)** phosphorus, **(C)** potassium, **(D)** calcium, **(E)** iron, **(F)** sulphur, **(G)** zinc and **(H)** total carbon contents in tomato plants uninoculated (yellow) or inoculated with *Diaporthe* EB4 (blue), with two different water treatments (well-watered or drought stress). Different letters indicate different means (Tukey $p<0.05$) for the [*Diaporthe* inoculation x Drought] interaction. Values are means +SE (n=5). Level of significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Interestingly, *Diaporthe* enhanced the chlorophyll content and the CO_2 assimilation rate under both water treatments. The net CO_2 assimilation rate in inoculated plants under drought was highest and correlated with the highest content of N and chlorophyll in leaves. This information can be used to predict that the maximum carboxylation rate by Rubisco (Vcmax) should also be the highest in inoculated plants under drought stress (Wang et al., 2021). Similar effects have been reported in other symbiotic systems. For example, *Diaporthe liquidambari* improved N accumulation in rice (Yang et al., 2014; Yang et al., 2015) and an increase in chlorophyll content was observed in *Trichoderma*–inoculated *Theobroma cacao* and *Neotyphodium*–inoculated *Elymus dahuricus* under drought stress (Zhang and Nan, 2007; Bae et al., 2009), whereas an enhancement of net CO_2 assimilation was reported in *Neotyphodium*–infected tall fescue (Newman et al., 2003). Likewise, an improved adaptation to drought stress was observed in barley inoculated with *Piriformospora indica* as a result of enhanced activity of key enzymes of the N metabolism and a better distribution of N in the plant (Ghaffari et al., 2019).

Diaporthe EB4 was shown to increase the IAA content of *Lolium perenne* and tritordeum plants exposed to salt stress (Toghueo et al., 2022), and exogenous application of IAA was reported to increase the chlorophyll content in maize exposed to salt stress, and to stimulate stomatal aperture due to improved concentration of K in cells (Kaya et al., 2013). In this regard, the accumulation of macronutrients like K in leaves, together with an increase in IAA, seems to optimize leaf CO_2 assimilation and water use. In our study, *Diaporthe*-inoculated plants under drought stress exhibited the greatest WUE, even though the stomatal conductance increased. In contrast, plants of *Lolium arundinaceum* symbiotic and non-symbiotic with *Epichloë coenophialum* (growing in the aboveground plant parts) held similar transpiration rates (Swarthout et al., 2009). In our study, the improvement of the relationship between the assimilated CO_2 molecules and the loss of H_2O molecules by transpiration was mainly attributed to a higher Rubisco activity (higher Vcmax) in the leaves of inoculated plants under drought, instead of a decrease in stomatal opening. Indeed, water movement through the xylem vessels could be enhanced in inoculated plants under drought stress because of the higher soil uptake of K by *Diaporthe*–colonized roots. Therefore, *Diaporthe* EB4 might promote tomato plant growth and confer tolerance to drought stress by improving soil uptake of mineral nutrients, chlorophyll content, leaf photosynthesis, and K–mediated stomatal dynamics (Figure 7).

In response to ROS production caused by drought stress, plants have developed an intricate antioxidant defense network composed of enzymatic and non–enzymatic antioxidants that scavenge ROS and maintain cellular redox homeostasis (Ahmad et al., 2010; Muhammad et al., 2021). In our study, APX and CAT, both H_2O_2 scavenging

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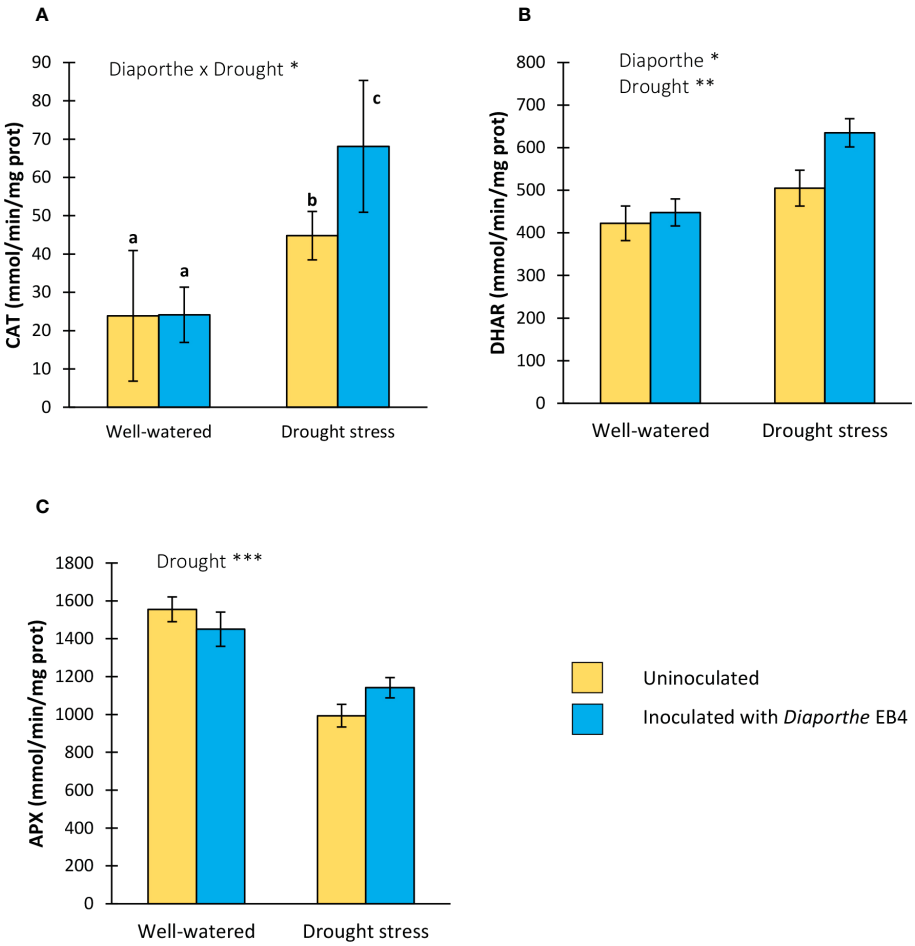

FIGURE 4
Activity of the antioxidant enzymes **(A)** catalase (CAT), **(B)** dehydroascorbate reductase (DHAR), and **(C)** ascorbate peroxidase (APX) of tomato plants uninoculated (yellow) or inoculated with *Diaporthe* EB4 (blue), with two different water treatments (well-watered or drought stress). Different letters indicate different means (Tukey $p < 0.05$) for the [*Diaporthe* inoculation × Drought] interaction. Values are means +SE (n=5). Level of significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

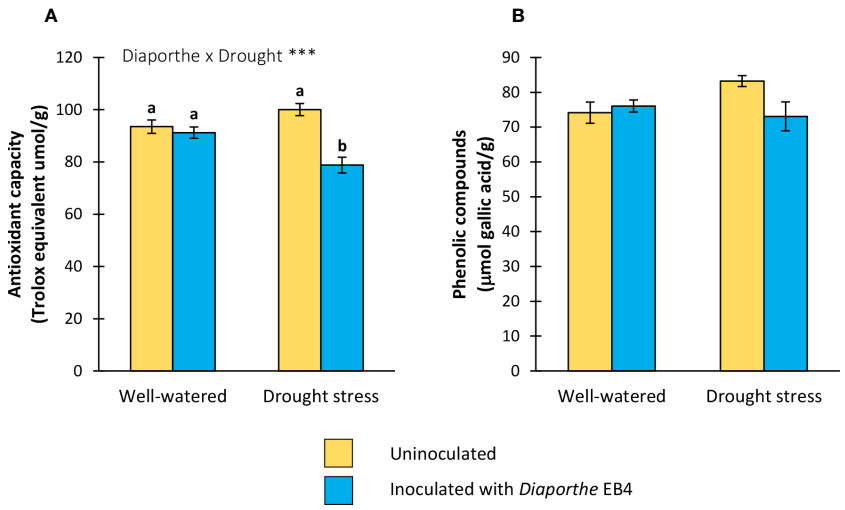

FIGURE 5
(A) Antioxidant capacity, and **(B)** total phenolic compounds content of tomato plants uninoculated (yellow) or inoculated with *Diaporthe* EB4 (blue), with two different water treatments (well-watered or drought stress). Different letters indicate different means (Tukey $p<0.05$) for the [*Diaporthe* inoculation × Drought] interaction. Values are means ± SE (n=5). Level of significance: $***p < 0.001$.

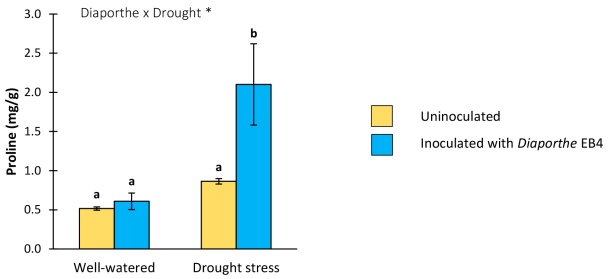

FIGURE 6
Proline content in leaves of tomato plants uninoculated (yellow) or inoculated with *Diaporthe* EB4 (blue), with two different water treatments (well-watered or drought stress). Different letters indicate different means (Tukey $p<0.05$) for the [*Diaporthe* inoculation x Drought] interaction. Values are means +SE (n=5). Level of significance: $*p < 0.05$.

enzymes, varied their activities under drought stress regardless of inoculation treatment, although in different ways. The activity of CAT increased under drought stress, implying that H_2O_2 accumulated in the plant cells, and this activity was notably higher in inoculated plants under drought. We thus propose that *Diaporthe* EB4 could similarly confer tolerance to drought through an induced mechanism, in which the activity of some antioxidant enzymes like CAT could be modulated.

Intriguingly, under drought stress the APX activity decreased, while the DHAR activity increased. Both APX and DHAR belong to the ascorbate-glutathione cycle. The decrease in APX activity is probably due to a lower content of ascorbate in leaf cells, which is

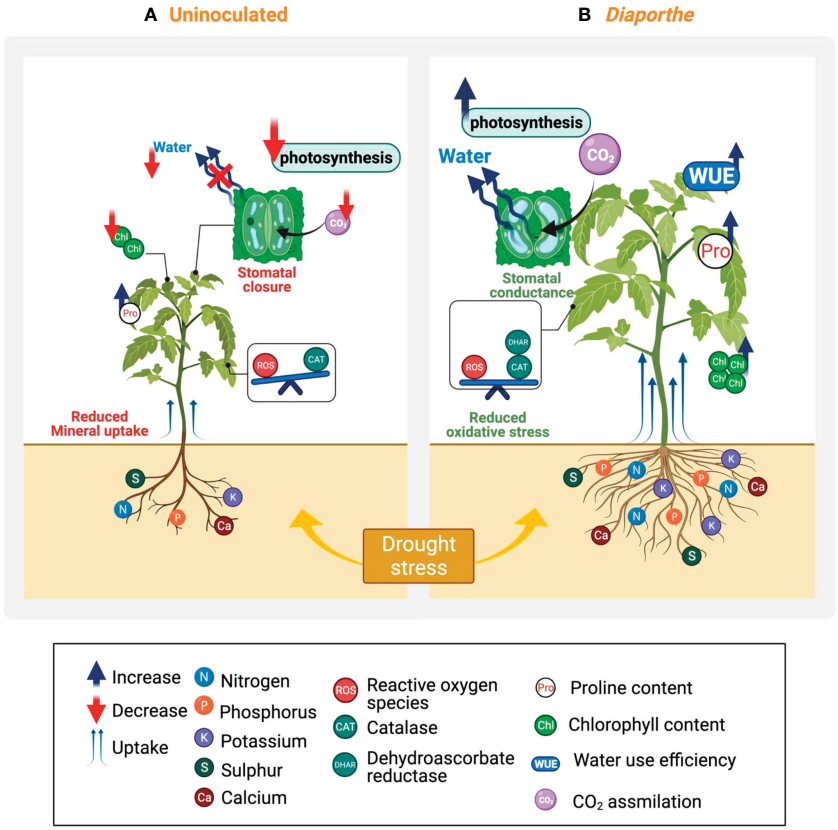

FIGURE 7
Overview of the effect of drought stress in uninoculated and *Diaporthe*-inoculated tomato plants. **(A)** Drought stress had a deleterious effect on tomato plant growth and biomass production. This biomass reduction can be associated with a reduction in photosynthetic activity caused by a reduction in stomatal conductance and consequently in the CO_2 assimilation rate, and also by a decline in the chlorophyll content. The stomatal closure decreased the water movement on the plant which can be also associated with a decrease in the mineral uptake. In response to drought stress, the activity of CAT and proline content increased to reduce oxidative damage and for an osmotic counterbalance, however, this increase does not seem to be enough to alleviate the negative effect. **(B)** *Diaporthe* significantly mitigated the harmful impact of drought stress through combined mechanisms, which include an increase in the chlorophyll content, an optimal stomatal conductance that facilitates the CO_2 assimilation, and a greater WUE, indicating the plant maintains its stomata open and subsequently preserves an optimal photosynthesis activity. *Diaporthe* stimulated the increase of antioxidant defense system, e.g., CAT and DHAR, suggesting a reduction of the oxidative stress caused by water limitations; significantly enhanced the proline content that can participate in the osmotic adjustment or in the structure protection, and increased the mineral uptake. All together favor plant growth under drought stress.

consistent with the lower growth of tomato plants under drought stress and the role of ascorbate in cell expansion and cell division (Foyer, 2018). APX was not significantly affected by *Diaporthe* inoculation. However, the significant increase in DHAR activity in inoculated plants under drought suggested that cellular ascorbate regeneration was better in the presence of *Diaporthe* EB4, although the content of ascorbate in inoculated plants probably did not reach levels similar to those under well-watered conditions on the basis of plant biomass. Altogether, *Diaporthe* EB4 could improve the enzymatic antioxidant response of tomato plants and confer protection against ROS generation during drought stress (Figure 7).

Additionally, fungal endophytes can induce the formation non–enzymatic antioxidant metabolites such as phenolic compounds (White and Torres, 2010; Bacon and White, 2016; Varela et al., 2016). In our previous studies, *Diaporthe* EB4 did not enhance the total phenolic content in grasses under control or salt stress conditions (Vázquez de Aldana et al., 2021; Toghueo et al., 2022). In the present study, we obtained rather similar results and *Diaporthe* seemed to induce a decline in the non–enzymatic antioxidant capacity under drought stress and to have no significant effect on the total phenolic content.

Osmotic adjustment through the accumulation of solutes such as proline is an important mechanism of plant adaptation to salinity and drought (Munns, 2011; Kaur and Asthir, 2015). In fact, an enhanced accumulation of proline due to inoculation with *Diaporthe* EB4 also occurred in plants of tritordeum under salt stress (Toghueo et al., 2022). In addition to its role as osmolyte, proline interacts with protein and membranes stabilizing their structures and activities (Farooq et al., 2009; Zivcak et al., 2016) and deters oxidative damage through scavenging of ROS, such as hydroxyl radicals formed during H_2O_2 decomposition within the Fenton reaction (Das and Roychoudhury, 2014). In this study, the highest proline accumulation was detected in inoculated plants under drought, a result in line with previous studies in which fungal endophytes like *Penicillium* sp., *Trichoderma harzianum*, DSE, or *Piriformospora indica* conferred drought tolerance to several crops and increased accumulation of proline as osmoprotectant (Molina-Montenegro et al., 2016; Alwhibi et al., 2017; Valli and Muthukumar, 2018; Swetha and Padmavathi, 2020). This accumulation of proline did not seem to notably reduce the loss of water molecules on the basis of the stomatal conductance. This led us to propose, together with its role as an osmoprotectant and ROS scavenger, that proline is also a source of reducing power (NADPH) that plants can use to produce ATP in the dark, showing an oscillating day/night content pattern (Signorelli, 2016) as they also use the accumulation of osmoprotectant sugars under drought stress to produce cell energy when the stress ceases (Ghaffari et al., 2019).

In conclusion, this study shows the capacity of *Diaporthe atlantica*, a fungus symbiotic with plants adapted to a saline environment, to promote growth and adaptation to drought stress on tomato. *Diaporthe* played a positive role in the modulation of tomato responses to drought stress through the combination of various processes. *Diaporthe* could confer drought stress tolerance to tomato by improving soil uptake of mineral nutrients, chlorophyll content, leaf photosynthesis and K-mediated stomatal dynamics. In addition, *Diaporthe* could improve the enzymatic antioxidant response of tomato, through an induced mechanism in which the activity of some enzymes like CAT could be modulated and confer protection against ROS generation during drought stress. An enhanced accumulation of proline could also play an important role in the response of plants to water stress, acting as osmoprotectant, ROS scavenger, and a source of reducing power to produce energy. In general, these results indicate that symbiotic fungi can enhance tomato tolerance to drought stress.

Data availability statement

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

Author contributions

EP performed experiments and analyses. All authors designed the experiments, worked on the analyses of data, wrote the manuscript, and approved the submitted version.

Funding

This research was supported by the European Union's H2020 research and innovation program under the Marie Sklodowska-Curie grant agreement no. 676480; by grant PID2019-109133RB-I00 funded by MCIN/AEI/10.13039/501100011033, and by project 'CLU2019-05—IRNASA/CSIC Unit of Excellence' funded by the Junta de Castilla y León and co-financed by EU (ERDF 'Europe drives our growth').

Acknowledgments

Thanks are due to Virginia González for technical help.

Conflict of interest

Author UU was employed by company Biofungitek Limited Society.

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.

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 28 February 2023
DOI 10.3389/fpls.2023.1125942

OPEN ACCESS

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

RECEIVED 16 December 2022
ACCEPTED 13 February 2023
PUBLISHED 28 February 2023

CITATION
Macaya-Sanz D, Witzell J, Collada C, Gil L and Martín JA (2023) Core endophytic mycobiome in *Ulmus minor* and its relation to Dutch elm disease resistance. *Front. Plant Sci.* 14:1125942. doi: 10.3389/fpls.2023.1125942

Core endophytic mycobiome in *Ulmus minor* and its relation to Dutch elm disease resistance

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The core microbiota of plants exerts key effects on plant performance and resilience to stress. The aim of this study was to identify the core endophytic mycobiome in *U. minor* stems and disentangle associations between its composition and the resistance to Dutch elm disease (DED). We also defined its spatial variation within the tree and among distant tree populations. Stem samples were taken i) from different heights of the crown of a 168-year-old elm tree, ii) from adult elm trees growing in a common garden and representing a gradient of resistance to DED, and iii) from trees growing in two distant natural populations, one of them with varying degrees of vitality. Endophyte composition was profiled by high throughput sequencing of the first internal transcribed spacer region (ITS1) of the ribosomal DNA. Three families of yeasts (Buckleyzymaceae, Trichomeriaceae and Bulleraceae) were associated to DED-resistant hosts. A small proportion (10%) of endophytic OTUs was almost ubiquitous throughout the crown while tree colonization by most fungal taxa followed stochastic patterns. A clear distinction in endophyte composition was found between geographical locations. By combining all surveys, we found evidence of a *U. minor* core mycobiome, pervasive within the tree and ubiquitous across locations, genotypes and health status.

KEYWORDS

fungal endophytes, metabarcoding, plant-fungal interactions, Dutch elm disease, core microbiome, tree microbiome

1 Introduction

The endophytic assembly in deciduous plant tissues (e.g. annual plants, and deciduous leaves) is largely configured each season through horizontal transmission, when priority effects appear to be crucial (Toju et al., 2018b; Ridout et al., 2019; Debray et al., 2022). However, the assembly of endophytes in perennial organs (e.g. tree stems) is likely more complex (Saikkonen, 2007). Studies in crop plants and forest trees have reported consistent

co-occurrence of endophytic assemblages known as core microbiomes, i.e., assemblages of microbes that constantly reside in the plant and are shared among conspecific hosts (Shade and Handelsman, 2012; Thomas et al., 2019; Noble et al., 2020). These core microbes are part of functional networks that positively or negatively affect host performance (Bonito et al., 2019). However, little is understood about core microbes of perennial organs and the extent to which their assembly is shaped by random colonization, environmental cues or active host recruiting factors (Müller et al., 2016). Perhaps because sampling in large tree crowns presents methodological difficulties, the diversity and spatial distribution of endophytes in long-lived trees remain largely unexplored.

Numerous environmental factors can potentially affect plant colonization by endophytes, including age, light availability, spatial distance from soil, and microclimate within the crown (Johnson and Whitney, 1989; Helander et al., 1993; Bahram et al., 2022). The endophytic composition can be also affected by host geographical location and host vitality (Agostinelli et al., 2018). Indeed, some endophytes that colonize long-lived trees are facultative saprotrophs or necrotrophs living in a cryptic phase (Carroll, 1988; Baum et al., 2003). Through environmental filtering, local climatic conditions (e.g. temperature, humidity and rainfall) can strongly influence the production and release of microbial propagules with potential to invade tree tissues (Zimmerman and Vitousek, 2012; Giauque et al., 2019). Furthermore, host-specific traits can drive an active recruitment of microbes (Cregger et al., 2018; Gallart et al., 2018). For instance, a genotype-dependent production of defense compounds against pathogens was shown to alter endophyte community assembly in maize (Saunders and Kohn, 2009). As a consequence of host and environmental effects on microbiomes, the composition of the surrounding vegetation and changes in land use can alter endophyte community at stand level (Li et al., 2019). In sum, endophyte assembly is conditioned by complex interactions among plants, microbes and the environment.

The current pandemic of Dutch elm disease (DED) is caused by *Ophiostoma novo-ulmi*. Since the beginning of the past century, DED has caused massive loss of elm trees native to Europe and North America (Martín et al., 2019b). The disease is vectored by elm bark beetles in the genera *Scolytus* and *Hylurgopinus*, or transmitted through root contacts. After inoculation, the fungus establishes in internal plant tissues, where it sporulates and spreads systemically, causing massive occlusion and embolism of xylem vessels. In most cases, infection ultimately leads to a wilt syndrome and tree death (Ouellette and Rioux, 1992), although some individuals are able to survive as recruiting trees through disease-resprouting cycles (Brasier and Webber, 2019). The composition of endophytic fungi in elms remains largely unexplored. A previous study showed that endophyte diversity in elms was influenced by host location and genotype (Martín et al., 2013), and that the diversity of the mycobiome in the xylem (but not in leaves or bark) of elm trees susceptible to DED was higher than in resistant trees. However, this study addressed only the culturable fraction of endophytes, which account for less than 5% of the total fungal richness within a tree (authors, personal observation).

Elm resistance to DED is affected by multiple factors, including the genetic make-up of hosts and pathogens, and their interaction with the environment (Martín et al., 2021). The role of microbiome in tree resistance remains poorly understood, although in ash dieback complex associations between endophytes and host genotypes seem to condition the outcome of disease (Griffiths et al., 2020). It is becoming clearer that certain endophytic infections trigger systemic responses in plants (Mejía et al., 2014) in certain cases priming plant defense against pathogens, as was recently evidenced in the case of the elm-*O. novo-ulmi* pathosystem (Martínez-Arias et al., 2021a). Some endophytes may also produce antimicrobial metabolites, enzymes, hormones and other bioactive compounds, enhancing host resistance (Hardoim et al., 2015; Busby et al., 2016; Martínez-Arias et al., 2021c). In particular, the core microbiome of a plant seems to exert key effects on plant performance and resistance to various stressors (Shade and Handelsman, 2012; Toju et al., 2018a). Following this concept, core taxa associated with elms probably perform essential functions, including protection against disease.

The general aim of this study was to identify the core endophytic mycobiome in *U. minor* stems as a first step to unravelling the ecology of elm microbial consortia. To address this aim we studied: i) the spatial variation of endophyte composition within the aerial part of a mature tree and between distant geographical locations; ii) the endophyte composition of ten *U. minor* trees showing a gradient of resistance level to *O. novo-ulmi*; and iii) the fungal composition of six large *U. minor* trees showing different vitality levels but growing in the same location.

2 Materials and methods

2.1 Plant material

To determine how tree stem fungal microbiome is structured, we sampled wood tissue from twigs (1-2 cm diameter) and trunks (5-cm cores at breast height) from trees at four locations in Spain in the spring of 2012. We focused on stem endobiome because it is a perennial tissue, in which microbiome interactions have time to evolve and mature, and because the agent responsible for DED is a vascular pathogen and therefore mostly interacts with the xylem microbiome. To prevent inclusion of epiphytic flora, the external layer of the bark (periderm) was manually extirpated after the collection. The stem tissues analyzed were xylem and the remaining phloem.

2.1.1 Within-tree mycobiome variation

Ten spots were sampled within the crown and on the stem of a landmark *Ulmus minor* tree (Somontes, Madrid, Spain; Figure 1; 'landmark tree'). The samples comprised eight twigs from the crown at four heights (3, 8, 13 and 18 m) and two orientations (north and south), and two trunk cores (same orientations). Cores were extracted using a sterilized core drill. The 25-m tree was a lingering monumental elm. Common garden tests on clones generated from its cuttings showed that the tree was not genetically resistant to DED (data not shown), and in 2014 it died after an exceptionally harmful DED outbreak.

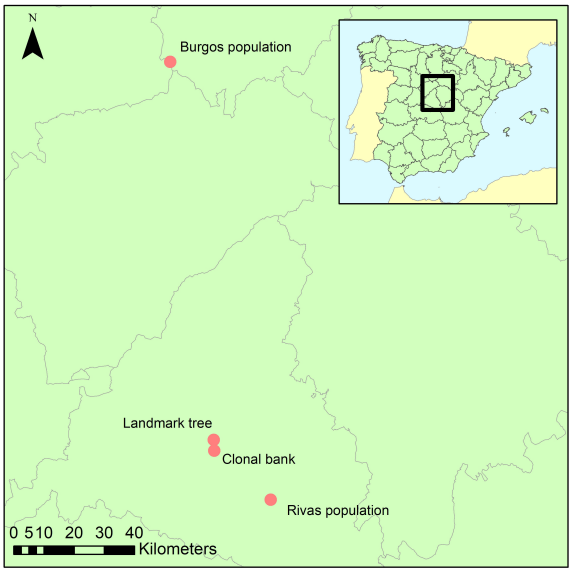

FIGURE 1
Geographical location of the four collection sites.

2.1.2 Wood mycobiome and elm DED resistance

The second sampling was at the elm clonal bank (common garden setup) at the Puerta de Hierro Forest Breeding Centre (Madrid; Figure 1), the headquarters of the Spanish elm breeding program. The clonal bank has around 250 genotypes from Spain, including seven DED-resistant genotypes (Martín et al., 2015). Four twigs were collected from scaffold branches in 10 trees (Supplementary Table 1) catalogued as resistant (n=3; V-AD2; M-RT1.5; M-DV5), intermediately susceptible (n=4; CR-RD2; GR-HL2; J-CA2; MA-PD2), or susceptible (n=3; GR-DF3; M-DV1; TO-PB1). Samples were collected at four spots per tree to ensure accurate representation of the endophyte composition and mitigate any effect of local infections (see below). All twigs were collected from the lower half of the crown, to a height of 4 m.

The level of resistance to DED of the 10 *U. minor* clones sampled at the clonal bank was determined during screening tests at the Spanish elm breeding program at Puerta de Hierro Forest Breeding Centre (Madrid, Spain) (Supplementary Table 1, Supplementary Text). The 10 trees sampled have been never artificially inoculated with the DED pathogen.

2.1.3 Variation in trees differing in vitality phenotype

Following the same protocol as in the clonal bank, twigs from six trees were collected from a natural *U. minor* stand in the municipality of Rivas-Vaciamadrid (Rivas population; 'Madrid province'; Figure 1). This population lacks genetically resistant clones (tested in a common garden) but has not been eradicated by DED. The reasons behind this elusion are unclear but could be due to phenotypic avoidance due to the effect of biotic or abiotic factors. The stand is nonetheless showing clear signs of dieback, in part because of DED infections but various other undetermined causes might be playing a role. Most trees in this stand belong to the susceptible *U. minor* var. *vulgaris*. This taxon presents very low genetic variability, because it originated from a single *U. minor* tree, the Atinian elm (Gil et al., 2004). Indeed, these trees are genetically similar to the clone TO-PB1, another *U. minor* var. *vulgaris* specimen held at the Breeding Centre (and included in the clonal bank collection). We collected samples from trees ranging various health statuses (Supplementary Figure 1). Those health statuses (named RIV1 to RIV6) were scored visually from 1 (no symptoms) to 6 (profuse dieback symptoms).

2.1.4 Variation among geographical locations

Using the same protocol as in the clonal bank, three trees from a small, natural stand in the province of Burgos (approximately 150 km north of the other locations; Figure 1) were sampled to provide a background reference of endophyte diversity and composition of the populations in Madrid province.

2.2 DNA isolation, amplification and NGS

After the collection, samples were sterilized, peeled, frozen and ground. All these steps were carried out in a laminar flow cabinet to minimize contaminations. The four twig samples taken from each individual tree at the clonal bank, Rivas and Burgos populations were combined and milled together, resulting in one pool of wood powder per sampled tree. DNA was isolated from the powder after enzymatic digestion to improve recovery of fungal DNA. Zirconium oxide beads were added during vortexing to increase cell wall lysis. Endophyte composition was profiled by high throughput sequencing of the first internal transcribed spacer region (ITS1) of the ribosomal DNA. Sequencing effort was uneven among experiments, prioritizing the landmark tree samples, which were also the first to be processed to determine the level of resolution needed in subsequent experiments. The clonal bank experiment followed in sequencing effort, to attain accurate values of endophyte abundance for identifying potential associations with DED resistance. The Burgos population was only shallowly sequenced since, as an outgroup, was only intended to test for ubiquity of microbiome elements detected in the other populations. DNA amplification was performed in two steps: (1) to cover the target region with oligonucleotides that contained the specific fungal primer ITS1-F (Gardes and Bruns, 1993) or the non-specific primer ITS2 (White et al., 1990); (2) to attach the adaptors for the sequencing platform. After the second PCR, the product of all the samples was quantified, pooled equimolarly and pyrosequenced in a 454 GS FLX Titanium platform (Roche, Basel, Switzerland). A negative control sample was created by autoclaving collected twigs three times and then applying to them the same protocols previously described. A more detailed description of these methods is available in the Supplementary Text.

2.3 Bioinformatic pipeline

The bioinformatic treatment of pyrosequencing output was performed following the guidelines of Lindahl et al. (2013).

Demultiplexing, denoising, dereplication, dechimerization and sequence truncation processes were carried out using the default values of the RunTitanium script developed in AmpliconNoise v1.29 (Quince et al., 2011; Supplementary Text). The ITS1 region was then extracted from the sequences using FungalITSextractor (Nilsson et al., 2010).

Although AmpliconNoise creates OTUs (Operational Taxonomic Units) by collapsing identical sequences, we further clustered them with the grammar-based software GramCluster 1.3 (Russell et al., 2010) in greedy mode to build new OTUs, allowing higher variation among sequences. This program was run on the whole dataset (i.e. pooling the output of all samples) to build OTUs across all samples, allowing subsequent among-sample comparisons.

2.4 Taxonomic assignment

Taxonomic composition was investigated using the naïve Bayesian classifier method implemented in R package dada2 v. 1.22.0 (Wang et al., 2007; Callahan et al., 2016). We used the last available UNITE release (16/10/2022) (Kõljalg et al., 2005; Nilsson et al., 2019; Kõljalg et al., 2020) as the reference curated database. For OTUs of special interest, we carried out BLAST searches on the NCBI database to double-check the assignment provided by dada2 using the UNITE database.

2.5 Diversity estimates and hypothesis contrasts

Commonly used diversity indices were estimated for each sample collected, using the counts per OTU as taxonomic information. Shannon's H and Simpson's λ indices, and species richness on counts rarefacted to 500, were calculated using R package "vegan" v. 2.6.4 (Oksanen et al., 2015). Statistical analyses were performed taking into account that count data in these types of studies follow a negative binomial distribution as in RNA-seq experiments (McMurdie and Holmes, 2014). As suggested by these authors, R package DESeq2 v. 1.34.0 (Love et al., 2014), which is designed to construct negative binomial models, was used to examine the data and test for associations between taxonomic group abundance and resistance to DED. In order to explore the structure of the samples, DESeq2 was used to perform a variance-stabilizing transformation of the OTU counts to conduct a standard Principal Components Analysis. Tests for associations were run on the clonal bank samples, setting crown wilting percentage (as a proxy of resistance) as the only explanatory variable. Significance was calculated with a Wald test and adjusted for multi-testing using the default DESeq2 approach that estimates False Discovery Rate adjusted P-values (more details in Supplementary Text). Given the unreliable taxonomic certainty of OTU formation through clustering and the possible redundancy in ecological function of closely related species and genera, we decided to focus on the higher taxonomic levels (such as family and order).

2.6 Core microbiome demarcation

The distributions of number of samples in which each OTU was present (OTU incidence distribution) were used to determine which OTUs were putatively from the core microbiome, following the concept of Shade and Handelsman (2012). The expected pattern of incidence of OTUs, if their occurrence probability is low and mostly based on randomness (i.e. local infections rather than core microbiome), must agree with a Poisson or negative binomial distribution. Therefore, if the OTU incidence distribution departs from that hypothesized behaviour, it can be assumed that non-local infections are occurring. Consequently, we selected more than seven samples as the threshold value in both the landmark tree and the clonal bank because it was where the distributions clearly diverged from Poisson distributions (see Results). Thus, OTUs present in more than seven spots of the landmark tree or in more than seven trees of the clonal bank, and also present in at least two out of the four locations, were considered core members.

3 Results

3.1 Sampling effort and saturation

After running the bioinformatic pipeline, we obtained 106,047 informative reads (considered counts). These were grouped by GramCluster into 435 clusters (considered OTUs henceforth). Out of these, 74 were singletons, 40 doubletons and 23 tripletons. A further 263 OTUs were represented by more than five reads. Five OTUs belonged to kingdoms other than fungi. Those OTUs plus the ones represented by singletons or doubletons were discarded for further analyses. To ensure a more accurate OTU richness comparison, we rarefied the count data to 500 reads per sample. The mean values (± s.e.) of rarefied OTUs ranged from 64.4 ± 3.3 in one of the lower resprouted branches of the Somontes tree to 15.6 ± 2.1 in one sample from the Rivas stand (RIV2, with advanced dieback). Rarefaction curves supported the figures observed by the rarefaction to 500 reads and indicated that the sampling effort was sufficient to capture the richness trends of each sample (Supplementary Figures 2, 3). Principal Component Analysis showed a separation between sites (Figure 2).

Across the total sample set, 103 families, 48 orders, 17 classes and 3 phyla were detected. Out of the 317 OTUs not discarded, 293 were assigned to a phylum, 267 to a class, 256 to an order and 228 to a family. Genus was provided for 203 OTUs, and species for 131. However, both genus and species assignments cannot be considered reliable due to the reduced taxonomic resolution of the ITS1.

3.2 Within-tree distribution of endophytes

The Somontes tree had 68,612 reads passing filtering, clustered into 231 OTUs (8 singletons, 2 doubletons and 14 tripletons, just considering the landmark tree counts). Regarding incidence, 11 OTUs were present in all the in-tree spots sampled and 22 were

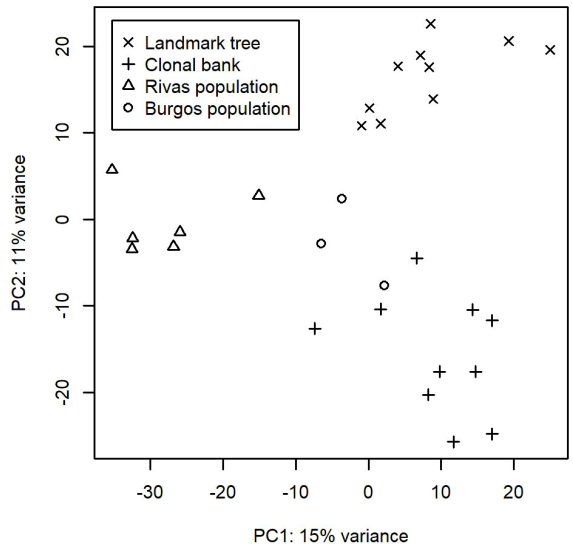

FIGURE 2
First two axes from the Principal Component Analyses performed on the OTU counts after variance stabilizing transformation.

present in at least eight (Table 1, Figure 3A). A further 80 OTUs were present in just one spot and 58 were present in two (Figure 3A). The number of OTUs at higher abundance in the tree did not follow a purely rare event distribution such as the Poisson or negative binomial distribution, as seen in the smooth but distinguishable peak at the end of the distribution (Figure 3A). Three phyla, 15 classes, 41 orders and 81 families were detected within the tree (Figure 4). Across the tree, the levels of diversity (measured as Shannon's H, Simpson's λ and rarefied OTU richness) were generally high, with the following deviations: (i) the two lowest branches, produced from resprouts from the trunk, displayed remarkably higher levels of diversity; (ii) one sample from the trunk and one from the middle crown exhibited low values of both H and λ.

3.3 Endophyte diversity in relation to DED resistance

High-throughput sequencing on the 10 trees of varying levels of resistance to DED from the clonal bank at Puerta de Hierro breeding center produced 20,534 sequences after filtering. The sequences were clustered into 173 OTUs: 20 singletons, 11 doubletons and 19 tripletons. Similar to the results in the Somontes tree, most OTUs were present in just one sample (67), two samples (27) or three samples (17). However, the counts did not drop at a rate consistent with a Poisson process, and reached a stable level beyond five samples (Figure 3B). In total, two phyla, 15 classes, 34 orders and 68 families were detected (Figures 5A, C).

Clone TO-PB1 (susceptible) displayed the lowest levels of diversity (H = 1.03). Conversely, the resistant clone M-RT1.5 showed the highest overall diversity estimates (H = 2.94). GR-HL2 (susceptible) and MA-PD2 (moderately resistant) also displayed high diversity values. Wilting after DED inoculation (used as a proxy of susceptibility) was not significantly correlated with any of the diversity estimates, indicating the absence of a strong correlation between diversity estimates and resistance to DED. However, the limited sample size (n = 10) may have prevented detection of a more subtle correlation.

The tests of association between wilting and taxa abundance produced unambiguous hits (Table 2). Three families and three orders were significantly associated with resistance and one family and order was associated with susceptibility. The family with the highest association was Buckleyzymaceae (Figure 6A), a Basidiomycota of the Cystobasidiomycetes class and undefined order (*Incertae sedis*). It had lower support at OTU level, represented by the genus *Buckleyzyma* (OTU_71). The next most significant hit was from the family Trichomeriaceae, Ascomycota (Figure 6B), a recently circumscribed family in the order Chaetothyriales, excised from family Herpotrichiellaceae. It was also supported, but to a lesser degree, by the hit at OTU level, in OTU_41 assigned to the genus *Knufia*. The next and least significant hit at family level was Bulleraceae (Figure 6C), echoing at order level as Tremellales (Basidiomycota). Two OTUs (OTU_70 and OTU_55) were significant and belonged to the genera *Genolevuria* (based on UNITE) or the related *Cryptococcus* (based on NCBI). All these taxa were negatively associated with susceptibility (proxied as wilting). Family Diatrypaceae was positively associated with susceptibility, and this result was reproduced with stronger support at order level (Xylariales) and at class level (Sordariomycetes). Also, OTU_1 and OTU_19 (Sordariomycetes) were positively associated to DED susceptibility, being the former assigned by dada2 to the genus *Anthostoma* and by BLAST into NCBI's GenBank to *Lopadostoma* but both with suboptimal identity (< 95%, due to a 11-bp indel), and the latter assigned *via* dada2 only at order level (Hypocreales), but *via* BLAST into NCBI's GenBank to *Annulohypoxylon multiforme*, Xylariales (>99% identity). These findings hint at a general relationship between the Sordariomycetes and susceptibility.

3.4 Endophytic mycobiome in trees representing a gradient of vitality

The six samples collected in the natural riparian stand at Rivas-Vaciamadrid municipality from trees at varying stages of dieback produced 13,408 reads, clustered into 92 OTUs: 16 singletons and 11 doubletons. Forty-eight were represented by more than five reads. Only six OTUs were present in all trees and 10 were present in five samples (Figure 3C). The secondary peak found in the OTU incidence distribution was not in the total number of samples (n = 6) but in n = 5.

None of these OTUs was identified as genus *Ophiostoma* or order *Ophiostomatales*, even though the UNITE database included several accessions for both *O. ulmi* and *O. novo-ulmi*, and it was undoubtedly detected as singleton in two trees of the clonal bank (GR-DF3 and V-AD2). The most affected tree (RIV2) and two trees with moderate dieback (RIV1 and RIV4) were dominated by Sordariomycetes: RIV1 was rich in Diatrypaceae and RIV2 in Bionectriaceae (Figures 5B, C). Both RIV4 (moderate dieback)

10.3389/fpls.2023.1125942

TABLE 1 OTUs present in at least eight samples of the landmark tree or the clonal bank.

OTU id	Phylum	Order	Class	Family	Genus	NL	NC	NT	Npop
OTU_0	Ascomycota	Dothideomycetes	Myriangiales	NA	NA	7	**9**	21	4
OTU_2	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	**8**	7	23	4
OTU_6	Ascomycota	Dothideomycetes	Dothideales	Saccotheciaceae	*Aureobasidium*	7	**10**	26	4
OTU_7	Ascomycota	Dothideomycetes	Myriangiales	Endosporiaceae	*Endosporium*	**10**	**10**	26	4
OTU_8	Ascomycota	Dothideomycetes	Pleosporales	Cucurbitariaceae	NA*	2	**9**	19	4
OTU_10	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	NA	**10**	**10**	29	4
OTU_13	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Retiarius*	6	**8**	15	3
OTU_14	Ascomycota	Dothideomycetes	Mycosphaerellales	Teratosphaeriaceae	*Lapidomyces*	3	**8**	11	2
OTU_15	Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*	**10**	**9**	25	4
OTU_16	Ascomycota	Dothideomycetes	Mycosphaerellales	Extremaceae	*Petrophila*	4	**8**	13	3
OTU_18	Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis	*Trichothecium*	0	**10**	16	3
OTU_21	Ascomycota	NA	NA	NA	NA	**8**	**8**	19	4
OTU_23	Ascomycota	NA	NA	NA	NA	**10**	7	17	2
OTU_24	Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	NA*	**10**	**9**	23	4
OTU_25	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	**10**	5	17	4
OTU_27	Ascomycota	Lecanoromycetes	Caliciales	Physciaceae	*Rinodina*	**10**	**10**	21	3
OTU_29	Ascomycota	Dothideomycetes	NA	NA	NA	**9**	**8**	20	3
OTU_32	Ascomycota	Dothideomycetes	Mycosphaerellales	NA	NA	2	**8**	11	3
OTU_33	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	**10**	**10**	28	4
OTU_34	Ascomycota	Sordariomycetes	Xylariales	Leptosilliaceae*	*Leptosillia**	**8**	3	13	3
OTU_35	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	*Neofusicoccum*	**9**	7	16	2
OTU_38	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Entoleuca**	0	**9**	15	3
OTU_40	Ascomycota	Eurotiomycetes	Chaetothyriales	Trichomeriaceae	NA	**9**	**9**	18	2
OTU_41	Ascomycota	Eurotiomycetes	Chaetothyriales	Trichomeriaceae	*Knufia*	**10**	**10**	29	4
OTU_46	Ascomycota	NA	NA	NA	NA	**10**	**9**	26	4
OTU_51	Ascomycota	NA*	NA*	NA*	NA*	6	**8**	17	4
OTU_65	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	**9**	6	23	4
OTU_66	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	NA	**10**	7	25	4
OTU_71	Basidiomycota	Cystobasidiomycetes	Incertae sedis	Buckleyzymaceae	*Buckleyzyma*	**9**	**8**	22	4
OTU_80	Ascomycota	Eurotiomycetes	Chaetothyriales	NA*	NA*	**9**	5	22	4
OTU_102	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	**8**	2	11	3
OTU_178	Ascomycota	NA	NA	NA	NA	**8**	1	9	2

Taxonomic assignment is based on ITS1 DNA similarity with UNITE database. Star (*) indicates assignment change after check in the NCBI database. The final columns show the number of samples in the landmark tree (*NL*), the clonal bank (*NC*) and the total sample set (*NT*) and the number of geographical locations (*Npop*) where the OTUs were detected. Bold numbers indicate presence in eight or more collected samples. NA indicates Not Assigned

and RIV6 (incipient dieback) had Nectriaceae as the most abundant family, although it was also abundant in the healthy RIV3. The two healthy trees (RIV3 and RIV5) were more infected than the other trees by Dothideomycetes and Eurotiomycetes. For diversity, RIV5 exhibited the highest values in all three indices calculated (Shannon's H, Simpson's λ and rarefied OTU richness). The affected RIV1 and RIV6 displayed high values of H and richness, and RIV3 (healthy) and RIV6 had high values of λ. The tree with lowest vitality (RIV2) had the lowest diversity values.

The healthiest tree (RIV5) displayed a clearly distinct pattern that was much richer in Basidiomycota (Figure 5B). Trichomeriaceae was the most common family in this tree,

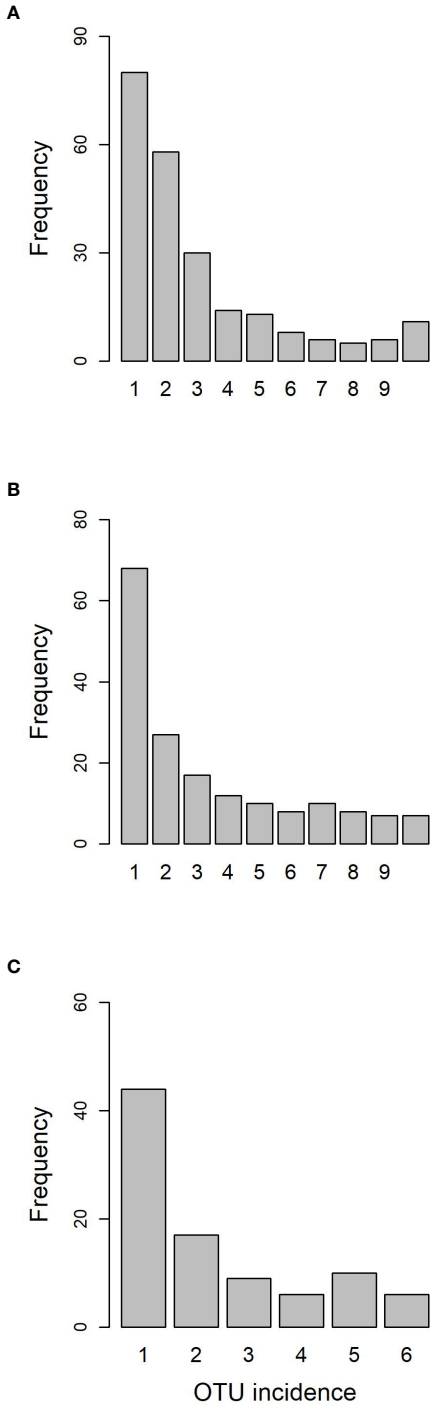

FIGURE 3
OTU frequency spectra for **(A)** landmark tree, **(B)** clonal bank and **(C)** Rivas population.

followed by Saccotheciaceae. The microbiome of RIV2, a tree with low vitality, was dominated by Bionectriaceae (OTU_147, identified as genus *Geosmithia* both in UNITE and NCBI; 100% of identity). This OTU was virtually absent in the other samples, except in the healthiest (RIV5), where it was not abundant but had a significant presence.

Regarding the taxa significantly associated with DED resistance, Buckleyzymaceae (represented mostly by OTU_71) was virtually absent from the population. Trichomeriaceae (represented mostly by OTU_41) was present in all trees but was much more abundant in RIV1 (dieback) and RIV5 (very healthy). Bulleraceae was slightly present in the healthiest tree RIV5. The single OTU associated with increased DED susceptibility (OTU_1; Diatrypaceae) was very abundant in RIV1 (dieback).

3.5 Patterns across the four sites – core fungal endobiome of *U. minor*

To assess the extent of ubiquity of the most common OTUs, we examined the patterns of OTU incidence pooling the global sample set (n = 29). Of the 317 OTUs passing filtering, 88 were present in only one sample, 64 in two samples and 34 in three samples. Distribution then reached a local maximum at six samples. Two clusters were present in all 29 samples (OTU_10, Didymellaceae, Dothideomycetes; and OTU_41, Trichomeriaceae, Eurotiomycetes, associated with DED resistance, see above), one was present in all but one (OTU_33, Cladosporiaceae, Dothideomycetes), and three others were present in all but two (Table 1). Beyond the category of "presence in nine samples" distribution was effectively flat. In other words, the number of OTUs present in 10 to 29 samples always ranged from 1 to 5. Note that not all samples were taken under the same conditions (single twig vs. pooled twigs).

To detect core mycobiome members, we used the independent distributions of each experiment presented in previous sections, and the incidence across all of collection sites. In that regard, 37 OTUs were found in the four sampled populations, 44 in three, 88 in two, and 153 were private to a single population. Both the pooled samples and the across-sites distributions concur with the distributions of OTUs in the clonal bank and, to a lesser extent, with that of the OTUs in the landmark tree. The OTUs present more frequently in our sampling than could be expected by chance are very likely members of the core microbiome (see Discussion). In total, 32 OTUs passed the criteria for core microbiome membership: 29 belonging to Ascomycota and three to Basidiomycota.

4 Discussion

4.1 Within-tree variation in species richness and diversity

Analyses on the landmark tree endophytic mycobiome did not reveal a clear structure, but allowed to draw some interesting conclusions: (i) although most of the samples collected displayed a similar taxonomic composition, some were remarkably different. For instance, a southern mid-height branch (H1S) was massively infected by a single OTU (Figure 4). (ii) The two lowest branches, resprouts from the trunk (epicormic shoots) aged a few years old, displayed higher taxonomic richness than any other branches, with a relatively higher representation of Basidiomycota. (iii) Finally, samples from the trunk showed a richness comparable to that of the crown branches. Taking this into consideration, when sampling trees to characterize their overall stem endophytic flora and to avoid

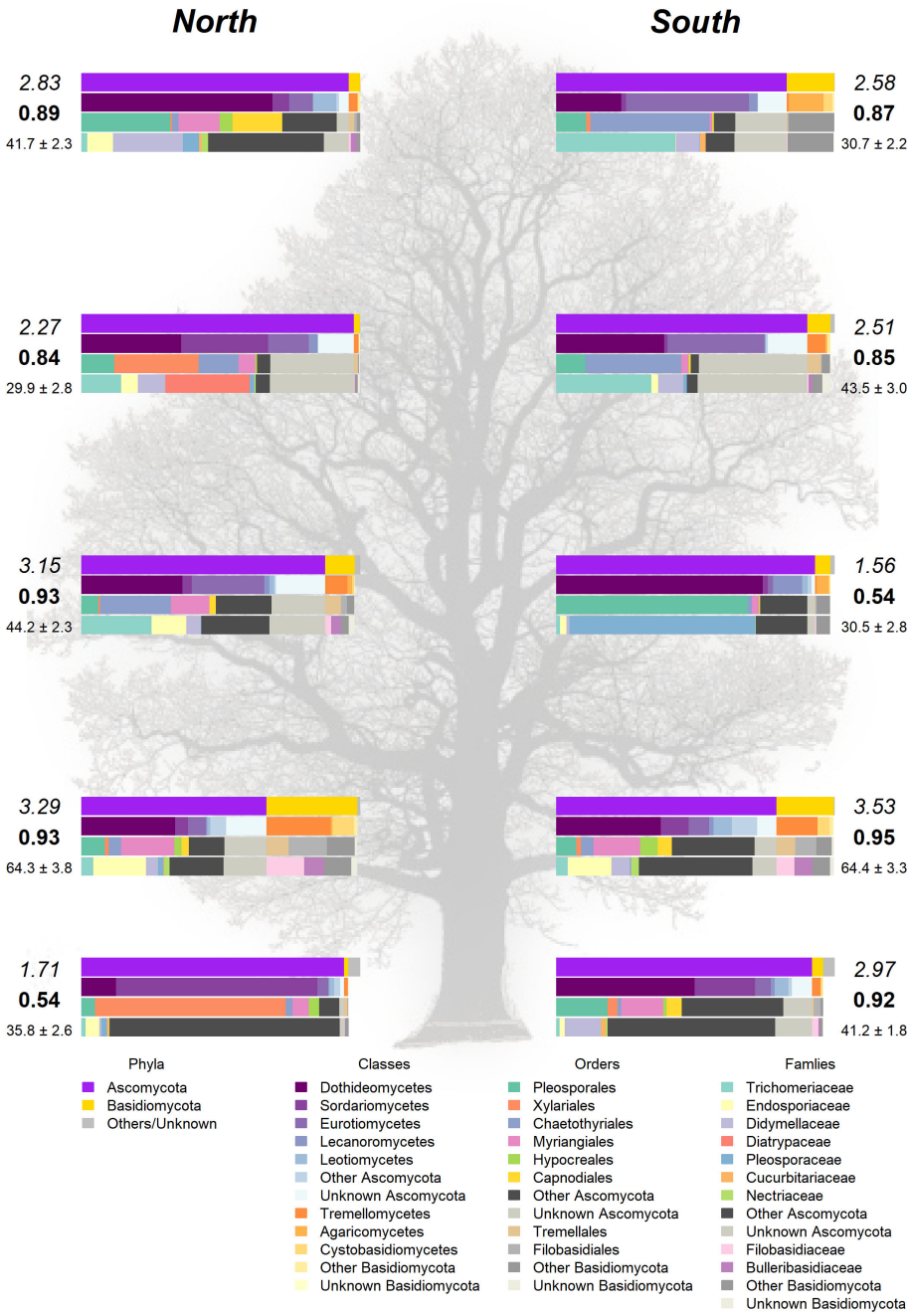

FIGURE 4
Taxonomic composition in the landmark tree. Only the most relevant taxa are shown. Colored bars represent the frequency of taxa at the levels of phylum, class, order and family (top to bottom). Numbers next to the bars indicate the Shannon (italics) and Simpson (bold) indices and the OTU richness rarefacted to 500 reads (with standard error). (Background image source: Tree Silhouette copy by Bob G in flickr, licensed under CC BY-NC-SA 2.0).

considerable biases due to abnormally high local infections, we recommend pooling tissue from at least two branches. However, mixing samples from epicormic and crown branches should be avoided, because they are likely to represent different endobiome compositions. The greater richness found in the lower branches supports previous research (Andrews et al., 1980; Johnson and Whitney, 1989) and could be partly attributed to the high density of inoculum in the ground with ability of entering into the stems through roots, bark surface and stomata in leaves (Bahram et al., 2022). Similarly, as a substrate for fungi, epicormic shoots may differ in anatomy and vigor from proleptic shoots (Negrón et al., 2013).

4.2 Endobiome and resistance to DED

The abundance of three distinct fungal endophytic taxa was associated with higher host resistance to DED (Table 2).

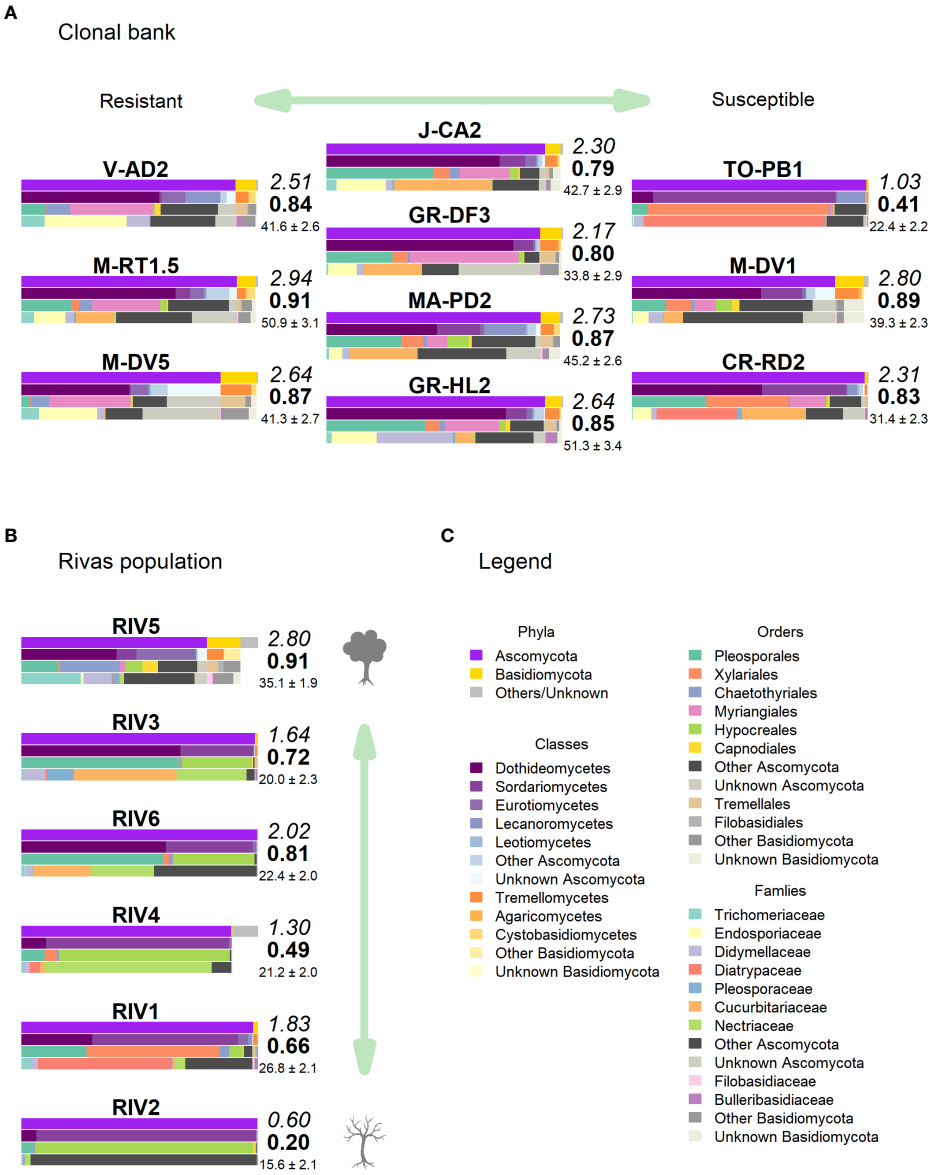

FIGURE 5
Taxonomic composition in **(A)** clonal bank and **(B)** Rivas population. Only the most relevant taxa are shown. Colored bars represent the frequency of taxa at the levels of phylum, class, order and family (top to bottom), following legend color code **(C)**. Numbers next to the bars indicate the Shannon (italics) and Simpson (bold) indices and the OTU richness rarefacted to 500 reads (with standard error). (Tree icon sources: minimal tree simple SVG Silh, licensed under CC0 1.0 and tree-304418 by Clker-Free-Vector-Images in pixabay under Pixabay licence).

Interestingly, the two highest associations at family level (Buckleyzymaceae, in Cystobasidiomycetes; Trichomeriaceae, in Eurotiomycetes) were mostly driven by OTUs considered to be members of the core microbiome (OTU_71 and OTU_41, respectively). Moreover, a trait of two out of the three taxa (Buckleyzymaceae and Bulleraceae) is that they grow, or are able to grow, as yeasts. Yeasts have the ability to systemically colonize plants and produce phytohormones and siderophores that promote plant growth and alleviate stress (Joubert and Doty, 2018; Martínez-Arias et al., 2021c). The greater abundance of these yeasts in resistant trees could improve tree resilience to DED infection, promoting resistance mechanisms to the physiological disorders caused by the pathogen. *O. novo-ulmi* also spreads systemically through the plant's vascular system in a yeast-like phase (Nigg et al., 2015) (blastospores), even in resistant trees (Martín et al., 2019a), inducing vessel embolism. Our results suggest that resistant trees benefit from harboring a high proportion of two fungi from the core endobiome (OTU_71 and OTU_41), which have the capacity to extensively colonize the plant. Extensive or systemic spread of an endophyte could allow higher interaction with the pathogen throughout the plant, and possibly a higher level of interaction with the plant's physiological functions.

The first endophyte was assigned to *Buckleyzyma aurantiaca*, based on the sequence similarity to the accessions in the database UNITE. When the ITS sequence of this OTU was run against Genbank, equal hits were returned for several accessions identified

TABLE 2 Taxa with significant positive or negative associations (*padj* < 0.1; *p-value* < 0.05 for OTUs) with resistance to DED.

Taxon	*baseMean*	*log2FC*	*lfcSE*	*stat*	*p-value*	*padj*	*Family*	*Order*
Class								
Cystobasidiomycetes	24.343	-2.038	0.475	-4.292	0.00002	0.00027		
Sordariomycetes	762.750	2.178	0.538	4.051	0.00005	0.00038		
Eurotiomycetes	71.577	-0.979	0.327	-2.994	0.00275	0.01375		
Order								
Xylariales	676.281	2.719	0.560	4.852	0.00000	0.00004		
Cystobasidiomycetes *incertae sedis*	19.118	-1.896	0.488	-3.886	0.00010	0.00153		
Chaetothyriales	59.560	-0.881	0.365	-2.410	0.01595	0.15951		
Tremellales	64.275	-0.977	0.426	-2.294	0.02180	0.16351		
Family								
Buckleyzymaceae	11.154	-2.128	0.572	-3.723	0.00020	0.01102		
Diatrypaceae	784.043	5.423	1.557	3.484	0.00049	0.01385		
Trichomeriaceae	49.146	-1.170	0.362	-3.233	0.00123	0.02288		
Bulleraceae	32.251	-2.889	0.982	-2.943	0.00325	0.04551		
OTU								
OTU_1	762.255	5.413	1.552	3.488	0.00049	0.05298	Diatrypaceae	Xylariales
OTU_70	19.432	-3.625	1.171	-3.097	0.00196	0.09598	Bulleraceae	Tremellales
OTU_71	13.338	-2.251	0.751	-2.998	0.00272	0.09598	Buckleyzymaceae	*Incertae sedis*
OTU_19	4.591	2.510	0.878	2.860	0.00424	0.09598		Hypocreales
OTU_55	20.021	-3.811	1.338	-2.848	0.00440	0.09598	Bulleraceae	Tremellales
OTU_41	49.449	-1.475	0.567	-2.602	0.00928	0.16857	Trichomeriaceae	Chaetothyriales

The test of association was performed by a Wald test. Column *baseMean* shows the mean of normalized counts; *log2FC*: estimate of the effect size scaled to the log2 of fold change; *lfcSE*: standard error of this estimate; *stat*: value of the Wald test statistic; and *p-value* and *padj*: respectively, the raw and the adjusted (for multiple tests) probabilities that the observed statistic is part of the null distribution. These columns correspond to the output of the function DESeq from R package DESeq2. A positive fold change indicates association with susceptibility to DED.

as *Buckleyzyma* and *Rhodotorula*, both cultured and uncultured, but with a level of identity of 97.22% (140/144 bp). This OTU is likely to be an undescribed species. Cystobasidiomycetes is a group of basidiomycetous yeasts with unclear systematics that includes strains previously isolated from plants (Oberwinkler, 2017), soils and waters (Jones, 2011; Duarte et al., 2015; Jones et al., 2015). An elm endophytic yeast from Cystobasidiomycetes was shown to reduce *O. novo-ulmi* growth *in vitro*, partly due to the release of volatiles (Martínez-Arias et al., 2021c). Furthermore, its inoculation into elm plantlets in tandem with a Chaetothyrial yeast, favored root development, photosynthesis and survival against abiotic stress (Martínez-Arias et al., 2021b).

The second endophyte (OTU_41) was assigned to *Knufia* by our pipeline. In Genbank, it did not retrieve perfect identities, obtaining a maximum identity of 97.55% (196/201 bp) and three gaps to *Knufia* but also to genus *Exophiala*. Most accessions were derived from uncultured strains, and some from molecular studies in soils and plants. This OTU could therefore also belong to an undescribed species. The Trichomeriaceae (Chaetotyriales) were formerly part of the Herpotrichiellaceae, which have been reported to grow in the sexual phase in dead plants and wood (Geiser et al., 2006). Members of Chaetotyriales can be classified as dark septate endophytes, which can provide important benefits to their hosts as reducers of biotic or abiotic damages (Punja and Utkhede, 2003; de Tenório et al., 2019).

The third associated taxon was represented by two OTUs (OTU_70 and OTU_55) of the genus *Cryptococcus* (via BLAST to NCBI; 100% and 97% of identity, respectively) or *Genolevuria* (via dada2 to UNITE), both Tremellal yeasts frequently found in plants and water (Jones et al., 2015). Albrectsen et al. (2018) found *Cryptococcus* as an endophyte in beetle-damaged *Populus tremula* leaves. In addition, *Cryptococcus* apparently outcompetes the Rosaceae pathogen *Botrytis cinerea* due to niche occupancy (Zambell and White, 2017).

4.3 Phenotypic vitality and wood mycobiome

The study of the natural population with varying degrees of dieback brought out some notable taxa. Firstly, *Geosmithia* spp. was extremely abundant in the declining tree RIV2. Concurringly, it was

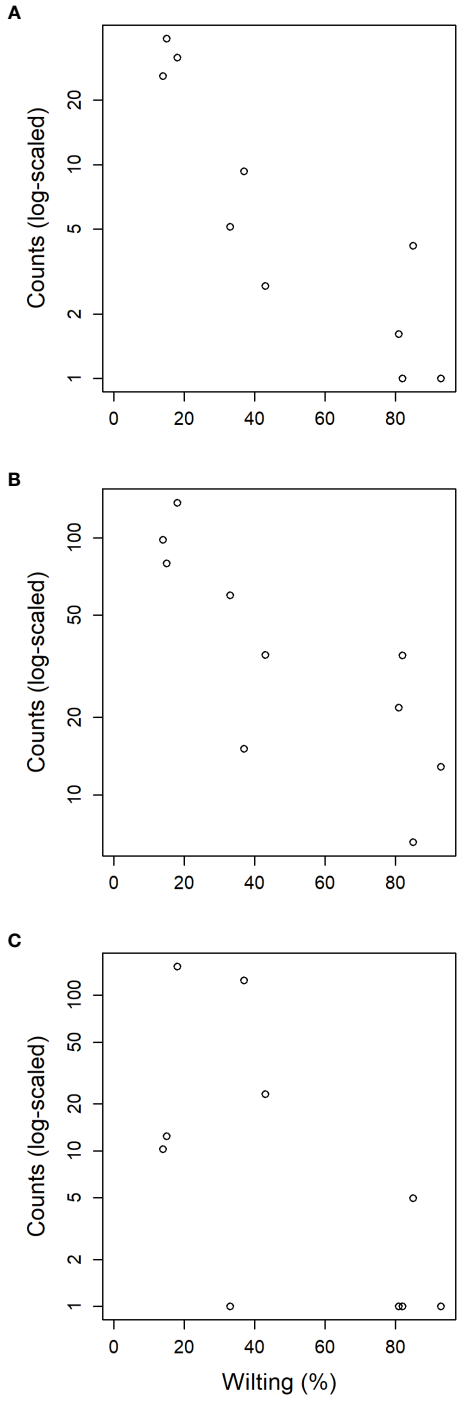

FIGURE 6
Relation between susceptibility to DED (measured as leaf wilting percentage) of the ten clonal bank genotypes and the normalized counts detected from reads of endophytic fungal families **(A)** Buckleyzymaceae, **(B)** Trichomeriaceae and **(C)** Bulleraceae.

identified as the dominant fungi in a *U. minor* tree with extensive dieback symptoms in the absence of DED pathogens (Hänzi et al., 2016). Certain *Geosmithia* fungi could therefore act as opportunistic or latent pathogens in elms, as previously reported by Hänzi et al. (2016). The presence of this genus in the healthy tree (RIV5) suggests that it is able to live as an endophyte in latent pathogenicity. Pepori et al. (2018) found that elms inoculated with *Geosmithia* fungi remained largely asymptomatic, and joint inoculation of *Geosmithia* and *O. novo-ulmi* reduced wilting symptoms compared to inoculation with *O. novo-ulmi* only. They also found parasitic behaviour of *Geosmithia* towards *O. novo-ulmi*. In elms, *Geosmithia* was frequently found in DED-infected trees (Pepori et al., 2015), most likely carried there by the beetles that are also the vectors of DED pathogens. Further research is needed into the potential contribution of *Geosmithia* to tree dieback in Rivas or, in contrast, the potential role of this taxon in the phenotypic avoidance of DED found in this elm stand.

Secondly, two other trees with dieback symptoms (RIV6 and RIV4) were dominated by Nectriaceae (especially RIV4). OTU_92 (*Fusarium*) was responsible for this signature and was also very abundant in the healthy RIV3. The family Nectriaceae (Sordariomycetes) includes facultative parasites that cause stem cankers, and saprobes. In elms, dieback symptoms have been associated with colonization by *Nectria* sp. (Heybroek, 1993; Plante and Bernier, 1997).

4.4 Core microbiome and among-site variation

Sampling from different spots in a single tree and from genetically different trees enabled the detection of robust signatures of a core microbiome. Out of the 231 OTUs found in the landmark tree, 11 were present in all samples (10) and 22 in more than seven samples (Table 1). In the clonal bank, eight OTUs were present in eight trees, seven were present in nine trees and another seven were in all trees (10). In the landmark tree and the clonal bank, the number of OTUs did not decrease following the pattern expected by randomness. The number of OTUs reached a tableau beyond five samples in both distributions (Figures 3A,B), and a relative maximum at the end of the distribution in the landmark tree (Figure 3A). Therefore, the probability that a given sample would contain a specific OTU depended on the OTU in question. Thus, not all OTUs can be considered rare events (i.e. events that would display Poisson distributions). Others with high probabilities of occurrence displayed different distributions (Poisson distributions, but with "absence of OTU" as rare event). Although not appreciable, perhaps due to their low numbers, other OTUs may have behaved as "medium frequency events", retrieving binomial distributions. Thus, the lack of agreement between the observed distributions and the expected monotonic decrease, characteristic of pure Poisson processes, shows that OTU occurrences range from rare to highly frequent. OTUs that follow a pattern of occurrence consistent with a Poisson distribution could be considered local infections with arguably different but low likelihoods of infecting a stem. Highly frequent OTUs, on the other hand, are likely to be members of the core microbiome. It is unclear why this latter group of endophytes is pervasive, but it could be explained by a high infective capacity (Griffin and Carson, 2018) (e.g. through insect vectors, rain and wind) and/or systemic propagation within the plant, as occurs in some endophytic yeasts (Joubert and Doty, 2018). Shallower sampling may not have allowed us to distinguish between the two trends in OTU occurrence, because the distributions would have overlapped, obscuring the underlying pattern. The most

commonly found fungal taxa both in the landmark tree and the clonal bank were the ascomycetous classes Dothideomycetes, Eurotiomycetes, Sordariomycetes, Leotiomycetes and Lecanoromycetes, and the basidiomycetous classes Tremellomycetes and Cystobasidiomycetes.

We identified 32 core OTUs by defining the core microbiome as the OTUs that are present in at least eight out of 10 samples in either the landmark tree or the clonal bank, and present in at least two populations. Although most of them were present in most samples across the four populations, some were abundant in the clonal bank but rare or absent in the landmark tree (e.g. OTU_18 and OTU_38). Considering that the clonal bank includes trees from various provenances across Spain (Supplementary Table 1) and a few are from the same provenance as the landmark tree, it is conceivable that these OTUs are controlled mostly by environmental cues (Zimmerman and Vitousek, 2012). Conversely, a few OTUs were widespread in the landmark tree, but rarer in the clonal bank (e.g. OTU_66, OTU_80 and OTU_102). OTU_66 and OTU_80 were present in the four populations and most of the samples but surprisingly lacking in some trees from the clonal bank. This pattern hints at an implication of host genotype (see Bálint et al. (2013)). However, physiological status and microscale environmental variation could also explain this pattern. The clear separation of samples by site shown in the Principal Component Analysis (Figure 2) indicates the important role of geographical location in shaping fungal endobiome communities. New targeted experiments are needed to confirm or refute these hypotheses.

5 Concluding remarks

We found clear evidence of the existence of a core endophytic mycobiome in elm stems, which account for circa 10% of the total endophyte richness. Our study strongly suggests that some core endophytes are associated to DED resistant genotypes. Recent works have shown the beneficial role of some endophytic yeasts in *U. minor* resilience against stress and in priming defenses against *O. novo-ulmi* (Martínez-Arias et al., 2021a). Therefore, resistant trees could not only display inherent genetic mechanisms of resistance, such as narrow earlywood vessels (Martín et al., 2021) or an early molecular response against the pathogen (Sherif et al., 2016), but could also benefit from mechanisms of resistance provided by their symbiotic microbiome. If this microbiome were heritable, new possibilities for elm breeding could arise directed to improve microbial functioning. Otherwise, the possibility of transplanting beneficial microbiomes could open new prospects for the fight against the disease.

Data availability statement

The datasets of the demultiplexed raw reads for this study can be found in the European Nucleotide Archive with the accession number PRJEB58145. R scripts used for this study and some processed datasets are stored at https://github.com/dmacaya/core-elm-mycobiome.

Author contributions

DM-S and JM contributed to the conception and design of the study. DM-S and JM performed the sampling and sample processing. CC supervised the molecular work at the lab. DM-S performed the molecular work, bioinformatics and statistical analysis. DM-S and JM wrote the draft of the manuscript. JW, CC, and LG revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the project CTQ2011-28503-C02-02 (Ministerio de Economía y Competitividad, Spain). The position of DM-S was partly funded by the program "Atracción de Talento Investigador" of the Community of Madrid (ref. 2019-T2/BIO-12780).

Acknowledgments

We acknowledge the support of Ministerio para la Transición Ecológica y el Reto Demográfico in the sampling of elms at Puerta de Hierro Forest Breeding Centre. A preliminary version of this study was released as a preprint in Macaya-Sanz et al. (2020) with doi: 10.1101/2020.06.23.166454.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 09 March 2023
DOI 10.3389/fpls.2023.1082480

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Plant Symbiotic Interactions, a section of the journal Frontiers in Plant Science

RECEIVED 28 October 2022
ACCEPTED 20 February 2023
PUBLISHED 09 March 2023

CITATION
Irshad K, Shaheed Siddiqui Z, Chen J, Rao Y, Hamna Ansari H, Wajid D, Nida K and Wei X (2023) Bio-priming with salt tolerant endophytes improved crop tolerance to salt stress *via* modulating photosystem II and antioxidant activities in a sub-optimal environment. *Front. Plant Sci.* 14:1082480. doi: 10.3389/fpls.2023.1082480

Bio-priming with salt tolerant endophytes improved crop tolerance to salt stress *via* modulating photosystem II and antioxidant activities in a sub-optimal environment

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Abiotic stress is one of the major constraints which restrain plant growth and productivity by disrupting physiological processes and stifling defense mechanisms. Hence, the present work aimed to evaluate the sustainability of bio-priming salt tolerant endophytes for improving plant salt tolerance. *Paecilomyces lilacinus KUCC-244* and *Trichoderma hamatum Th-16* were obtained and cultured on PDA medium containing different concentrations of NaCl. The highest salt (500 mM) tolerant fungal colonies were selected and purified. *Paecilomyces* at 61.3×10^{-6} conidia/ml and *Trichoderma* at about 64.9×10^{-3} conidia/ml of colony forming unit (CFU) were used for priming wheat and mung bean seeds. Twenty- days-old primed and unprimed seedlings of wheat and mung bean were subjected to NaCl treatments at 100 and 200 mM. Results indicate that both endophytes sustain salt resistance in crops, however *T. hamatum* significantly increased the growth (141 to 209%) and chlorophyll content (81 to 189%), over unprimed control under extreme salinity. Moreover, the reduced levels (22 to 58%) of oxidative stress markers (H_2O_2 and MDA) corresponded with the increased antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) activities (141 and 110%). Photochemical attributes like quantum yield (F_V/F_M) (14 to 32%) and performance index (PI) (73 to 94%) were also enhanced in bio-primed plants in comparison to control under stress. In addition, the energy loss (DI_O/RC) was considerably less (31 to 46%), corresponding with lower damage at PS II level in primed plants. Also, the increase in I and P steps of OJIP curve in *T. hamatum* and *P. lilacinus* primed plants showed the availability of more active reaction centers (RC) at PS II under salt stress in comparison to unprimed control plants. Infrared thermographic images also

showed that bio-primed plants were resistant to salt stress. Hence, it is concluded that the use of bio-priming with salt tolerant endophytes specifically *T. hamatum* can be an effective approach to mitigate the salt stress cosnequences and develop a potential salt resistance in crop plants.

KEYWORDS

Chlorophyll 'a' fluorescence, bacterial priming, wheat, mung bean, ionic stress, response

Introduction

The twin goals of ensuring global agricultural productivity and its execution in a sustainable manner are challenged due to the increased incidence of ecological catastrophes (Ebert and Engels, 2020). As a result, our agriculture system is frequently subjected to both biotic and abiotic stress. In the last few decades, a number of studies have been reported the effect of abiotic and biotic stressors on crops (Chinnusamy et al., 2005; Kwon et al., 2009; Fizza et al., 2021; Ansari et al., 2022), highlighting the alternate means of controlling the negative impacts of such stressors and sustain plant growth in a sub-optimal environment. Moreover, out of many environmental fluctuations, soil salinization has become a fundamental enigma as it has been encountered in all climates. The assault of this salinity stress, which is mainly caused by sodium ions, can be observed in the germination, growth, development, and reproduction of the crop (Mahmood et al., 2021). Hence, soils are rendered hypersaline due to the prevalence of NaCl by natural or anthropogenic means, which decreases crop production by more than 20% (Porcel et al., 2012). In response to salt stress, plants show plasticity in terms of periodic adjustment like osmolyte synthesis due to physiological modifications in their defensive metabolism (Nephali et al., 2021). However, the strategies to adapt salt tolerance in crops have become insufficient to overcome extreme salinity (Augé et al., 2014). Thus, to mitigate the salt stress and sustain the modern agriculture system, various biotechnological approaches have been employed to ensure crop productivity.

Among such approaches, bio-priming has been considered an innovative and sustainable method for alleviating plant salt stress. Seed bio-priming is a strategy of seed treatment (seed priming) for regulating plant growth, managing stress, and improving seed germination (Sarkar et al., 2021). Moreover, seed priming alone (osmo-priming, matrix priming) or in combination with a low dosage of biocontrol agents have been reported to increase the germination rate, uniformity and sustainability of plant growth and development under sub-optimal environment (Johnson and Puthur, 2021). However, Seed priming *via* conventional and specifically chemical means impaired the soil ecosystem, which creates fluctuations in the food chain. Therefore, seed bio-priming with plant growth-promoting microbes (PGPM) that are naturally colonized around the root zone of the plants has a great potential to increase the plant's performance in a suboptimal environment (Dimkpa et al., 2009).

In addition, it is currently being recognized that the application of endophytes offers a great potential to reduce the abiotic and biotic stress in plants. Lately, the application of endophytes to reduce the hypersaline stress in plants has also been reported (Sandhya et al., 2009; Yao et al., 2010; Verma et al., 2021). Several studies suggested that the endophytes sustained growth by increasing the uptake of nutrients such as zinc, phosphorus, boron and copper and making other nutrients available to plants in a saline-sodic soil (Sarma et al., 2015; Liu et al., 2017).

Paecilomyces lilacinus and *Trichoderma hamatum* are endophytic saprophyte fungus that can be found in different soil types and have the ability to grow in a broad range of soil pH having sodium ions. *P. lilacinus* is effectively used to control nematode growth as it has the ability to penetrate and destroy the embryo. Similarly, *T. hamatum* is a beneficial endophytic plant symbiont, compared to *P. lilacinus* which is widely used to control fungal diseases in crop plants (Afzal et al., 2013). Some reports indicate that that *Trichoderma* enhanced the tolerance to abiotic stress in plants (Shoresh et al., 2010; Estrada et al., 2013). However, the role of *P. lilacinus* in plants to enhance stress tolerance against abiotic stress has not been reported so far. Hence, the present study aimed to probe the application of *P. lilacinus* and *T. hamatum* as an effective bio-priming agent in crop plants against hypersaline environment.

Plant photosynthesis coupled with defense mechanisms are the prime physiological modulations that indicate the health status of the crops. The thorough analysis of the photosynthetic apparatus *via* non-destructive approach like chlorophyll fluorescence can mimic the real time changes in perturbation and light harvesting efficiency of the photosynthetic membrane. Furthermore, light harvesting complexes and reaction centers of PS II are not only true source of energy production but also plays a crucial role to stress tolerance under abiotic stresses. Therefore, the present study evaluated the sustainable role of isolated endophytes through seed-priming on photo-physiology, light harvesting efficiency, energy fluxes, and subsequent antioxidant system in two important crops, under a suboptimal environment. Also knowing that the energy exploitation in the photosynthetic apparatus of bio-primed plants during salt stress tolerance has not been documented so far. Likewise the application of *T. hamatum* and *P. lilacinus* as a

bio-priming agent to enhance salt tolerance in plants is yet to be studied In essence, the current research was designed to scrutinize the energy distribution inside the photosynthetic membrane by non-destructive means to explicate the energy source for the induction of salt tolerance in plants due to bio-stimulating natural colonizers i.e., *T. hamatum* and *P. lilacinus*.

Materials and methods

Seed source and selection

Seeds of Wheat *(Triticum aestivum)* and Mung bean *(Vigna radiata)* were collected from the Stress Physiology Phenomic Centre, Department of Botany, University of Karachi, and surface sterilized into 10% NaClO (sodium hypochlorite) for 3 min to remove the surface fungus and dust. Seeds were then thoroughly washed with distilled water to remove NaClO traces.

Collection and purification of beneficial endophytic fungi

The plant-beneficial fungal endophytic fungi *P. lilacinus* and *T. hamatum* were obtained from Karachi University Culture Collection (KUCC) and purified on PDA (Potato Dextrose Agar) with several replicates. Saline medium of PDA was prepared to examine the salt tolerance of *P. lilacinus* and *T. hamatum*, having several concentrations of NaCl (100, 200, 300, 400, and 500 mM) in its composition. These sets were kept at room temperature 30-34 ± 2°C for 7 days to select salt-tolerant endophytic strains and later it was used for further study (Figure 1). The Colony-forming unit (CFU) was maintained at 61.3×10^{-6} Conidia/ml of *Paecilomyces* and about 64.9×10^{-3} Conidia/ml of *Trichoderma* colony forming units (CFU) per milliliter for liquid as:

$$Cfu/ml = \frac{No.\ of\ colonies \times\ dilution\ factor}{The\ volume\ of\ the\ culture\ plate}$$

Inoculation of fungal endophytes by seed priming technique

The endophytic fungi *P. lilacinus* and *T. hamatum* were inoculated in plants by seed bio-priming technique as described by Saeid et al. (2018). Seeds of Wheat and Mung bean were selected for the inoculation of endophytes. The fungal suspension was prepared from pure PDA cultures by adding 10 ml of sterile distilled water into fungal plates. Plates were slightly scratched by a wire loop and fungal suspension was poured into a beaker (the process was repeated twice). The final volume was made up to 100 ml with sterile distilled water to make the stock. From the fungal spore stock, 25 ml was taken and made up the volume up to 100 ml with sterile distilled water to prepare 25% fungal suspension. Later, the surface sterilized and dried seeds of both crops were treated by soaking in the spore suspensions prepared for different time intervals (5, 10, and 15 min). The seeds were dried under a sterile air stream in laminar air flow for 2 h (Singh et al., 2013).

Experimental design and stress application

The experiment was conducted at the Stress Physiology Phenomic Center, Department of Botany, University of Karachi, Pakistan. Under natural environmental conditions, the experiments were organized in a completely randomized design to analyze

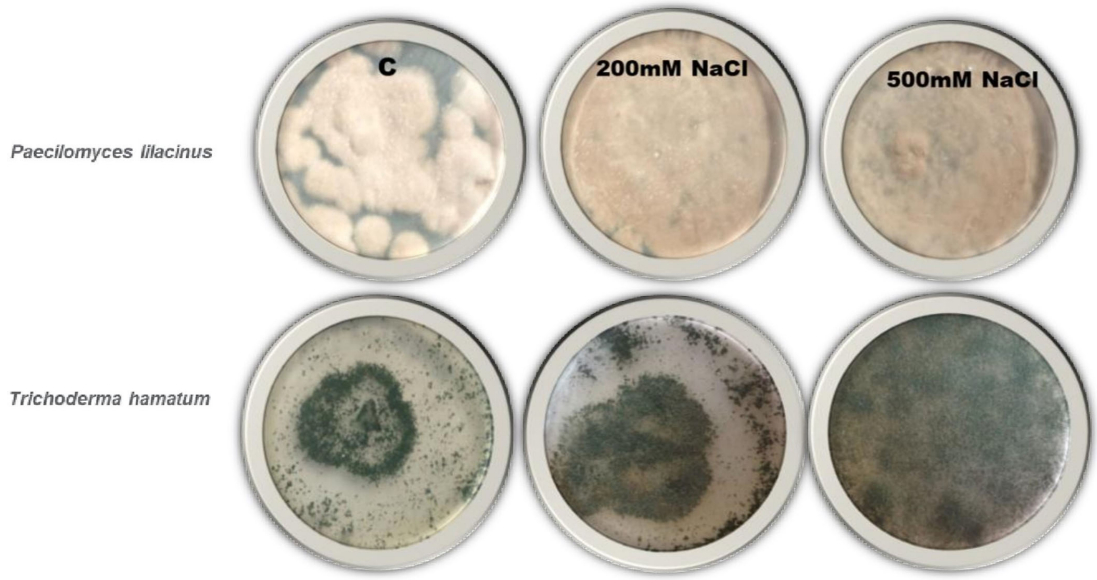

FIGURE 1
Endophytes culture on high saline medium to use in plant. Highest salt tolerance endophytes culture was used in further study.

endophytic symbiosis with the crop plant. Two sets of experiments were conducted, 1) Seeds without inoculation of *P. lilacinus* and *T. hamatum* and 2) Seeds with inoculation of *P. lilacinus* and *T. hamatum*. Ten treated seeds were sown per pot having 1 Kg of soil and allowed to germinate. The composition of the soil is 80.5% sand particles, 7.1% silt and 8.1% clay, 4.10% organic carbon, 0.83% total nitrogen, pH 7.6, and electrical conductivity was 1.7 $dS.m^{-1}$. Wheat and Mung bean were allowed to grow at an average day-night temperature of 33 ± 4°C to 22 ± 3°C. Twenty days old inoculated and un-inoculated seedlings were treated with different salt concentrations by gradual increment method to reach 100 and 200 mM NaCl. In this regard, 50 mM for 200 mM and 25 mM for 100 mM was given on alternate days. The moisture level was maintained by adding up water as stated by Umar and Siddiqui (2018). The whole setup of the experiment was repeated with four replicates of treatments and controls. The plants were exposed to saline treatments for 7 days and later plants were harvested.

Relative water content

For the calculation of Relative water content (RWC) Barrs and Weatherley (1962) method was applied with some modifications. Randomly selected leaves of each control and treated samples of an area of 4 × 2 cm^2 of wheat and 1.2 cm^2 of Mung bean were excised from the mid-veins and the edge section and fresh weight (FW) was recorded. Later, leaves were kept in Petri plates of 90 mm diameter for12 h, which contain distilled water. Afterward, the leaves samples were taken off the Petri plate and turgid weight (TW) were recorded. For the measurement of the dry weight (DW), leaves samples were oven dried at 80°C for 48 hours. RWC was calculated by using the formula:

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

Stomatal conductance and chlorophyll content index

For the observation of stomatal conductance, young randomly selected leaves of Wheat and Mung bean from each treated and control intact plant was used between 9:00 A.M. and 11:00 A.M. For this investigation, Decagon Leaf Porometer (Model SC-1) was used, and data were recorded from the middle and lower part of the leaf surface. The stomatal conductance of leaf was expressed as mmole $m^{-2}s^{-1}$. Similarly, the chlorophyll content index (CCI) of young leaves of each treated and control intact plant leaf was recorded between 9:00 A.M. and 11:00 A.M. Chlorophyll Content Meter CCM-200; Opti-Sciences Inc., Hudson, NH, USA was used. The average values of ten leaves of each replicate were used to show in bar graphs.

Photochemical traits of photosystem II

For the photochemical traits of Photosystem II assessment, chlorophyll fluorescence was recorded by using as Opti-Sciences Fluorometer (Model OS-30 p^+; Hudson, USA). For the analysis, the youngest and fully expended leaves were selected between 9:00 A.M. and 11:00 A.M. From intact plants, leaves were clipped for 60 min for dark-adapted measurement from each treatment and control plant. Light-adapted quantum yield was recorded under a normal day-light environment. Performance index (PI_{ABS}), Original (F_O), and maximum (F_M), the dark-adapted quantum yield of PS II photochemistry was calculated by the ratio of variable to maximum fluorescence (F_V/F_M), photochemical quenching (qP), and JIP test data was used to calculate as described by Strasser et al., 2010; Stirbet and Govindjee, 2011 (Supplementary Table 1).

IR thermal images

FLIR-E5 (FLIR Systems, USA) was used before harvesting. IR thermal sensor observed the infra-red thermography from each Wheat and Mung bean treated and control plant. Before the measurement, the system was optimized for 60-90 min, and later on, images were taken. A computerized report was generated using FLIR Software 2.10 after transferring the images into the computer.

H_2O_2 content

Total hydrogen peroxide (H_2O_2) content was estimated according to the method described by Velikova et al. (2000). Freshly harvested leaf samples were homogenized in 3 ml of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath. Afterward, homogenate was centrifuged at 12000 rpm for 15 min. Later on, 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide (KI) were mixed with 0.5 ml of supernatant. Optical density of the supernatant was taken at 390 nm. H_2O_2 content was estimated with reference to a standard curve and expressed in mmole g^{-1} FW.

$$H_2O_2 \ \ Content = Ve \times R \times \frac{D.F}{Vs} \times \ \ W$$

Where,

Ve = Volume used for the estimation, R = Reading from the standard curve, D.F = Dilution factor, Vs = Volume of extract, W = Weight of leaf sample.

Malondialdehyde content

Lipid peroxidation in the leaf tissues was observed by Dhindsa et al. (1981), the amount of malondialdehyde (MDA) produced by the reaction of Thio-barbituric acid (TBA). Freshly harvested leaves samples of 0.25 g were homogenized with 0.1% trichloroacetic acid (TCA) in a pestle and mortar and centrifuged at 10,000 rpm for 5 min. 1mL supernatant was added into 4 ml of 20% TCA containing 0.5% TBA. The mixture was heated for 30 min in a water bath at 95 °C and allowed to cool. Absorbance was recorded at 532 and 600 nm. MDA-TBA extinction co-efficient was recorded at 532 nm.

$$Conc.\ \ of\ \ \ MDA\ \ (\mu M) = \frac{(A_{532} - A_{600})}{155}$$

Antioxidant enzymes activity

Leaf sample of 500 mg in liquid nitrogen (5°C) was homogenized with 10 ml of abstraction buffer (Tris-HCl pH 6.8, 10 ml DDT, 0.1 mM EDTA, 50 mg PVP) for enzymatic antioxidant evaluation. The mixture was centrifuged at 15,000 rpm for 10 mins to estimate total protein by the method described by Bradford (1976). The antioxidant enzymes i.e., Superoxide Dismutase (EC # 1.15.1.1) and catalase (EC # 1.11.1.6) was measured by the method of Beyer and Fridovich (1987) and Patterson et al. (1984), respectively.

Statistical analysis

The data generated from the treated and control groups were subjected to statistical analyses using the software SPSS Version 20 (IBM, United States). The Bonferroni *Post- hoc* test was applied to differentiate significant differences among the mean values of different treatments and presented as small alphabets on the bar graphs ($p< 0.05$).

Results

Morphological response of plants against different priming treatments

In the sub-optimal environment, seedling length of wheat and mung bean plants was significantly reduced compared to control (Figure 2). It was evident from the data that the maximum reduction in root and shoot length was observed in wheat (13.83 and 17.4 cm) and mung bean (6.77 and 13.5 cm) plants when exposed to 200 mM salt stress. However, bio-priming with *T. hamatum* and *P. lilacinus* alleviates the salt stress and thus increases the seedling length of wheat from 26 to 149% and mung bean from 5 to 216% (Supplementary Table 2). It was observed that bio-priming agents results in a more profound increase in the root length as compared to the shoot length. However, general trend shows that the increase in priming duration such as 5, 10, and 15 minutes had a positive impact on the shoot length in both plants compared to root length. Unlike, the percentage of root length with

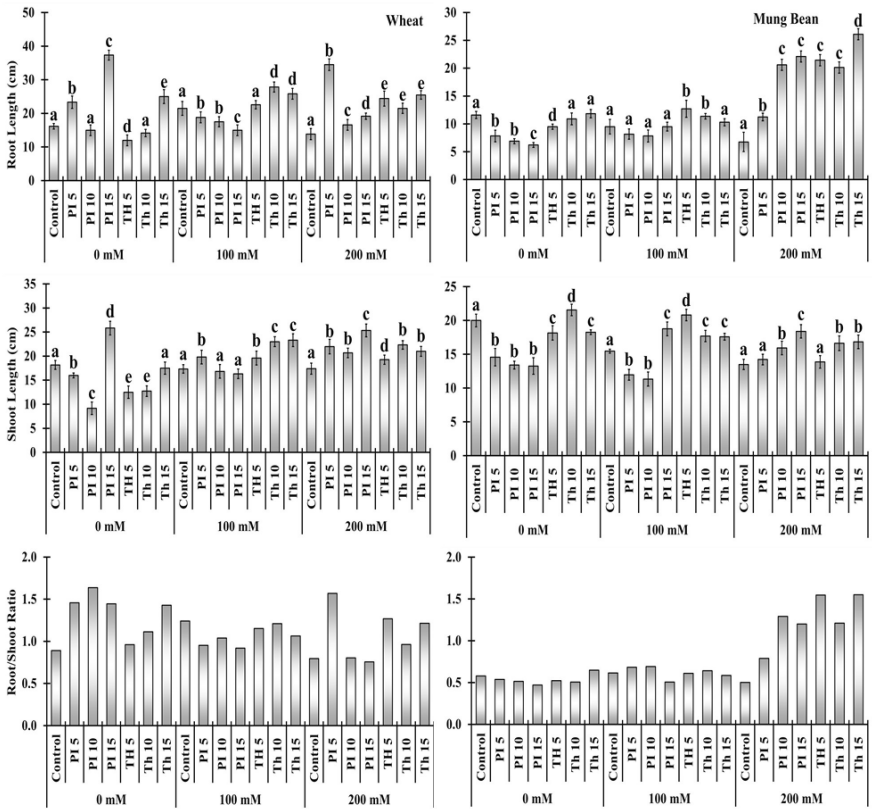

FIGURE 2
Effects of bio- priming with fungal endophytes on Root Length (RL), Shoot Length (SL) and Root/Shoot ratio of wheat and mung bean grown under saline environment *Note*: The symbols on the horizontal axis represents: Control: Seeds without priming, Pl _ Seed priming with *Paecilomyces lilacinus*, *Th* _ Seed priming with *Trichoderma hamatum* 5, 10, 15 = duration of bio-priming in minute, 0, 100 & 200 mM NaCl concentration. On bars, vertical lines represent ± Mean Standard Error (S.E) and similar alphabets represents non-significant difference between the the means of treatment at $p<0.05$.

respect to time duration was slightly increased in bio-primed treated wheat (20 to149%) and substantially increased in mung bean plants (66 to 285%) under 200 mM salt stress (Figure 2, Supplementary Table 2). Among all the treatments, the highest root-to-shoot ratio was observed in mung bean plants when it was primed with *T. hamatum* (141 to 209%) salt stress, followed by *P. lilacinus* (57 to 157%) under 200 mM salt stress. However, the root to shoot ratio was comparatively much lower in wheat plants compared to mung bean (Figure 2).

Chlorophyll content index and stomatal conductance

Salt stress substantially reduced the chlorophyll content index (CCI) and stomatal conductance of the unprimed plants in comparison to the primed. Bio-priming with *T. hamatum* significantly increased CCI over control in wheat plants with an increase in priming duration, which was about 141 to 285% under 100 mM and 81 to 189% in 200 mM salinity (Figure 3, Supplementary Table 2). Moreover, *P. lilacinus* priming had a substantially negative effect on wheat plants at 100 mM salt stress, displaying a decline in CCI percentage over control (-43, -42 and -44%) but substantially increased the CCI content of wheat plants over control under 200 mM salt stress (44, 83, and 362%). *P. lilacinus* expressed more profound effect on the mung bean plants compared to wheat, had significantly increased the CCI at both 100 and 200 mM salt stress (47 - 170%and 35 - 61%).

Two types of the consequential stimulated regime by priming agents in wheat and mung bean plants regarding stomatal conductance were observed under extreme salinity (200 mM). Stomatal conductance was significantly decreased in wheat plants over the control when primed with *T. hamatum*, (-47, -32, and -16%) and with *P. lilacinus* (-28, -17, and -14%). In contrast, in mung bean plants, both priming agents substantially increased the stomatal conductance over the control (9, -8, 3, 159, 65 and -6%) with some exceptions under 200 mM salt stress respectively (Figure 3, Supplementary Table 2).

Oxidative damage markers

Elevated level of H_2O_2 and MDA in un-primed plants indicates that salt stress relatively increased the oxidative stress. Bio-priming alleviates the stress in wheat and mung bean plants as the oxidative damage was relatively lower than in control plants. Under 100 mM salt stress, H_2O_2 was relatively lower in wheat plants primed with *T. hamatum* (-15, 23, and -22%) and *P. lilacinus* (-52, -21, and -12%) with some exceptions. (Figure 4, Supplementary Table 2). Moreover, the MDA content among the primed plants was considerably lower in both wheat and mung bean plants in comparison to the control plants. It was evident from the data that MDA content was considerably decreased with the priming of *T. hamatum* (-47, -39, and 58%) than with *P. lilacinus* (-29, -32, and 4.98%) in wheat plants under high salinity (200 mM).

Photochemical attributes

Salt stress results in a significant decrease in the performance index (PI) and an increase in the dissipation per reaction center (DI_O/RC) in wheat and mung bean plants, which was later overcome by bio-priming. Results showed that under 200 mM salt stress, the highest PI was observed in mung bean plants primed

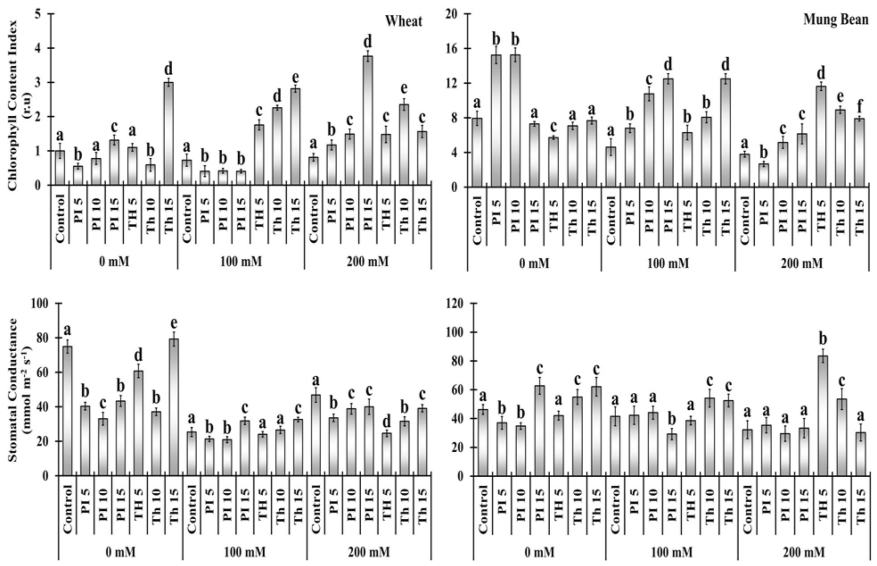

FIGURE 3
Effects of bio-priming on chlorophyll content index (CCI) and stomatal conductance (gs) of wheat and mung bean plants grown under saline environment. The symbols on the horizontal axis represents: Control: Seeds without priming, Pl _ Seed priming with *Paecilomyces lilacinus*, *Th*_ Seed priming with *Trichoderma hamatum* 5, 10, 15 = duration of bio-priming in minute, 0, 100 &200 mM NaCl concentration. On bars, vertical lines represent ± Mean Standard Error (S.E) and similar alphabets represents non-significant difference between the means of treatment at $p<0.05$.

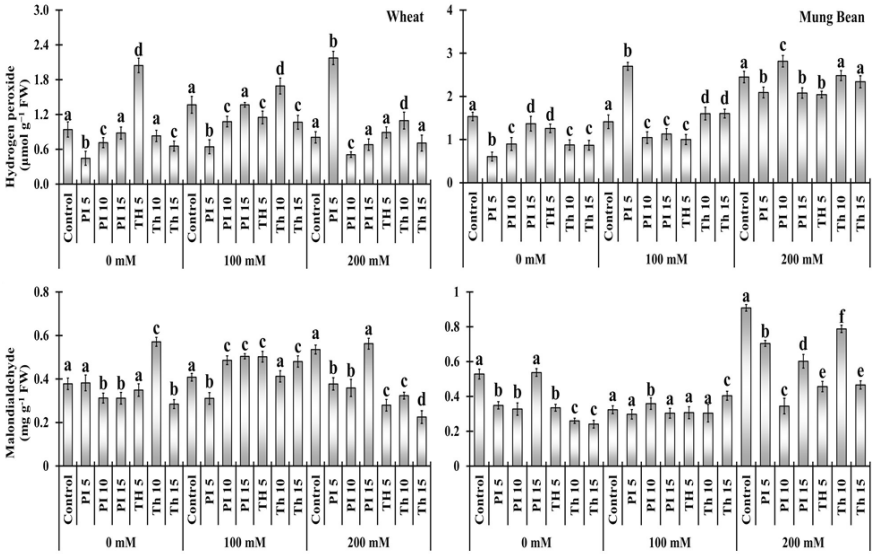

FIGURE 4
Effects of bio-priming with fungal endophytes on hydrogen peroxide (H_2O_2) and Malondialdehyde (MDA) contents of wheat and mung bean grown under saline environment. The symbols on the horizontal axis represents: Control: Seeds without priming, PI _ Seed priming with *Paecilomyces lilacinus*, *Th* _ Seed priming with *Trichoderma hamatum* 5, 10, 15 = duration of bio-priming in minute, 0, 100 & 200 mM NaCl concentration. On bars, vertical lines represent ± Mean Standard Error (S.E) and similar alphabets represents non-significant difference between the means of treatment at $p<0.05$.

with *T. hamatum* (94%) followed by *P. lilacinus* (73%) over the control (unprimed plants). Likewise, a similar trend was observed regarding the maximum quantum yield of PS II (F_V/F_M) in mung bean plants (32 and 26%) in comparison to the un-primed stress plants. In wheat plants, priming of *P. lilacinus* caused the highest PI and F_V/F_M (455 and 18%), followed by *T. hamatum* (357 and 14%) under 200 mM salt stress. However, one way to assess the plant's performance is to observe the release of absorbed energy, which indicates the performance of the plant under stress conditions. In the present study, we found that dissipation per reaction center (DI_O/RC) was significantly decreased due to the priming in both wheat (-31, -42, and -35%) and mung bean (-39, -42, and -46%) under the extreme salinity level (200 mM) (Figure 5, Supplementary Table 2).

The OJIP induction curve analysis showed the effect of salt stress as the increase in salinity level (from 0, 100, and 200 mM) caused the decline in the fluorescence intensity (OJIP curve) of the un-primed wheat plants. Highest peaks of the induction transients were observed among the bio-primed plants under both non-stress and stress conditions (*T. hamatum* and *P. lilacinus)*, while the lowest curve was displayed by the unprimed 200 mM stress plants. However, one striking pattern was observed among the OJIP curve of plants primed with *T. hamatum* (10 min priming duration) in wheat and mung bean plants. In wheat plants under control (unstressed) conditions, the aforementioned treated plants showed the lowest induction curve, which was moderately increased under 100 mM salt stress and led to the highest peak of all under 200 mM salt stress. In contrast, a complete revert pattern was observed in mung bean plants. *T. hamatum* (10 mins) primed plants had the highest induction curve values in the control environment, which then decreased to moderate values and then further decreased to a lower curve in the high salinity (200 mM) environment (Figure 6). Moreover, in mung bean plants, the lowest curves were still attributed to the un-primed plants, showing the stress retardation in the photosynthetic machinery of the mung bean plants. The highest curves were exhibited by the plants primed with *P. lilacinus* under 200 mM stress.

Antioxidant enzymes

Antioxidant enzymes including super oxide dismutase (SOD) and catalase (CAT) activities were measured at different NaCl concentrations with and without endophytes i.e. *P. lilacinus* and *T. hamatum* application. In comparison to the control condition, SOD and CAT activities were stimulated by the degree of salinity stress at 100 mM (44 to 141%) and 200 mM (27 to 110%) in both varieties. However, among the two varieties, the increment of SOD and CAT in wheat was greater in comparison to mung beans. Moreover, among the priming treatments, *T. haamatum* (15 min) prompted the highest SOD (141, 151, 74 and 110%) and CAT (141, 71, 62 and 62%) activity under increasing salt stress over the control, in which the least antioxidant activity was observed. Besides, among different treatments of *P. lilacinus* the highest increment in SOD (44 to 72%) and CAT (40 to 101%) activities was attributed to the 15 min of priming in both varieties. (Figure 7).

Discussion

Due to the changing climate and the increasing assault of abiotic stress, agricultural productivity is heavily curtailed. In the present

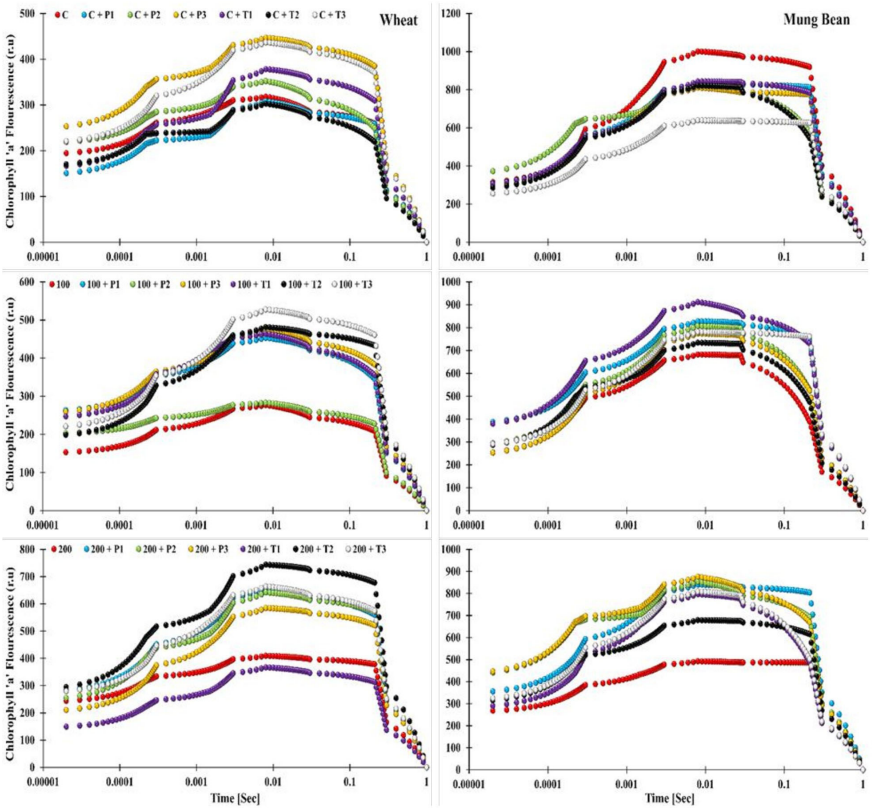

FIGURE 5
Effects of bio-priming with fungal endophytes on OJIP transient curve of wheat and mung bean grown under saline environment. The symbols on the horizontal axis represents: Control: Seeds without priming, Pl = Seed priming with *Paecilomyces lilacinus*, Th= Seed priming with *Trichoderma hamatum* 5, 10, 15 = duration of bio-priming in minute, 0, 100 & 200 mM NaCl concentration.

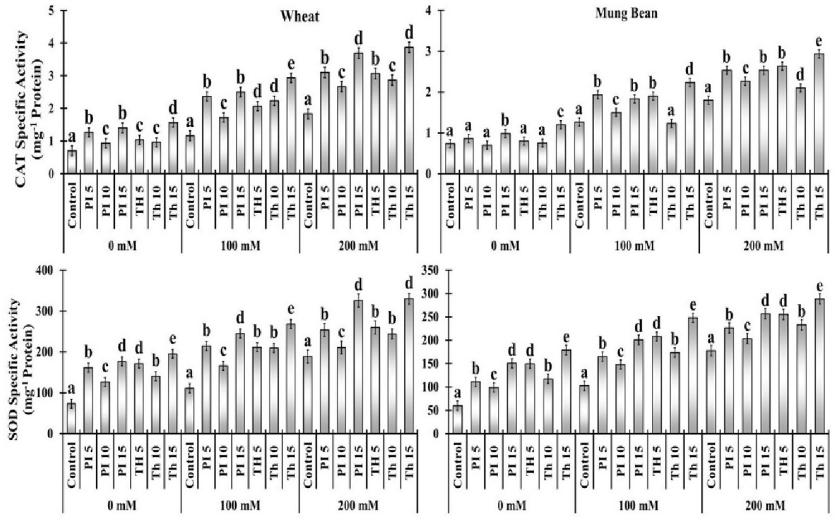

FIGURE 6
Effects of bio-priming with fungal endophytes on Catalase Specific Activity (CAT) and Superoxide Dismutase Specific Activity (SOD) contents of wheat and mung bean grown under saline environment. The symbols on the horizontal axis represents: Control: Seeds without priming, Pl = Seed priming with *Paecilomyces lilacinus*, Th= Seed priming with *Trichoderma hamatum* 5, 10, 15 = duration of bio-priming in minute, 0, 100 & 200 mM NaCl concentration. On bars, vertical lines represent ± Mean Standard Error (S.E) and similar alphabets represents non-significant difference between the means of treatment at $p<0.05$.

study two sodium-tolerant biological priming agents, namely *T. hamatum* and *P. lilacinus*, with three priming durations (5, 10, and 15 min) were used. Later seeds were allowed to germinate and grow in a salt-stress environment. It was observed that the root and shoot length of both wheat and mung bean plants declined with the elevating salt stress. It is evident from the literature that salt stress inhibited plant growth in a sub-optimal environment (Dey et al., 2004; Azooz et al., 2013; Fizza et al., 2021; Ansari et al., 2022). The decrease in plant growth is attributed to nutrient imbalance, osmotic, and ionic stress (Iqbal and Ashraf, 2013; Rasool et al., 2013; Alqarawi et al., 2014). In the present study, it was observed that the priming with *T. hamatum* and *P. lilacinus* increased the root and shoot length of both wheat and mung bean plants in a sub-optimal environment (Figure 2). The highest and most significant amelioration was observed in mung bean plants by virtue of *Trichoderma* priming. Our findings are in accordance with those of Mastouri et al. (2010) and Rawat et al. (2013), who found that *Trichoderma* isolates mitigate the negative effects of salt stress in several plants. It was reported that *Trichoderma* is symbiotically associated with plants and thus enhances plant growth due to hormonal modulation or molecules closely related to GA_3 (Iqbal and Ashraf, 2013; Rawat et al., 2013). Thus, *Trichoderma* association also elongates roots, which aids plants in absorbing nutrients and water from the soil and improves their ability to withstand salt stress (Arora et al., 1992). Likewise, some *Paecilomyces* spp. has also enhanced plant growth *via* growth-regulating metabolites like IAA and GA that could work to ameliorate the stress (Bashri and Prasad, 2016; Liu et al., 2019).

Our results, with respect to the decrease in chlorophyll content index (CCI) under salt stress are supported by the findings of Ahmad et al. (2016) for *Cicer arietinum*, and Alqarawi et al. (2014) for *Ephedra alata*. The decrease in pigment content is attributed to several factors, including the detrimental effects of salt stress on chloroplast (Zörb et al., 2009), increased activity of chlorophyllase and the consequent reduction in chlorophyll synthesis (Sultana et al., 1999), and instability of the pigment protein complex (Levitt, 1980). The outcomes also demonstrated the potential of *T. hamatum* and *P. lilacinus* in curtailing the detrimental effects of

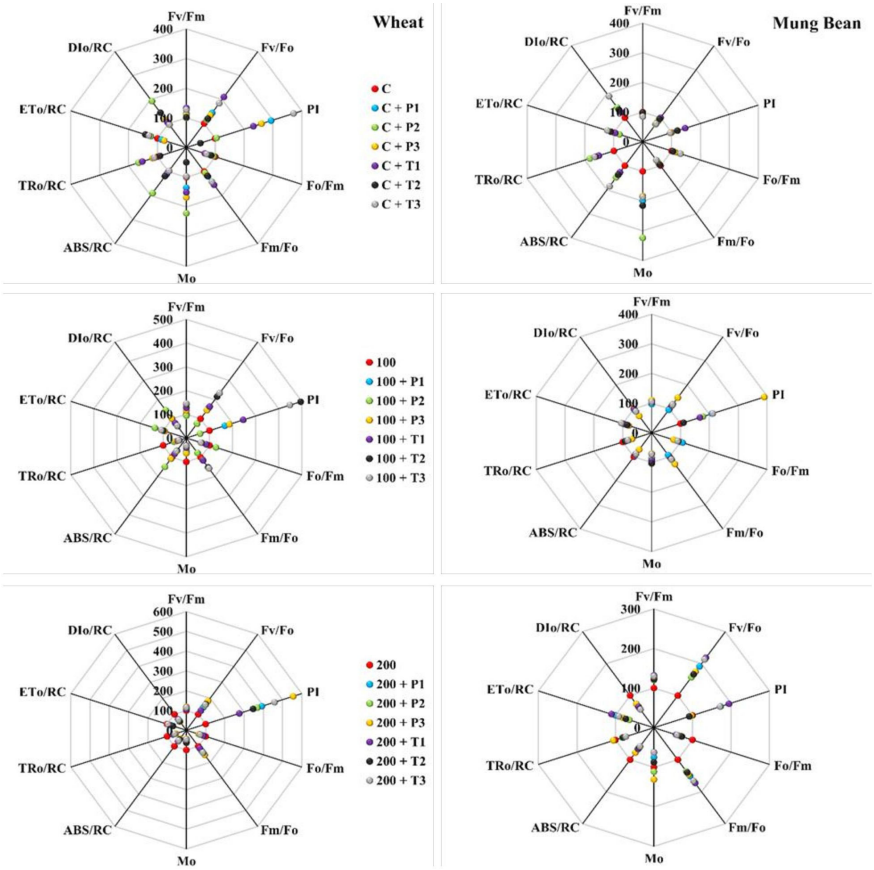

FIGURE 7
Effects of bio-priming on maximum quantum yield of PSII (*FV/FM*), activity of water splitting complex on donor site of PSII (FV/F_O), performance index (PI), quantum yield of energy dissipation (F_O/FM), electron transport rate through PSII (FM/F_O), approximated initial slope of fluorescence transient (M_O), absorption per reaction centre (*ABS/RC*), trapping per reaction centre (TR_O/RC), electron transport per reaction centre (ET_O/RC) and dissipation per reaction centre (DI_O/RC) of wheat and Mung bean grown under saline environment. The values of the parameters are expressed as percentage increase or decrease over the control (considered as 100). The symbols on the horizontal axis represents: Control: Seeds without priming, PI = Seed priming with Paecilomyces lilacinus, Th= Seed priming with *Trichoderma hamatum* 5, 10, 15 = duration of bio-priming in minute, 0, 100 & 200 mM NaCl concentration. On bars, vertical lines represent ± Mean Standard Error (S.E) and similar alphabets represents non-significant difference between the means of treatment at $p<0.05$.

NaCl on the CCI and induced a significant rise in chlorophyll content in both salt-treated plants and control plants (Figure 1). *P. lilacinus* has also been reported to increase the chlorophyll content in carrot plants (Nesha and Siddiqui, 2017). Moreover, *Trichoderma* spp. has also been linked to improvements in the pigment system and the reduction of harmful effects of NaCl, according to Rawat et al. (2011) and Zhang et al. (2013). Compared to control, plants that are primed with *T. hamatum* showed improvement in photosynthetic pigments could be attributed by the synthesis of phytohormones such auxin, gibberellins, and cytokinins (Martínez-Medina et al., 2014; Resende et al., 2014).

Salt stress reduced the stomatal conductance of wheat and mung bean plants which is one of the most common responses of plants to prevent excessive water loss and controls the passage of carbon and water between plants and the atmosphere (Brodribb and McAdam, 2011). However, the priming of *T. hamatum* significantly increased the stomatal conductance over control (un-primed) under extreme salt stress (Figure 1). While in wheat plants, stomatal closure was observed to reduce transpiration and conserve water during salt stress. This closure is regulated through the ABA level as well as extensive signal transduction of guard cells induced by *T. hamatum* (Efetova et al., 2007; Joshi-Saha et al., 2011). Therefore, two different behavior of *T. hamatum* priming was observed under high salt stress. In wheat plants, it fosters higher stomatal conductance which could be a strategy to fix more CO_2 due to a fast growth strategy before the onset of salt stress consequences compared to mung bean plants.

In salt stress, H_2O_2 can serve both as a measure of toxicity or that damaged plant cells permanently or it may be a secondary messenger that controls the plant's antioxidant defense (Gechev et al., 2006). In the current investigation, we discovered that salt stress led to a considerable rise in H_2O_2 levels. However, in primed wheat plants, the level of H_2O_2 was significantly lower than in mung bean plants. Moreover, the more decrease in H_2O_2 level was observed among the plants primed with *T. hamatum*, therefore, we proposed that priming of *T. hamatum* promoted lesser oxidative or cellular damage caused by salt stress which is in accordance with the finding of Güler et al. (2001). Likewise, the other damage marker, MDA content was also lower among the wheat plants over the mung beans, hence, the priming was more effective among the wheat plants. As suggested by earlier studies, salt stress may have an impact on altering the composition of membrane lipids since it caused lipid peroxidation (Samadi et al., 2019). The decrease in MDA content suggested that *T. hamatum* prevented the plant from oxidative damage in comparison to unprimed plants. These findings strongly concur with those of Zhang et al. (2013) who discovered lower levels of lipid peroxidation in cucumber plants under salt stress that had been treated with *T. harzianum*.

Salt stress adversely affects the photosynthetic apparatus of the plants which can be observed through chlorophyll a fluorescence parameter. Chl fluorescence is frequently employed as a measure of photosystem efficiency because it offers important information about the quantum efficiency of photochemistry and heat dissipation (Lichtenthaler and Burkart, 1999). Quantum yield (F_V/F_M) and PS II functionality gradually decreased with the increase in exposure time and salt concertation, which negatively affected the membrane stability. This suggests that the PS II reaction center deteriorated under higher stress levels (Lu and Zhang, 2000). However, *T. hamatum* priming significantly enhanced the F_V/F_M and PS II efficiency of stressed plants over control and *P. lilacinus* priming. These outcomes are indicative of *T. hamatum* efficacy to enhance salt tolerance which is linked with the improved PS II functionality in stressed plants. The increase in energy loss (DI_O/RC) among the control plants exhibited stress damage at the PS II level which was quite higher among the control plants while bio-primed plants had considerably very low dissipation hence, lower damage at PS II level.

According to the findings of Ran et al. (2021), the OJIP curve of the present work showed a decline in I and P values with elevated salt stress. However, the increase in I and P steps in *T. hamatum* and *P. lilacinus* primed plants showed the availability of more active reaction centers (RC) PS II under salt stress in comparison to control (unprimed plants) (Kalaji et al., 2011). This indicated that bio-primed plants were more tolerant to salt stress as their absorbed energy was more efficiently transferred to reaction centers for photochemistry (Tsimilli-Michael and Strasser, 2008; Stirbet and Govindjee, 2011). The decrease in I and P phase under salt stress control (unprimed) plants was due to a bottleneck in electron transfer at the electron acceptor side of the PSI, the increase in cyclic electron flow (CEF) around the PS I is revealed by the decrease in I-P phase (Kono et al., 2014; Hamdani et al., 2015). This has been alleviated *via T. hamatum* priming that mitigate the smooth electron flow between PS II and PS I which resulted in high photosynthetic yield of the stressed and unstressed plants (Figure 6).

According to the leaf energy flux model (Figure 8) the highest absorption per reaction center (ABs/RC) and dissipation per reaction center (DI_O/RC) were observed among the un-primed plants (wheat and mung bean) which was due to more inactive reaction centers (RC) to active reaction center ratio. Hence, this explains that the controlled plants were able to absorb more photons, but the trapped energy was not used to reduce the plastoquinone pool and absorbed photon was rather dissipated in the form of energy or heat. However, bio-priming enhanced the active to inactive RC ratio among the wheat and mung bean plants which helped to increase the rate of Q_A reduction by trapped exciton (TR_O/RC) under high salt stress (200 mM). This increment led to the enhanced electron transport (ET_O/RC) which reflected the increased activity of active RC to reoxidize the reduced Q_A (Grieco et al., 2015). This combined increased in trapping and transport of exciton displayed the stress tolerance induced by bio-priming agent which reflected in the enhanced photosynthetic yield (PI) and least energy dissipation (DI_O/RC) of the primed plants.

The infra-red thermographic images also evidently supported the results. A significant color change was observed among the leaves of primed and un-primed plants indicating a rise in leaf temperature of the control plants under high salt stress (Figure 9). This rise in temerature reflects the decline in water contents of the

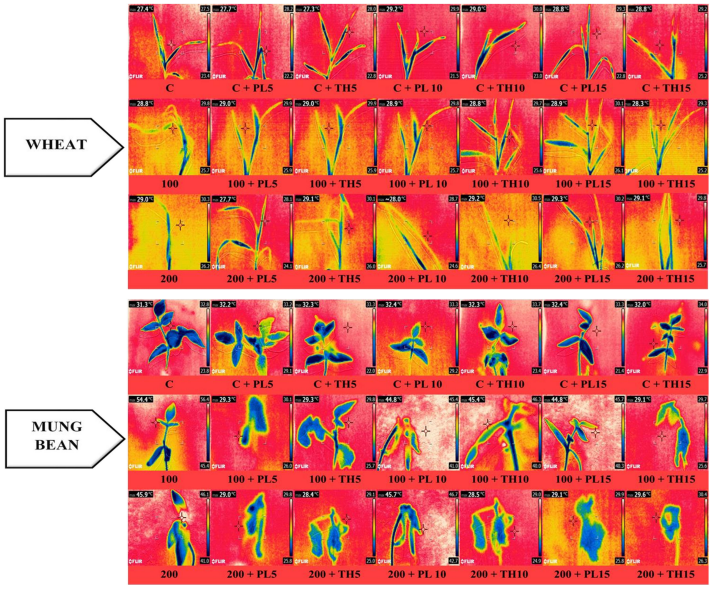

FIGURE 8
Effects of seed priming with fungal endophytes on infra-red thermal images of wheat and mung bean grown under saline environment. The symbols on the horizontal axis represents: C = Seed without priming, PL5= Seed priming with *Paecilomyces lilacinus* for 5 mins, PL 10= Seed priming with *Paecilomyces lilacinus* for 10 mins, PL 15= Seed priming with *Paecilomyces lilacinus* for 15 mins, TH 5= Seed priming with *Trichoderma hamatum* for 5 mins, TH 10= Seed priming with *Trichoderma hamatum* for 10 mins, TH 15= Seed priming with *Trichoderma hamatum* for 15 mins. 0 (C), 100 and 200 mM represents different salinity (NaCl) levels.

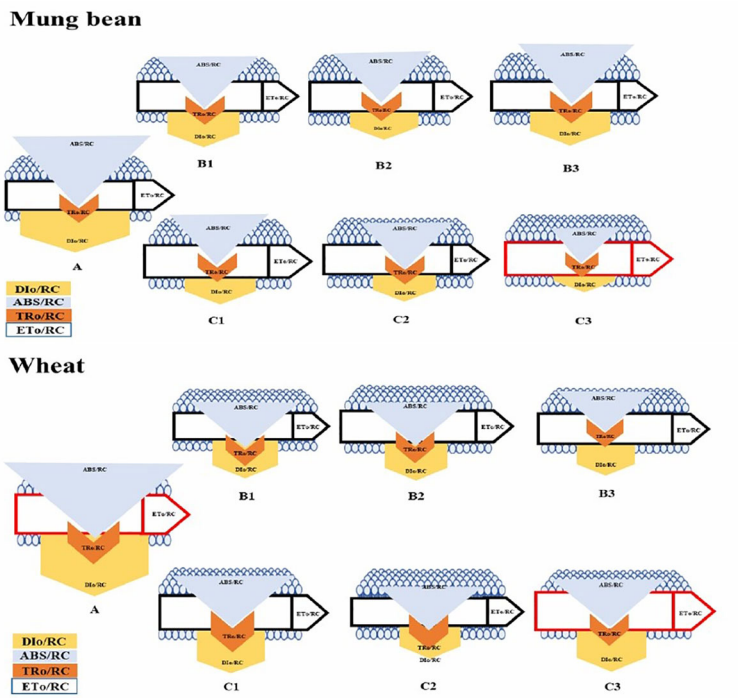

FIGURE 9
Membrane pipeline model showing the proportion of specific energy fluxes in treated plants. In the membrane, ABS/RC, TRO/RC, ETO/RC, and DIO/RC indicate absorption, maximum trapped exciting flux per active PSII, electron transport, and dissipation flux, respectively. The value of each parameter can be seen in relative changes in the width of each arrow (see the color legend). The diagram exhibits the variation of ABS/RC, TRO/RC, ETO/RC, and DIO/RC, for seven treatments, namely, A=200mM, B1=200mM and *Paecilomyces lilacinus* strain with 5 minutes time interval, B2=200mM and *Paecilomyces lilacinus* strain with 10 minutes time interval, B3=200mM and *Paecilomyces lilacinus* strain with 15 minutes time interval, C1=200mM and *Trichoderma harzianum* strain with 5 minutes time interval, C2=200mM and *Trichoderma harzianum* strain with 10 minutes time interval, and C3=200mM and *Trichoderma harzianum* strain with 15 minutes time interval. The model displays fluxes in different shapes; the size of each shape was developed by the different values of four fluxes in each treatment.

leaves. It was evident from the data that bio-primed plants demosntrate lesser increase in leave temperature corresponding with higher water content. Moreover, under the water stress, leaf temperature somewhat mimicked the gas exchange rates and grain output, perhaps due to other changes brought on by this stress factor in plants, like impairments in the rates of photosynthesis and partitioning of energy in plant leaves and canopy structures (resulting in variations in the absorption and/or dissipation of energy) (Casari et al., 2019). Therefore, the results were coherent that the bio-primed plants were more tolerant to varying levels of salt stress (0, 100, and 200 mM) in comparison to the control plants.

Antioxidant activities are important physiological aspects playing a key role in coping with salt stress (Guo et al., 2018). Abiotic stress causes an increase in ROS production that must be controlled in a homeostatic pool, yet excessive levels of ROS can produce oxidative stress, which can damage plant physiology and cause plant death by causing denaturation of protein structure, lipid peroxidation, and nucleotide disruption (Demidchik, 2015). In this context, an increase in antioxidant activity protects cells against environmental challenges like salinity and drought. *P. lilacinus &* specifically *T. hamatum* treated plants showed a remarkable increase in antioxidant enzyme activities like SOD and CAT under high salt stress (200 mM). which significantly reduce the production of ROS like H_2O_2 that is potent enough to induce lipid peroxidation in cell membrane. Hence, increasing antioxidant activities ultimately brings down the level of MDA in treated plants as compared to control by scavenging ROS (Figure 7).

It is concluded that bio-priming with endophytes produces resistant in crop plants to salt stress through modulation in physiological and photosystem II functionality which was further supported by the infrared thermographic images of the stress and control plants. Endophytes not only sustain better quantum absorption and energy flow in plants but also contribute to sustaining photosystem II performance and lower down the stress markers production and energy loss in a sub-optimal environment. Further our current findings suggest that the use of bio-priming with salt tolerant and bio-stimulating natural colonizers specifically with *T. hamatum* could be a suitable approach in mitigating salt stress in wheat and mung bean plants.

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

All authors contributed to the study's conception and design. Material preparation, search, and collection of relevant articles and reviews were performed by KI, ZS, JC, XW, YR, HA and DW thoroughly checked the first draft and decisively improved the manuscript. All authors contributed to the article and agreed the submitted version. All authors contributed to the article and approved the submitted version.

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.2023.1082480/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 22 March 2023
DOI 10.3389/fpls.2023.1048762

OPEN ACCESS

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

RECEIVED 20 September 2022
ACCEPTED 07 March 2023
PUBLISHED 22 March 2023

CITATION
Gomes T, Pereira JA, Moya-Laraño J, Poveda J, Lino-Neto T and Baptista P (2023) Deciphering plant health status: The link between secondary metabolites, fungal community and disease incidence in olive tree.
Front. Plant Sci. 14:1048762.
doi: 10.3389/fpls.2023.1048762

Deciphering plant health status: The link between secondary metabolites, fungal community and disease incidence in olive tree

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Plant-associated microorganisms are increasingly recognized to play key roles in host health. Among several strategies, associated microorganisms can promote the production of specific metabolites by their hosts. However, there is still a huge gap in the understanding of such mechanisms in plant-microorganism interaction. Here, we want to determine whether different levels of olive leaf spot (OLS) disease incidence were related to differences in the composition of fungal and secondary metabolites (*i.e.* phenolic and volatile compounds) in leaves from olive tree cultivars with contrasting OLS susceptibilities (ranging from tolerant to highly susceptible). Accordingly, leaves with three levels of OLS incidence from both cultivars were used to assess epiphytic and endophytic fungal communities, by barcoding of cultivable isolates, as well as to evaluate leaf phenolic and volatile composition. Fungal and metabolite compositions variations were detected according to the level of disease incidence. Changes were particularly noticed for OLS-tolerant cultivars, opposing to OLS-susceptible cultivars, suggesting that disease development is linked, not only to leaf fungal and metabolite composition, but also to host genotype. A set of metabolites/fungi that can act as predictive biomarkers of plant tolerance/susceptibility to OLS disease were identified. The metabolites α-farnesene and p-cymene, and the fungi *Fusarium* sp. and *Alternaria* sp. were more related to disease incidence, while *Pyronema domesticum* was related to the absence of disease symptoms. Cultivar susceptibility to OLS disease is then suggested to be driven by fungi, volatile and phenolic host leaves composition, and above all to plant-fungus interaction. A deeper understanding of these complex interactions may unravel plant defensive responses.

KEYWORDS

***Venturia oleaginea*, epiphytes, endophytes, volatile compounds, phenolic compounds**

1 Introduction

In natural ecosystems, the aboveground parts of plants come across a myriad of fungal species that can colonize the surface (epiphytes) or internal plant tissues (endophytes), with beneficial or detrimental outcomes (Zeilinger et al., 2016). For example, different fungal groups (e.g. *Trichoderma* and *Epicoccum*) have demonstrated the ability to protect host plants from pathogens, while others (e.g. *Colletotrichum*) are well-recognized pathogens causing plant diseases (Poveda and Baptista, 2021). Despite the ecological and agricultural importance of plant-fungal associations, the complex interaction network occurring among fungi and plants is still not fully understood (Chaudhry et al., 2021). There are still many open questions that remain unanswered regarding how plant-associated microorganisms contribute to the health status of their host. Recent studies have provided strong evidences about the capacity of endophytes to improve plant protection against pathogens by supplying several bioactive metabolites to their host (Fadiji and Babalola, 2020). From the wide range of secondary metabolites that are induced during plant-microbial interactions, both phenolic and volatile organic compounds (VOCs) seem to be particularly important, due to their recognized antimicrobial activity and ability to induce plant defenses against pathogens (Tilocca et al., 2020; Wallis and Galarneau, 2020; Poveda, 2021). However, the elucidation of plant associated microorganism potential to improve plant health through the synthesis of bioactive metabolites in host tissues is a challenging task, especially if studied in the nature. In fact, the microorganisms that interact with plants are ubiquitous in nature and can contribute to metabolite production in different ways. Microorganisms can produce their own secondary metabolites (which will be mixed with those produced by plant host), change the biosynthesis of plant host metabolites, or even metabolize plant host secondary metabolites and produce new metabolites (Pang et al., 2021). Probably due to such complex aspects occurring during plant-microorganism interactions, few studies have focused on the mechanisms employed by microorganisms in protecting host plant from pathogens.

The olive leaf spot (OLS) disease, caused by the fungus *Venturia oleaginea* (Castagne) Rossman & Crous (syn. *Fusicladium oleagineum*, *Spilocaea oleaginea*), is one of the most damaging diseases of olive tree (*Olea europaea* L.) worldwide (Viruega et al., 2013). Fungal development is mostly restricted to olive leaf tissues, including leaf surface and subcuticular areas, causing scab lesions and leaf-drop symptoms, leading occasionally to tree death (Viruega et al., 2013). Under the same agro-climatic conditions, the OLS disease is more severe in certain olive tree cultivars (*e.g.* "Madural" and "Verdeal Transmontana") than in others (*e.g.* "Cobrançosa") (Pereira, J.A., Per. Com.). In the present work, this biological system was chosen as a model for studying the impact of interactions occurring among fungi and plant hosts on the plant health status. Indeed, the availability of olive cultivars with distinct susceptibility levels to OLS, and with the possibility in displaying different levels of disease incidence, is an advantage. Detected differences on fungal communities or metabolites (volatile and phenolic compounds) of host plant leaves could thus be linked to the cultivar or disease incidence effect. Moreover, using this model, the simultaneous study of interactions occurring between plant and epiphytes or endophytes is possible. This is particularly relevant due to the recognized ability of *V. oleaginea* to develop in the surface and subcuticular spaces of the leaves. By considering these aspects, we hypothesized that plant interactions with fungi could modify plant secondary metabolites composition, thus affecting the incidence of OLS disease on these cultivars. Specifically, we address the following questions: (1) Is OLS incidence related to host-associated epiphytic and endophytic fungal communities composition in leaves? (2) Is OLS incidence related to host plant composition on phenolic and volatile compounds? (3) Is there any relation among fungal consortia and secondary metabolites composition that could explain different incidence levels of OLS disease? As far as we known, no previous investigation has addressed concerning fungal communities and chemical composition of leaves as a whole. The understanding of these complex associations (i.e. host plant, phytochemicals, fungal communities and disease incidence) might improve our knowledge on the role of different fungal taxa and metabolites in OLS disease incidence.

2 Materials and methods

2.1 Study site and olive leaves collection

The study was conducted in two olive orchards at Mirandela region (Northeast of Portugal), at coordinates N 41° 32.593' W 07° 07.445' (orchard 1) and N 41° 29.490' W 07° 15.413' (orchard 2). Each orchard comprises olive trees from three olive cultivars, *i.e.* "Cobrançosa", "Madural" and "Verdeal Transmontana", at the spacing of 7 x 7m, and is managed through integrated production guidelines (Malavolta and Perdikis, 2018). These three cultivars are considered tolerant ("Cobrançosa") and susceptible ("Madural" and "Verdeal Transmontana") to OLS disease. In each orchard, five trees per cultivar were randomly selected in close proximity to each other. Leaves were randomly collected in the four orientations of the tree canopy, at 1.5 meters above the ground, from March to May. The collected leaves were used to assess OLS disease incidence of each tree (% infected leaves), to determine epiphytic and endophytic fungal communities, as well as chemical composition (*i.e.*, phenolic and volatile compounds). The disease incidence (%) in each surveyed olive tree was assessed using a total of 60 randomly collected leaves. The number of observed symptomatic leaves was used for determining the percentage of infected leaves. For chemical evaluations, and to mimic natural conditions within the tree canopy, a mixture of ten randomly selected leaves per tree was used, comprising five leaves with visible spots (OLS-symptomatic leaves) and five leaves without visible spots (asymptomatic leaves). For fungal diversity assessment was used a similar procedure by using a mixture of six leaves per tree (three OLS-symptomatic leaves and three asymptomatic leaves). All evaluations were performed using fresh leaves (immediately upon their collection), with exception of assessment of phenolic compounds that used lyophilized leaves. For this, leaves were stored in a deep freezer at

-20°C, lyophilized, ground to a fine powder using an analytical mill, and stored in a dark room until phenolic analysis.

2.2 Assessment of foliar fungal communities

2.2.1 Fungal isolation

Both epiphytic and endophytic fungal communities in olive tree leaves were evaluated based on culture-dependent methods. The isolation of fungal epiphytes was performed by the dilution plate method, by using around 1-gram weight of leaf samples in 9 mL of sterile potassium phosphate buffer pH 7.0 (0.20 g/L KCl; 8 g/L NaCl; 1.4 g/L Na_2HPO_4; 0.24 g/L KH_2PO_4), according to the procedure described by Gomes et al. (2018). Briefly, aliquots (1 ml) of the resulting microbial suspension were separately plated in triplicate onto Potato Dextrose Agar (PDA, Difco) and Plate Count Agar (PCA, Himedia) media, supplemented with 0.01% (w/v) chloramphenicol (Oxoid, Basingstoke, Hampshire, UK). In total, 1,080 Petri plates were inoculated (30 trees x 6 leaves x 2 culture media x 3 replicates). Plates were incubated at 25 ± 2°C in the dark for fungal growth and colonies counting. The number of epiphytes (i.e. the number of individual colonies of fungi on the leaf surface) was expressed as log CFU/cm^2. For estimating the leaf surface, an ellipse equation ($A=\pi ab$) was used, being A the area, whereas a and b were the half-length of longitudinal and transverse axes of a leaf, respectively.

Endophytic fungi were isolated from the same leaves used to isolate epiphytes. Epiphytes were removed by surface disinfection of leaves, using the procedure previously optimized by Martins et al. (2016), which consisted in the sequential immersion of leaves in 70% (v/v) ethanol for 2 min, 3–5% (v/v) sodium hypochlorite for 3 min, 70% (v/v) ethanol for 1 min, and sterile distilled water (three times, 1 min each). After disinfection, each leaf was cut into five fragments (ca. 5 x 5 mm), which were transferred to the same culture media used to isolate epiphytes. Endophytic fungi were isolated from a total of 1,800 leaf tissue segments (30 trees x 6 leaves x 2 culture media x 5 fragments). Validation of the surface sterilization procedure was done by imprinting the surface of sterilized leaf tissues onto PDA and PCA media. Emerging fungal colonies were subcultured on fresh medium until pure epiphytic/endophytic cultures were obtained.

2.2.2 Fungal identification

Each fungal colony was identified by using morphological and molecular approaches, according to Gomes et al. (2018). Briefly, fungal isolates were firstly grouped according to their morphological similarity at colony level (colony appearances, mycelial textures, spore mass color, diffusible pigment and pigmentations on both obverse and reverse of colonies). Three representative isolates of each morphotype were further selected for molecular identification, using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA). Total genomic DNA was extracted from harvested mycelial/spores using the REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK) following manufacturer's instructions. The ITS region (ITS1, 5.8S, ITS2) was amplified using ITS1/ITS4 primers set (White et al., 1990). Amplifications occurred in a MyCycler™ (Bio-Rad) thermocycler, using 50 µL PCR reactions, which contained 5 µL of 10x complete PCR buffer (0.1% tween 20, 25 mM $MgCl_2$, pH 8.8), 1 µL dNTPs of 10 mM, 1 µL of each primer (10 µM), 4 µL of DNA, 0.2 µL of DFS-Taq DNA Polymerase (5 U/µL) (BIORON GmbH) and 37.8 µL of distilled sterile water. The PCR program was set for an initial denaturation step at 95°C for 5min, followed by 30 cycles of denaturation at 94°C for 40s, primer annealing at 48°C – 56°C (being 54°C the most used) for 50s and extension at 72°C for 45s, followed by a final extension step at 72°C for 7 min. The amplified products (~ 650 bp) were purified and sequenced using Macrogen Inc. (Madrid, Spain) services. The obtained DNA sequences were analysed with DNASTAR v.2.58 software and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST algorithm, according the procedure described by Gomes et al. (2018). The obtained sequences are available at GenBank with the following accession numbers: KU324941-KU325040; KU325041-KU325240; KU325241-KU325457. Each operational taxonomic unit (OTU) was taxonomically classified according to the Index Fungorum Database (www.indexfungorum.org).

2.3 Phenolic compounds identification and quantification

2.3.1 Standards and reagents

Used standards were purchased from Sigma (St. Louis, MO, USA) or Extrasynthèse (Genay, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use.

2.3.2 Extraction of phenolic compounds

Before the extraction of phenolic compounds, each lyophilized leaf sample was powered and sieved using a 900 µm sieve. The extraction was performed as previously described by Vinha et al. (2002). Briefly, about 1.5 g of the powdered leaf samples were weighed in quadruplicates. Each sample was separately mixed with 50 mL of methanol (99.96%, Aldrich) at 150 rpm for 1 h (room temperature). The obtained methanolic extracts were filtered through a Whatman No.4 paper and evaporated (Stuart RE3000, UK) to dryness under reduced pressure (35°C). After dissolution in 2 ml methanol (99.96%, Aldrich) and filtration (Whatman No. 2), an aliquot of 20 µl of the obtained extracts was analyzed by HPLC.

2.3.3 Analysis of phenolic compounds

Chromatographic separation was performed as previously reported by Vinha et al. (2002), with an analytical HPLC unit (Knauer Smartline), equipped with a Knauer Smartline autosampler 3800, and a Knauer Diode Array Detector (DAD). A reversed-phase Spherisorb ODS2 column was used during analysis (250 x 4.6 mm, 5 µm particle size, Merck, Darmstadt, Germany). The used solvent system was a gradient of water–formic acid (19:1) and methanol, applied at a flow rate of 0.9 mL min^{-1}. Spectral data from all peaks were accumulated within the 200–400 nm range. Chromatograms were recorded at 280, 320 and 350 nm, and data were managed on

ClarityChrom® software (Knauer, Berlin, Germany). Phenolic compounds were quantified through the comparison performed with known amounts of external standards: hydroxytyrosol, oleuropein, chlorogenic acid and rutin were quantified at 280 nm, caffeic acid at 320 nm, and verbascoside, apigenin-7-O-glucoside, luteolin-7-O-glucoside and luteolin at 350 nm. HPLC analyses were performed using two technical replicates for each extract. The means of the four replicates for each tree leaf sample were then calculated. Phenolic compounds were expressed as the amount of phenolics per dry weight (DW) of leaf extract (mg/g of DW).

2.4 Volatile identification and quantification

The extraction and analysis of volatile compounds from fresh leaves were performed according to the methodology described by Malheiro et al. (2015), with some modifications.

2.4.1 Extraction of volatile compounds

The extraction of leaf volatiles was performed by headspace solid phase microextraction (HS-SPME). Around 1-gram weight of fresh leaves was placed in 50 ml vials, containing 10 µl of 4-methyl 2-pentanol (10.65 ppm dissolved in methanol), which was used as an internal standard. The vials were sealed with a polypropylene cap with a silicon septum. Following an incubation in an ultrasonic bath at 40°C, for 10 min, the divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 µm) fiber was inserted into the vial headspace for more than 30 minutes, at 40°C, for volatile adsorption. The volatiles were desorbed by placing the fiber into the gas chromatographic (GC) injection port for 10 min, at 280°C. The HS-SPME analyses were performed using five replicates for each tree leaf sample.

2.4.2 Gas chromatography-mass spectrometry (GC-MS) conditions

Chromatographic analysis was performed on an Agilent 6890 series GC (Agilent, Avondale, PA, USA), with splitless injection, coupled to a MS detector (Agilent 5973), according to the conditions described by Malheiro et al. (2015). Volatile compounds were identified by comparing the experimental spectra with spectra from NIST data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.) and also by comparison of their GC retention index. Retention indices were determined as reported by Malheiro et al. (2015). Concentration of identified compounds were calculated by the ratio of each individual base ion peak area to the area of the internal standard. The obtained ratio was then converted to mass equivalents, on the basis on the internal mass standard added. Volatiles were represented as the amount of volatile compound per fresh weight (FW) of leaf tissue (mg/kg of FW).

2.5 Data analysis

Based on OLS disease assessment results, three ranges of disease incidence were defined: 0-5%, 5-10%, and 10-15%. Data was analyzed considering each group of disease incidence and each cultivar. Thus, a total of nine experimental units were established (three ranges of disease incidence per cultivar), each one with a sample size of three to four trees. The normality assumption of the data was verified using the Shapiro-Wilk test.

2.5.1 Differences on fungal communities and metabolite profiles among OLS incidence ranges and cultivars

The total number and abundance of fungal OTUs and metabolites (phenolic and volatiles compounds) for each olive tree are presented as the mean for each OLS disease incidence range (0-5%, 5-10%, 10-15%) and cultivar ("Cobrançosa", "Madural", "Verdeal Transmontana"). Differences between means were evaluated by one-way analysis of variance (One-way ANOVA) with SPSS v.20, followed by Tukey's *post hoc* test ($p < 0.05$). Non-metric Multidimensional Scaling (NMDS) plots, based on Bray–Curtis distances, were performed to assess the variation in the composition of foliar fungal communities and metabolite profiles, among different ranges of OLS disease incidence (0-5%, 5-10%, 10-15%). Kruskal's stress was used to estimate goodness of fit (commonly acceptable when <0.2). A one-way analysis of similarity (ANOSIM) was used to determine significant differences in fungal (or metabolite) compositions among OLS incidence groupings, using Bray–Curtis distance matrices. ANOSIM generates a P-value (significant level below to 0.05) and a R-value, which gives the degree of discrimination between groups and ranges from 0 (indistinguishable) to 1 (completely dissimilar) (Clarke and Gorley, 2015). NMDS plots and ANOSIM analyses were performed using *Community Analysis Package* v. 6.0 (Henderson and Seaby, 2019). Subsequent analyses were performed in R (R Core Team 2018). Using the 'heatmap 2' function of *gplots* package, with the Euclidean distance, heatmaps with hierarchical clustering were constructed for grouping host cultivars and OLS incidence ranges, according to the abundance of fungal OTUs (abundance >10) and metabolites (abundance >12). Each sample was transformed into a row Z-score and high relative values were colored differently from those with low relative values.

2.5.2 Relationship between OLS disease incidence, host cultivar, fungal OTUs and metabolites

Random forest analysis was firstly performed to identify the ranking importance of variables (fungal OTUs and metabolites) for predicting OLS incidence (Breiman, 2001; Cutler et al., 2007). This analysis was set through machine learning algorithms, using the R RandomForest package (Cutler et al., 2007). For each tree grown on a bootstrap sample, the error rate for observations left out of the bootstrap sample was monitored. The predictor variables explained 74.1% and 85.2% of the variation in fungal OTUs and metabolites, respectively. The Gini coefficient indicates the variable contribution (importance) for OLS disease incidence. Spearman correlations and redundancy analyses (RDA) were then performed using the most important fungal OTUs and metabolites, which were pre-selected by the random forest analysis (Gini index >100). The Spearman

correlations were computed using the R corrplot package (Wei et al., 2017), in order to check the correlation between fungal OTUs, metabolites and OLS incidence. RDA was performed using R vegan package (Oksanen et al., 2017), in order to find relationships among cultivars ("Cobrançosa", "Madural", "Verdeal Transmontana"), OLS disease incidence ranges (0-5%, 5-10%, 10-15%), fungal OTUs and secondary metabolites. One-way analysis of variance (ANOVA) was carried out with 'anova' function, to test significant differences between cultivars or OLS incidence groupings, previously obtained by RDA ordination based on fungal OTUs and metabolites.

3 Results

3.1 Differences on fungal communities and metabolite profiles among OLS incidence ranges and cultivars

Overall, 154 fungal operational taxonomic units (OTUs), 18 phenolic and 73 volatile compounds, were identified from all analyzed olive leaves (Figures S1–S3). Among the identified fungal genera, *Cladosporium*, *Alternaria* and *Fusarium* were the most frequently isolated, representing 35% of the total number of isolates. In what concerns metabolites, the phenolic compounds oleuropein, apigenin-7-O-glucoside, rutin and verbascoside, as well as the volatiles Z3-hexen-1-ol-acetate and Z3-hexen-1-ol, were the most abundant, accounting together for 78% and 82% of the total phenolic and volatile fraction, respectively.

In general, the number of both fungal OTUs and detected metabolites did not change significantly across the three levels of OLS disease incidence (Figure S4). In what concerns abundance, only the abundance of fungal isolates retrieved from the most OLS-susceptible cultivar ("Verdeal Transmontana") exhibited a 2-fold significant increase (p <0.05) in trees with the highest OLS disease incidence. The comparison among cultivars, showed only differences on the number of volatile compound (Figure S5). Indeed, with an increase of OLS disease incidence, the levels of volatiles decreased significantly (p<0.05) in cv. "Cobrançosa", while increased significantly (p<0.05) in cv. "Madural".

The comparison between the endophytic and epiphytic communities across distinct OLS incidences showed differences in terms of diversity and abundance (Figure S6). With the highest OLS disease incidence, endophytic fungi displayed a greater increase in abundance (up to 1.4-fold, p <0.05) and richness (up to 1.1-fold, p <0.05) than epiphytic fungi. Regarding the epiphytic community, only a significant increase was detected for epiphytes abundance in trees with the highest OLS incidence (up to 1.3-fold, p <0.05).

The composition of fungal communities and metabolite profiles differs significantly among trees with distinct disease incidence levels (Figure 1; Table S1). Hierarchical cluster analysis based on the most abundant fungal OTUs also separated fungal communities into two main groups, corresponding to the communities found in trees with low OLS incidence and communities with higher incidence levels (Figure 2A). However, such separation was dependent upon the olive cultivar. Fungal communities from cv. "Verdeal Transmontana" clustered together, regardless of tree disease incidence. In contrast, the fungal composition from cvs. "Cobrançosa" and "Madural" differed when considering trees exhibiting high and low OLS incidence levels (ANOSIM, R=0.40, p=0.001). Accordingly, fungal communities from cv. "Verdeal Transmontana" were less distinct in trees with different OLS incidence levels (ANOSIM, R=0.33, p=0.002). Nevertheless, the ANOSIM analysis could still distinguish all three incidence levels in this cultivar (R=0.31, p=0.016). Differences on fungal community composition between cultivars were always lower in trees displaying the highest OLS disease incidence, which was particularly detected in cvs. Cobrançosa" and "Madural". Indeed, the highest difference on fungal communities between cultivars was detected at the lowest OLS-disease incidence level (ANOSIM, R=0.72, p=0.001).

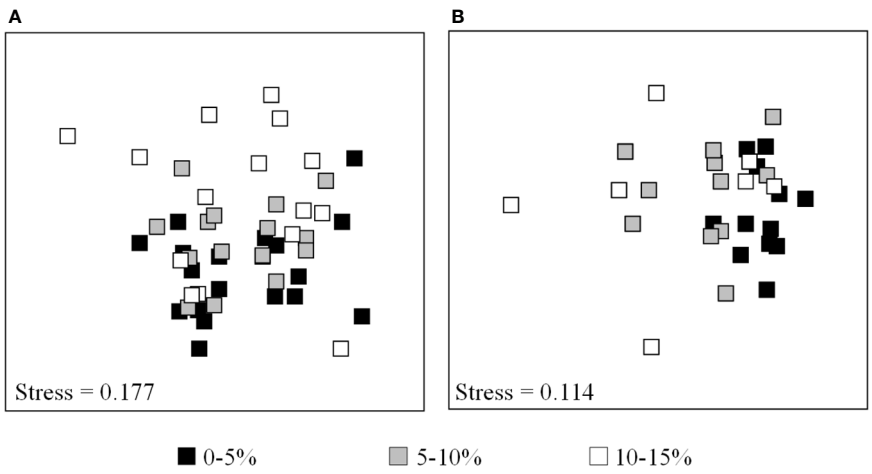

FIGURE 1
Non-metric multidimensional scaling (NMDS) plots of foliar fungal communities **(A)** and metabolite profiles **(B)** detected on olive trees displaying different levels of OLS disease incidence (0-5%, 5-10%, 10-15%). Clustering analysis was performed with Bray-Curtis distance. Kruskal's stress values are displayed.

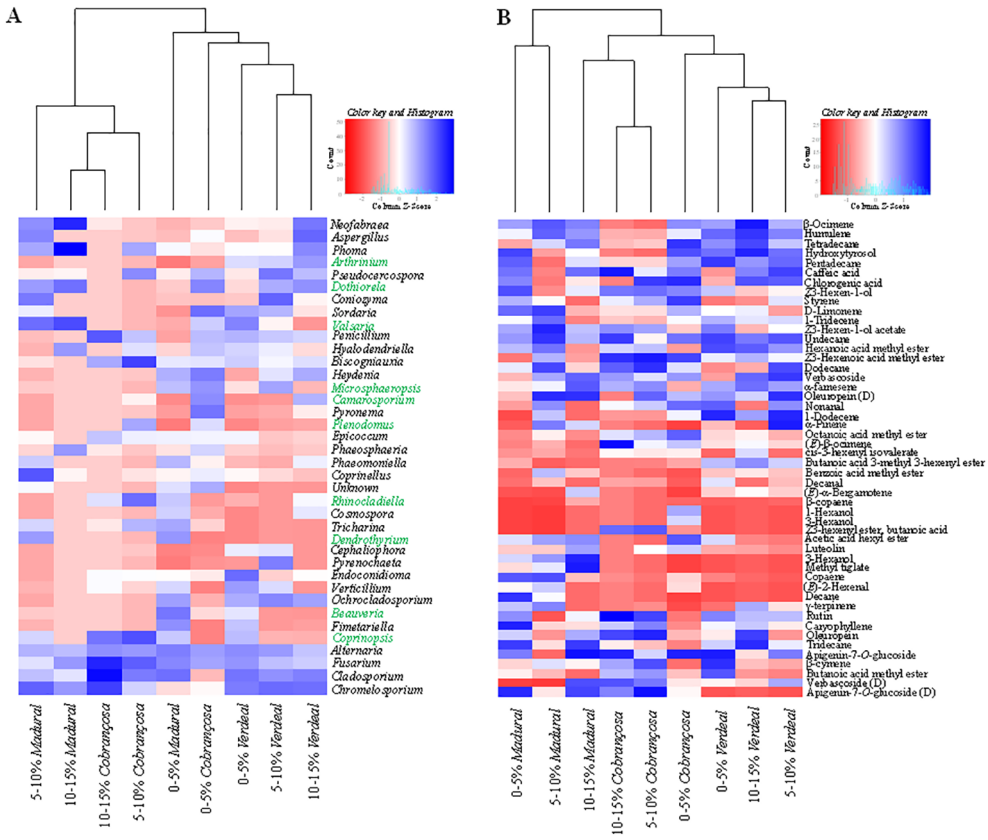

FIGURE 2
Variation of fungal communities **(A)** and metabolites profiles **(B)** in leaves of olive trees from distinct cultivars ("Cobrançosa", "Madural" and "Verdeal Transmontana"), displaying different levels of OLS disease incidence (0-5%, 5-10% and 10-15%). Heat maps indicate differences in the relative abundances of the most abundant fungal OTUs and metabolites. The color-scale ranges from red z < -3 to blue z >3, indicating the abundance of fungal OTUs and metabolites. Fungal isolates exclusively found on the episphere (leaf surface) and endosphere (leaf interior) are shown in green and purple color, respectively.

The metabolite profiles of trees from the three cultivars were distinct (ANOSIM, R=0.32, p=0.001), being these differences greater between cvs. "Madural" and "Verdeal Transmontana" (ANOSIM, R=0.35, p=0.001). Although less relevant than for fungal communities, leaf metabolite composition within each cultivar also varied with OLS-disease incidence, being the greatest differences observed among trees with the lowest and highest disease incidence levels (Figure 2B). This result was particularly observed for trees from cvs. "Madural" (ANOSIM, R=0.970, p=0.002) and "Cobrançosa" (ANOSIM, R=0.88, p=0.002), while cv. "Verdeal Transmontana" exhibited a similar metabolite composition among all trees (ANOSIM, R=0.125, p=0.079).

3.2 Relationship between host cultivar, foliar fungal community, metabolite profile and disease incidence

One of the goals of this study was the identification of a set of fungal OTUs and metabolites that could explain differences in susceptibility of different olive tree cultivars to OLS disease. The complexity of this biological system, in which multiple interaction effects can occur between host plant, fungi, and metabolites, together with the large amount of microbial/metabolite produced data, increases the difficulty of this task. Thus, to more accurately predict such relationships, a random forest analysis was employed to select the most relevant variables (i.e. fungi/metabolite) for the prediction of OLS incidence. The random forest ranks the importance of input variables measured by a *Gini* coefficient value. A higher *Gini* coefficient value represents a greater variable importance (Cutler et al., 2007). Eight fungal OTUs and ten metabolites (four phenolics and six volatiles) were identified as the most important variables for determining OLS disease incidence (*Gini* coefficient > 100; Figure S7). For testing the association between fungi, metabolites and OLS disease incidence, the selected variables were then used to perform *Spearman* correlations (Figure 3). The results revealed that the volatiles (E)-α-bergamotene, α-farnesene and p-cymene, the phenolic luteolin, and the fungal OTUs *Alternaria* sp., exhibited significant positive correlations with disease incidence. In contrast, *Cladosporium cladosporioides* and *Pyronema domesticum* were negatively correlated with OLS disease incidence. Other fungal OTUs were also found to be either negatively or positively correlated with some metabolites, as well as with other fungal OTUs. Specific significant inter- and intra-group metabolites correlations also existed, being particularly observed a strong positive correlation between the

10.3389/fpls.2023.1048762

volatiles α-farnesene and p-cymene, and between these two compounds and 1-octanol.

Since *Spearman* correlations only test the correlation between two variables, we have additionally performed a redundancy analysis (RDA). Beyond a simple ordination among the variables (unconstrained ordination, as in PCA), the explanatory variables are used in this analysis to test their predictive power on the multidimensional variable space (constrained ordination). The final outcome expresses how much of the variance in the set of response variables (fungal OTUs and metabolites) is explained by the set of explanatory variables (host cultivar and disease incidence level). Note that the order of explanatory to predictor variables was inverted relatively to the random forest analysis. Here, the question is how well OLS disease incidence levels or olive cultivars discriminate among metabolites or components of the fungal community. As for the previous *Spearman* correlations, only the most important variables preselected by the random forest analysis were used in RDA. OLS disease incidence ranges (p <0.01) and olive cultivars (p <0.01) were clearly discriminated among foliar fungal community and metabolite profiles (Figure 4). Several correlations were also detected between metabolites, fungal OTUs, host cultivars, and OLS disease incidence. The strength of such correlations was assessed by the arrow length and angle between arrows and axes. The lowest OLS disease incidence range is more closely associated to cv. "Cobrançosa", and to the presence of *P. domesticum* on leaves. In contrast, higher OLS disease incidence is more associated to cv. "Verdeal Transmontana". The segregation of trees with higher OLS disease incidence is based on their metabolite and fungal profiles. Disease incidence of 5-10% was mostly related with the metabolites α-farnesene, p-cymene, apigenin-7-O-glucoside and to the presence of *Alternaria* sp.; while the highest disease incidence (10-15%) was more closely associated to verbascoside, Z3-hexenoic acid, methyl ester, butanoic acid, methyl ester, and to the presence of *Fusarium* sp.

4 Discussion

To the best of our knowledge, the present study is the first work to address the relationship between host plant, foliar fungal communities, metabolic profiles and plant disease incidence under field conditions. We attempted to determine whether differences in susceptibility of different olive tree cultivars to OLS disease is linked to fungal communities and/or metabolite composition of host plant leaves.

Our results underline the importance of fungal communities inhabiting the leaves of each olive tree as possible determinants of disease incidence. Indeed, several significant correlations occurred between the abundance of specific fungi (*Alternaria* sp., *C. cladosporioides* or *P. domesticum*) and the incidence of OLS

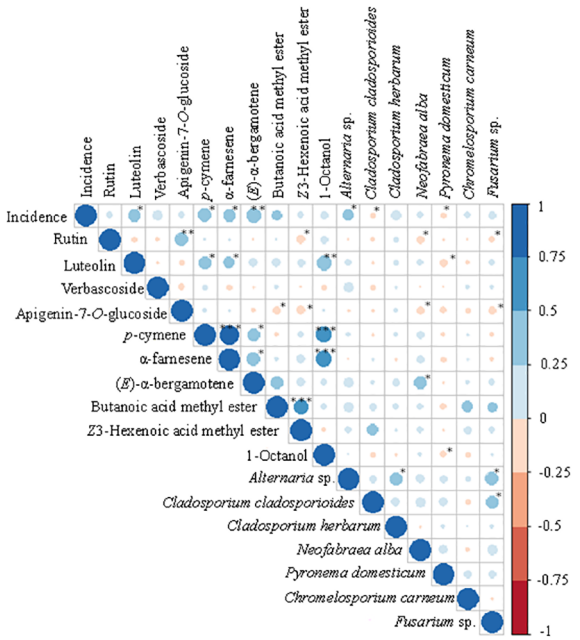

FIGURE 3
Spearman correlations between fungal OTUs abundance, metabolites concentration and OLS disease incidence. These correlations were only performed with variables (fungal OTUs and metabolites) preselected by the random forest analysis. Blue color represents positive correlations, while red color represents negative correlations. Circle size and color shading indicate correlation coefficient values. High coefficient values (maximum = 1) are represented by larger and darker circles, while smaller and lighter circles represent lower coefficient values (minimum = 0). Asterisks indicate statistically significant correlations at *p < 0.05, **p < 0.01 and ***p < 0.001.

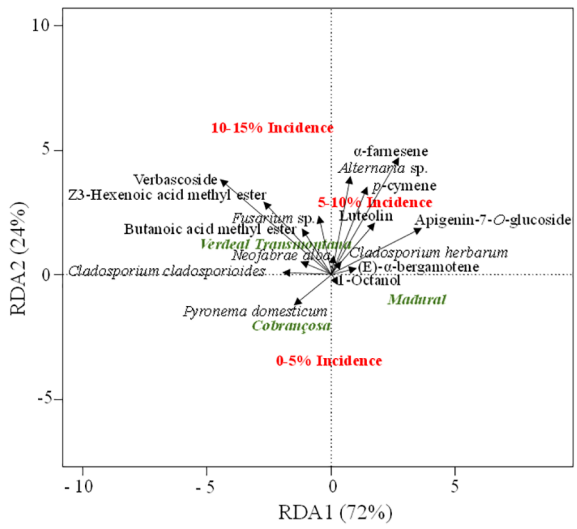

FIGURE 4
Redundancy analysis (RDA) ordination plot showing the association between the OLS disease incidence levels (0-5%, 5-10% and 10-15%), olive tree cultivars ("Cobrançosa", "Madural" and "Verdeal Transmontana"), leaf fungal community and metabolites composition. This analysis was only performed with variables (fungal OTUs and metabolites) preselected by the random forest analysis. In RDA analysis, a positive correlation between two variables is expressed by relatively long vectors pointing approximately in the same direction, whereas a negative correlation is indicated by arrows pointing in opposite directions. The longer the variable decline, the stronger the relationship of that parameter with the olive cultivar/disease incidence. The percentage of variation is explained by each axis.

disease. Moreover, the relation between fungal composition and disease incidence was found to be dependent on host cultivar (and thereby on genotype susceptibility). When analyzing the three levels of OLS disease incidence, a greater variation on the foliar fungal composition was detected in the OLS-tolerant cv. "Cobrançosa", in comparison with the susceptible cv. "Verdeal Transmontana". We hypothesize that fungal community changes could affect OLS disease incidence, probably due to the role of endophytic/epiphytic fungi that could act as biocontrol agents for olive diseases (Poveda and Baptista, 2021). A reduction on the abundance of those fungi able to provide host plant protection against OLS disease could determine higher disease incidence in the most tolerant cultivar. Contrasting with what occurs in the tolerant cultivar, the pathogen could be more adapted to the leaf fungal community on the most OLS-susceptible cultivar (cv. "Verdeal Transmontana").

The pathogen *V. oleaginea* itself is able to alter the fungal community of leaves during disease development. Accordingly, those trees displaying the highest OLS disease incidence exhibit a more similar foliar fungal community composition, regardless of the olive tree cultivar considered. The results from the present study are in line with the now accepted idea that disease development and progression depend on pathogen adaptation to the new environment, as well as on the interactions outcomes established with other microorganisms in the shared niche (McNally and Brown, 2015). Although microbiota studies can help to understand the role of other fungi for the development of olive diseases, it would be difficult to determine whether the reported changes are really due to the pathogen itself or are only a result from disease development. Taking this into consideration, changes in the fungal microbiota of olive in the presence of different diseases, as those caused by *Xylella fastidiosa* (Giampetruzzi et al., 2020), *Pseudomonas savastanoi* pv. *savastanoi* (Gomes et al., 2019), *Colletotrichum* sp. (Martins et al., 2021), and even *V. oleaginea* (Varanda et al., 2019), have been studied. For example, when studying OLS disease, Varanda et al. (2019) revealed a relation of OLS disease and the abundance of specific isolates, such as *Chalara* sp. and *Foliophoma* sp., while the absence of disease was related to the presence of *Alternaria* sp. and *Epicoccum* sp. isolates. These results contradict the findings from the present work, in which the presence of *Alternaria* sp. was strongly related to the development of the disease. These results suggest that other factors could be affecting plant disease development as well.

In the present study, leaf volatile emissions changed both quantitatively and qualitatively in leaves from trees exhibiting different incidences of OLS disease. Detected variations were different according to the host cultivar, suggesting that volatile compounds can probably contribute to plant OLS-resistance/tolerance. As far as we know, this is the first time in which such differences were detected according to the cultivar susceptibility to disease, leaving us to speculate on the underlying mechanism. Differences on cultivar susceptibility can be caused by multiple factors, including the activation of different plant defense pathways. Indeed, in a meta-analysis about induced plant volatiles, the effects of pathogenic infections caused by distinct fungi were attributed to differences in the induced defense pathways (Ameye et al., 2018). Curiously, on the most OLS-tolerant cultivar, a suppression rather than an induction of volatile emissions was observed in trees with increasing levels of disease incidence. Similar results were obtained following pathogen attacks in maize and potato plants (Seidl-Adams et al., 2015; Moreira et al., 2021). The reduction on volatile emissions has been associated with enhanced defense responses, suggesting that volatiles may also act as disease suppressors (Erb, 2018). However, little is known about such volatile capacity and mechanisms involved in the process (Erb, 2018). In the present work, specific volatile compounds (i.e. α-farnesene, p-cymene and 1-octanol) were found to be positively correlated with each other and with OLS incidence, suggesting that they may be integrated in a specific pathway and contribute to a higher OLS incidence. Given the capacity of volatiles to regulate different signaling cascades involved in plant defense, the integration of these volatile compounds through a signaling crosstalk is likely to occur (Erb, 2018).

The phenolic composition of olive tree leaves also changed with OLS disease incidence levels, displaying a variable pattern that depends on the cultivar. As for volatile compounds, the observed differences on phenolics might reflect the variation of olive tree cultivars on their susceptibility to disease. A relation between phenolic composition and susceptibility to infection was previously found in Norway spruce when attacked by the needle bladder rust (Ganthaler et al., 2017), or in maize after infection with *Fusarium verticillioides* (Bernardi et al., 2018). The possible contribution of phenolic compounds to OLS resistance/tolerance of host cultivar was further reinforced by the positive correlation found between some phenolic compounds (*i.e.* luteolin, rutin, verbascoside and apigenin-7-O-glucoside) and OLS disease incidence.

Previous works on plant defense responses to pathogen attacks mainly used reductionist approaches, by focusing on host plant protection conferred by either fungal (Collinge et al., 2022) or plant secondary metabolites (Zaynab et al., 2018). In the present study, disease incidence was interlinked for the first time to host cultivar, to fungal communities inhabiting leaves and to leaf metabolite composition. Different olive tree cultivars, grown in the same field, exhibited distinct fungal communities on their leaves and displayed diverse leaf metabolite compositions. Thus, host cultivar appears to affect, not only leaf fungal composition, but also metabolite profiles. Moreover, the interaction effects between fungi and metabolite compounds could also play an important role on the composition of each other. Accordingly, changes on fungal and metabolite composition in leaf samples from trees with different incidence levels of OLS disease revealed a similar trend, suggesting a possible link between fungi and metabolites. This relationship is further reinforced by the significant correlations found between certain fungal OTUs and metabolites. Although further analysis is required, we hypothesize that fungal communities residing in olive leaves could influence the metabolites of host plant, as previously observed by *Trichoderma* endophytes (Marra et al., 2020; Dini et al., 2021). Reciprocally, leaf metabolites could also affect fungal communities on olive leaves, as previously suggested for other plant species (Zambell and White, 2017).

A strength of our work is the identification of fungal OTUs and secondary metabolites strongly associated with OLS disease incidence. The lowest level of OLS incidence, which was found to

be associated to the most OLS-tolerant cultivar, was linked to the presence of *P. domesticum* that appears to suppress OLS disease. This possibility is worth investigating further in the future. Although *P. domesticum* has already been described to colonize the inner tissues of other plant species (Ghasemi et al., 2019), information about their role in conferring host plant protection against biotic stress is completely lacking. The highest level of OLS incidence, which was associated to the most OLS-susceptible cultivar (cv. "Verdeal Transmontana"), was found to be positively correlated with various fungal OTUs and metabolites. Among the fungal taxa positively correlated with OLS incidence, both *Alternaria* sp. and *Fusarium* sp. have been extensively described as important plant pathogens causing numerous diseases in several plant species (Hernandez-Escribano et al., 2018; Wei et al., 2018). In what concerns olive tree, only few reports described their capacity to infect olive fruits, causing fruit-rot (Moral et al., 2008; Trapero-Casas et al., 2009). Both genera have been described as making part of synergistic pathogen-pathogen interactions that often lead to increased disease severity (Lamichhane and Venturi, 2015). Thus, both fungi are likely to play a similar role in our pathosystem.

Besides the fungal role on OLS disease development, the positive correlation of specific secondary metabolites with OLS incidence could also implicate them on OLS disease development or as part of plant defense responses. Among the positively correlated metabolites, both α-farnesene and p-cymene seem to be the most important volatiles produced in leaves from trees with higher OLS disease incidence. Both sesquiterpenes have been described as important players on plant defenses against pathogen attacks (Runyon et al., 2020; Lemaitre-Guillier et al., 2021), suggesting a potential defensive role. In a similar way, other phenolic compounds, apigenin-7-O-glucoside and verbascoside, could play a role on OLS plant responses, since their levels have been previously described to increase after pathogen infection (Markakis et al., 2010; Schmidt et al., 2015). In addition, other phenolics (*e.g.* flavonoids and cinnamic acid derivatives) and volatile (*e.g.* ester) compounds were also positively correlated with OLS disease incidence, although without significance. Therefore, the role of positively correlated metabolites with OLS disease incidence is more likely to be part of plant defense responses to pathogen attack.

In conclusion, both fungal communities and metabolite compositions, in association with plant genotype, seem to play an important role on OLS disease incidence. The OLS-tolerant cv. "Cobrançosa" displayed greater variation in fungal and metabolite assemblages among trees with different OLS incidence, when compared to OLS-susceptible cv. "Verdeal Transmontana". Thus, differences on cultivar OLS-susceptibility are likely to be related with leaf fungal composition, metabolites (both phenolic and volatile compounds), and a combination of both. The complex interactions occurring between the host plant (cultivar), fungi and metabolite composition will influence the OLS disease incidence. Our work identified several key fungi and metabolites that could play an important role in the susceptibility/tolerance of cultivars to OLS disease. In this regard, future studies on the interactions of *Pyronema domesticum* with olive tree and *V. oleaginea* pathogen could provide functional roles of this fungus in host susceptibility/resistance to OLS disease.

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

PB and JP designed the experiments and together with TL-N supervised the study and revised the manuscript. TG performed most of the experiments, analyzed the data and drafted the manuscript. JM-L assisted with data analysis and together with JP revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work is funded by FEDER funds through COMPETE (Programa Operacional Factores de Competitividade), national funds through FCT (Fundação para a Ciência e a Tecnologia) and by Horizon 2020, the European Union's Framework Programme for Research and Innovation, within the project PRIMA/0002/2018 (INTOMED - Innovative tools to combat crop pests in the Mediterranean), and the Mountain Research Center - CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2020) as well as BioISI (UIDB/04046/2020) and CBMA (UIDB/04050/2020). TG thanks FCT, for PhD SFRH/BD/98127/2013 grant. JP thanks Grants for the Recualification of the Spanish University System for 2021-2023, Public University of Navarra. Recualification Modality. Funded by the European Union – NextGenerationEU.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1048762/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 05 June 2023
DOI 10.3389/fpls.2023.1196765

OPEN ACCESS

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RECEIVED 30 March 2023
ACCEPTED 08 May 2023
PUBLISHED 05 June 2023

CITATION
Wilberts L, Rojas-Preciado N, Jacquemyn H and Lievens B (2023) Fungal strain and crop cultivar affect growth of sweet pepper plants after root inoculation with entomopathogenic fungi. *Front. Plant Sci.* 14:1196765. doi: 10.3389/fpls.2023.1196765

Fungal strain and crop cultivar affect growth of sweet pepper plants after root inoculation with entomopathogenic fungi

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As endophytes, entomopathogenic fungi can protect plants against biotic and abiotic stresses and at the same time promote plant growth and plant health. To date, most studies have investigated whether *Beauveria bassiana* can enhance plant growth and plant health, while only little is known about other entomopathogenic fungi. In this study, we evaluated whether root inoculation of the entomopathogenic fungi *Akanthomyces muscarius* ARSEF 5128, *B. bassiana* ARSEF 3097 and *Cordyceps fumosorosea* ARSEF 3682 can promote plant growth of sweet pepper (*Capsicum annuum* L.), and whether effects are cultivar-dependent. Plant height, stem diameter, number of leaves, canopy area, and plant weight were assessed four weeks following inoculation in two independent experiments using two cultivars of sweet pepper (cv. 'IDS RZ F1' and cv. 'Maduro'). Results showed that the three entomopathogenic fungi were able to enhance plant growth, particularly canopy area and plant weight. Further, results showed that effects significantly depended on cultivar and fungal strain, with the strongest fungal effects obtained for cv. 'IDS RZ F1', especially when inoculated with *C. fumosorosea*. We conclude that inoculation of sweet pepper roots with entomopathogenic fungi can stimulate plant growth, but effects depend on fungal strain and crop cultivar.

KEYWORDS

***Akanthomyces muscarius*, *Beauveria bassiana*, *Cordyceps fumosorosea*, endophyte, plant growth promotion**

1 Introduction

Entomopathogenic fungi are well known for their ability to infect and kill insects (Shah and Pell, 2003; Islam et al., 2021). After invading a host, the fungus proliferates and invades the host's organs and tissues, leading to the death of the insect. Next, the fungus emerges from the insect cadaver and produces thousands of new spores, which then disperse and

infect a new host (Shah and Pell, 2003; Islam et al., 2021). Due to the fact that they are able to suppress natural insect populations and generally impose no or minimal adverse effects on humans and the environment (but see Hu et al., 2016), entomopathogenic fungi are commonly used as bioinsecticides, especially because virtually all insect orders are vulnerable to fungal diseases (Hajek and St Leger, 1994; Glare et al., 2012; Bamisile et al., 2021). There are several products based on entomopathogenic fungi commercially available for insect control, predominantly based on members of the genera *Akanthomyces* (previously *Lecanicillium* and *Verticillium*) (Hypocreales: Cordycipitaceae), *Beauveria* (Hypocreales: Cordycipitaceae), *Cordyceps* (previously *Isaria* and *Paecilomyces*) (Hypocreales: Cordycipitaceae) and *Metarhizium* (Hypocreales: Clavicipitaceae) (Faria and Wraight, 2007; van Lenteren et al., 2018).

In addition to colonizing insect hosts as pathogens, an increasing number of studies have shown that entomopathogenic fungi can associate with plants, often by colonizing plant tissues without causing disease symptoms as endophytes (Vega, 2008; Vidal and Jaber, 2015; Gange et al., 2019; Quesada-Moraga, 2020). Local or systematic colonization occurs mainly in the roots, stems, leaves and internal tissues of plants (Behie et al., 2015). The endophytic behavior of entomopathogenic fungi has been reported in numerous cultivated and non-cultivated plant species, both naturally colonized and artificially inoculated by diverse methods, and several of these fungi have the potential to improve the plant's response to biotic and abiotic stresses (Vega, 2008; Vidal and Jaber, 2015; Vega, 2018; Gange et al., 2019; Francis et al., 2022). For example, banana and common bean plants inoculated with entomopathogenic fungi showed reduced reproduction rates and higher mortality rates of the banana root borer (*Cosmopolites sordidus*), one of the most important pests on bananas (Akello et al., 2008), and the pea leaf miner (*Liriomyza huidobrensis*) (Akutse et al., 2013), respectively, while endophytic colonization of sweet pepper by entomopathogenic fungi had negative effects on the development and fecundity of aphids (*Myzus persicae*) (Jaber and Araj, 2018; Wilberts et al., 2022). Moreover, endophytic entomopathogenic fungi have been shown to reduce pathogen infestation (Jaber and Alananbeh, 2018; Jaber and Ownley, 2018) and provide plants with drought stress tolerance (Ferus et al., 2019).

Given their capability to increase plant resistance against biotic and abiotic stress, endophytic entomopathogenic fungi are being increasingly evaluated as biostimulants or biopesticides (Lacey et al., 2015; Lugtenberg et al., 2016; Jaber and Ownley, 2018; Vega, 2018; Quesada-Moraga, 2020). However, most studies exploring the potential of endophytic entomopathogenic fungi in agricultural sustainability have focused on their use as biocontrol agents to suppress insect pests (Vidal and Jaber, 2015; Vega, 2018; Mantzoukas and Eliopoulos, 2020) and less research has focused on their possible role as plant growth promoters, notwithstanding a number of studies have shown their potential to stimulate plant growth following endophytic colonization (Tall and Meyling, 2018; Canassa et al., 2019; Espinoza et al., 2019; Ahmad et al., 2020). Given that endophytic entomopathogenic fungi can persist for a long time in host tissues, growth-promoting effects can be expected to last for a long time (Brownbridge et al., 2012; Bamisile et al., 2020), although there are also examples of transient colonization that led to enhanced growth (Gurulingappa et al., 2010; Resquín-Romero et al., 2016), further enhancing their appeal as plant growth promoters.

Among endophytic fungal entomopathogens, *Beauveria bassiana* is the most frequently studied species to promote plant growth (Vega, 2018). It has been reported as early as 1990 as naturally occurring in maize (Vakili, 1990), and has since then been isolated from several other plant species (Márquez et al., 2007; Vega et al., 2010; Pimentel et al., 2016). The fungus has also been successfully established as an endophyte in several crops following artificial inoculation, benefiting plant growth and overall plant health (Espinoza et al., 2019; Saragih et al., 2019; Shaalan et al., 2021). By contrast, only little attention has been given to other fungal entomopathogens like *Akanthomyces* or *Cordyceps*, and their potential benefits on plant growth and plant health remain to be investigated. Furthermore, the effects of entomopathogenic fungi have been shown to vary between plant species (Gurulingappa et al., 2010; Sánchez-Rodríguez et al., 2018), suggesting that plant growth promotion may be affected by the host's genotype or cultivar. Because plant-fungus interactions comprise complex molecular dialogues that induce large-scale transcriptomic changes in both partners (Tucci et al., 2011; Pieterse et al., 2014; Alam et al., 2021; Mattoo and Nonzom, 2021), it can be assumed that both the entomopathogenic fungal strain and cultivar strongly determine the net result of the plant response, but evidence is still scarce.

The aim of this study was to assess the plant growth promoting capabilities of different species of entomopathogenic fungi and to assess whether plant responses are mediated by plant cultivar. Therefore, we tested the effects of root inoculation of two cultivars of sweet pepper (*Capsicum annuum* L.; Solanaceae) with *B. bassiana* (ARSEF 3097) and the fungal species *Akanthomyces muscarius* (ARSEF 5128) and *Cordyceps fumosorosea* (ARSEF 3682) on plant height, stem diameter, number of leaves, canopy area and plant weight. Experiments were performed in two different years.

2 Materials and methods

2.1 Plant and fungal material

Two cultivars of sweet pepper were used in this study: cv 'IDS RZ F1' (Rijk Zwaan, De Lier, the Netherlands) and cv 'Maduro' (Enza Zaden, Enkhuizen, the Netherlands). These cultivars are commonly used in commercial sweet pepper cultivation in Belgium. Both cultivars have crude, medium-size red fruits. IDS RZ F1 is resistant to Tobamovirus pathotypes P0, P1, P2 and P3, while Maduro is resistant to pathotypes P0, P1 and P2. Plants were sown in a 3:1 mixture of potting mix (Universal potting mix; Agrofino, Ghent, Belgium) and white sand, and incubated until fungal inoculation (see further) in a plant cabinet that was equipped with LED lights above the foliage, providing a photosynthetic flux density of 790 µmol photons $m^{-2}s^{-1}$ (23 ± 1°C, 65 ± 2% RH and a 16L:8D photoperiod) (MD1400, Snijders Labs, the Netherlands).

Three endophytic entomopathogenic fungi were used in this study: *Akanthomyces muscarius* ARSEF 5128 (Ve-6; previously known as *Lecanicillium muscarium*), *Beauveria bassiana* ARSEF 3097 (ATCC 74040), and *Cordyceps fumosorosea* ARSEF 3682 (Apopka 97; previously identified as *Isaria fumosorosea*). These three fungi are the active substance in the bioinsecticides Mycotal®, Naturalis® and PreFeRal®, respectively. Originally, *A. muscarius* ARSEF 5128 was isolated from a greenhouse whitefly in Littlehampton (UK) (Hall, 1982), *B. bassiana* ARSEF 3097 from a boll weevil in the Rio Grande Valley (USA) (Wright, 1996) and *C. fumosorosea* ARSEF 3682 from a mealy bug in a greenhouse in Apopka (USA) (Vidal et al., 1998). All strains have been shown to colonize plants as an endophyte upon artificial inoculation in various crops, including sweet pepper (Kuchár et al., 2019; Rondot and Reineke, 2019; Nicoletti and Becchimanzi, 2020; Doherty et al., 2021; Wilberts et al., 2022). The strains were acquired from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF; New York, USA), and were stored as agar plugs in glycerol at -80°C.

2.2 Fungal spore suspensions and plant inoculation

Stored agar plugs of each fungus were plated onto quarter-strength (¼) Sabouraud dextrose agar with yeast extract (Oxoid Holdings Ltd, United Kingdom) (SDAY), and once again replated onto the same agar medium before use. Conidial suspensions were prepared by culturing the fungi in darkness on SDAY for seven days at 25°C, followed by flooding the plates with sterile physiological water (0.8% NaCl) and scraping fungal tissue of the plates. Next, fungal fragments and spores were filtered through microcloth (Mira Cloth, Merck, Massachusetts, USA) to remove fungal hyphae, and the spore concentration was determined by using a Bürker hemocytometer under the microscope, and diluted to 1×10^7 conidia mL^{-1}. Before inoculation, a 100 µL aliquot of 1×10^3 spores mL^{-1} was plated on three SDAY plates to check spore viability. The number of germinated and ungerminated spores was determined under the microscope after 24 h of incubation at 25°C. Spores with germ tubes at least two times longer than their diameter were considered as germinated. The germination assays showed >90% viability rate for all fungal spore suspensions used in the experiments.

Plants were inoculated as described in Wilberts et al. (2022). Briefly, at the first true leaf stage seedlings were uprooted and roots were rinsed under running tap water. Next, roots were dipped in 10 mL of the conidial spore suspensions for 18h. Roots of a separate set of seedlings were submerged in 10 mL physiological water to be included as non-inoculated (control) plants. Seedlings were then placed individually in 17-cm-diameter plastic pots in a 3:1 mixture of potting mix (Universal potting mix; Agrofino, Ghent, Belgium) and white sand (for chemical characteristics of the potting medium, see Table S1; Supporting information), and put in the greenhouse according to a randomized complete block design. The experiment was performed with 10 replicates per treatment, yielding 2 cultivars × 4 treatments × 10 plants = 80 plants in total. The experiment was performed twice (February-March 2021 and February-March 2022, further referred to as "Exp 2021" and "Exp 2022", respectively). In both trials, plants were maintained at 23 ± 5°C, 65 ± 10% RH and a photoperiod of 16L:8D. Plants were watered daily with a nutrient solution for sweet pepper (Table S2; Supporting information). Temperature, relative humidity and solar insolation in the greenhouse were monitored throughout the experiments (Figure S1; Supporting information).

2.3 Plant growth

To assess plant growth, plant height (from lowest leaf node to the highest node), stem diameter, number of leaves, canopy area, and fresh and dry weight were measured for each plant. Plant height was measured at the start of the experiment (i.e. immediately after inoculation and potting) and subsequently every week for a total period of four weeks. All other variables were measured at the end of the experiment, i.e. four weeks after transplantation. Stem diameter was measured 1 cm above the lowest leaf node with a sliding caliper. Canopy area was calculated from top view images taken with a Canon EOS 1300d camera with Canon zoom lens EF-S 18-55mm f/3.5-5.6 III. The surroundings of the plants, including the plant pots, were covered with blue plastic as a contrast, while a red reference card of known size (15 × 10 cm) was put next to each plant. Then, canopy area was calculated by color segmentation with an R tool based on the EBImage (Pau et al., 2010) and imagemagick packages by separating the green plant pixels from the blue background. The red reference surface was used to calculate the green area (van Wesemael et al., 2019). To determine fresh and dry weight of the plants, plants were removed from the pots and roots were washed. Next, after air drying, fresh weight of the plants was determined. Subsequently, the plants were placed in individual paper bags and dried for five days at 80°C, after which the dry weight was determined. Before weighing the plants, the fifth leaf of every plant was collected, surface-sterilized (Landa et al., 2013) and subjected to DNA extraction and PCR amplification using the species-specific primer combinations ITS1F (Gardes and Bruns, 1993) and Am_Rv1 (5'-AGATGCTGATAATACAGAGTT-3'), ITS1F and Bb_Rv1 (5'-GATGCTGGAATACAAGAGTTTGAG-3') and ITS1F and Cf_Rv1 (5'-CGGATTCAGAAAGA CTGATAG-3') to detect *A. muscarius*, *B. bassiana* and *C. fumosorosea* respectively, as described in Wilberts et al. (2022).

2.4 Statistical analysis

Plant height was analyzed using a Generalized linear mixed model (GLMM) based on a Gamma distribution with a log link function using treatment, plant cultivar, and week as fixed factors, while plant was entered as random factor (performed with the 'glmer' function from the lme4 package). Plant height was entered as response variable, and the interaction factor between the fungal

treatment and cultivar was added to the model. Stem diameter, canopy area, fresh weight and dry weight were analyzed using a Generalized Linear Model (GLM) based on a Gamma distribution with a log link function using treatment, plant cultivar and their interaction as fixed factors (performed with the 'glm' function from the lme4 package). The number of leaves was analyzed using a GLM based on a Poisson distribution with a log link function using treatment, plant cultivar and their interaction as fixed factors. For this analysis, each plant was considered a biological replicate, giving a total of 10 replicates per treatment. To evaluate overall differences between the different treatments and cultivars, an analysis of variance (ANOVA) Type III test was performed on all models. When an overall difference was observed, a *post hoc* pairwise comparison (with estimated marginal means using the emmeans package) was performed to determine the pairwise differences between the different treatments and cultivars. The statistical analysis of the greenhouse experiments was performed for each dataset separately, as experiments were performed in different years. A significance level of $\alpha = 0.05$ was applied to establish significant differences. All analyses and visualization of the data (ggplot2 package) were performed using R version 3.6.1 (R Core Team, 2019).

3 Results

3.1 Plant growth

Cultivar had a strong effect on plant growth, while the effects of fungal strain were less pronounced and differed between the two experiments (Table 1). The effect of fungal strain on plant growth was strongest in the experiment performed in 2022 (Table 1). Plant height of IDS RZ F1 plants was significantly larger than that of Maduro plants over the course of both experiments (Figure 1; Table 1). In the experiment performed in 2021 (Exp 2021), fungal inoculation with the entomopathogenic fungi did not have a significant effect on plant height (Table 1). In the experiment performed in 2022 (Exp 2022), fungal inoculation resulted in higher IDS RZ F1 plants, especially when inoculated with *C. fumosorosea* ($P = 0.019$). For Maduro plants, fungal inoculation did not elicit an effect on plant height compared to control plants (*A. muscarius*: $P = 0.997$; *B. bassiana*: $P = 0.967$; *C. fumosorosea*: $P = 0.868$).

Stem diameter did not differ between cultivars in Exp 2021, while in Exp 2022 Maduro plants were thinner than IDS RZ F1 plants (Figures 2A, B; Table 1). In the first experiment, IDS RZ F1 plants inoculated with *A. muscarius* were significantly thicker than plants inoculated with *C. fumosorosea* ($P = 0.028$), while no other differences were observed among treatments (Figure 2A). In the second experiment, plants inoculated with *B. bassiana* and *C. fumosorosea* had significantly thicker stems than control plants for both cultivars (IDS RZ F1 - *B. bassiana*: $P = 0.037$; IDS RZ F1 - C. *fumosorosea*: $P = 0.020$; Maduro - *B. bassiana*: $P = 0.034$; Maduro - C. *fumosorosea*: $P < 0.001$) (Figure 2B). Likewise, Maduro plants inoculated with *C. fumosorosea* had significantly thicker stems than Maduro plants inoculated with *A. muscarius* ($P = 0.042$) (Figure 2B).

The number of leaves did not differ significantly between cultivars in both experiments (Table 1). Also fungal inoculation did not affect the number of leaves significantly (Figures 2C, D; Table 1). Canopy area of IDS RZ F1 plants was significantly larger than that of Maduro plants in both experiments (Figures 2E, F; Table 1). While fungal inoculation did not significantly affect canopy area in Exp 2021, clear effects were observed in Exp 2022 (Figures 2E, F). Specifically, in Exp 2022, fungal inoculation of IDS RZ F1 plants resulted in a wider canopy for all fungi compared to the control plants (*A. muscarius*: $P = 0.043$; *B. bassiana*: $P = 0.015$; *C. fumosorosea*: $P < 0.001$). Furthermore, IDS RZ F1 plants inoculated with *C. fumosorosea* had a significantly wider canopy than IDS RZ F1 plants inoculated with *A. muscarius* or *B. bassiana* (*A. muscarius*: $P < 0.001$; *B. bassiana*: $P < 0.001$) (Figure 2F). IDS RZ F1 control plants had a canopy area of 449.57 ± 72.50 cm^2 on average, compared to 574.98 ± 86.46 cm^2, 595.22 ± 129.37 cm^2 and 883.44 ± 116.90 cm^2 for IDS RZ F1 plants inoculated with *A. muscarius*, *B. bassiana* and *C. fumosorosea*, respectively. Maduro plants inoculated with *C. fumosorosea* also had a wider canopy than Maduro plants inoculated with *A. muscarius* ($P = 0.026$) and control plants ($P < 0.001$), although the difference was less pronounced than in IDS RZ F1 plants. Maduro plants inoculated with *C. fumosorosea* had a canopy area of 481.47 ± 94.04 cm^2 on average, while Maduro plants inoculated with *A. muscarius* and

TABLE 1 Effects of fungal strain, cultivar and their interaction on growth of sweet pepper plants[1].

	2021			2022		
	Fungal strain	Cultivar	Fungal strain × Cultivar	Fungal strain	Cultivar	Fungal strain × Cultivar
Plant height	5.372	9.269 **	2.426	8.945 *	32.321 ***	3.049
Stem diameter	8.115 *	1.594	7.315	10.362 *	5.802 *	1.02
Number of leaves	5.860	2.274	3.401	3.356	2.020	0.835
Canopy area	6.834	11.949 ***	3.233	54.902 ***	21.868 ***	4.847
Fresh weight	2.014	13.685 ***	14.314 **	34.132 ***	15.730 ***	0.560
Dry weight	5.704	14.426 ***	19.432 ***	39.289 ***	19.020 ***	1.947

[1]Chi-square distribution values from ANOVA on 10 plants per treatment measured four weeks after inoculation for all growth variables except plant height. Plant height was compared over the course of four weeks with weekly measurements (Generalized Linear Mixed Model). Asterisks indicate significant differences between the treatments (0.05 > P > 0.01: *; 0.01 > P > 0.001: **P < 0.001: ***).

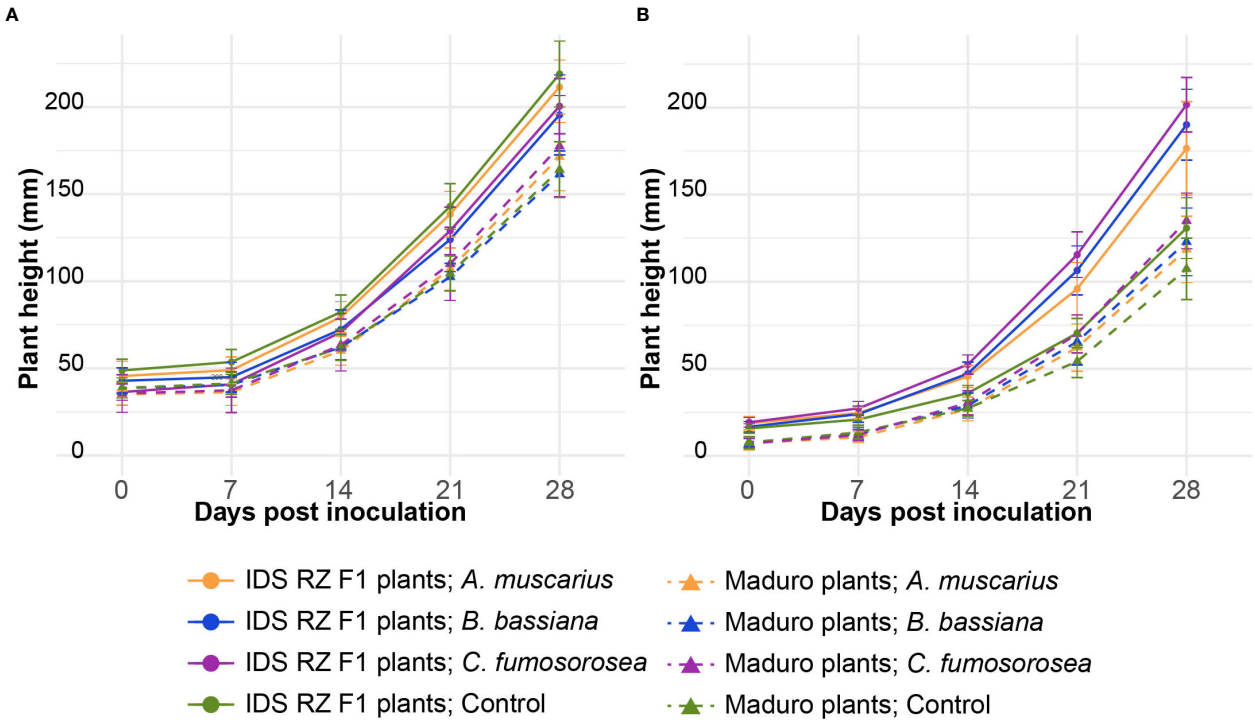

FIGURE 1
Average plant height of *Capsicum annuum* cv. IDS RZ F1 and cv. Maduro, inoculated with *Akanthomyces muscarius* ARSEF 5128, *Beauveria bassiana* ARSEF 3097 or *Cordyceps fumosorosea* ARSEF 3682, compared to control plants (n = 10). Plant height was measured weekly over a period of four weeks after fungal inoculation. The experiment was set up twice: in February-March 2021 **(A)** and in February-March 2022 **(B)**. Error bars represent standard error of the mean.

control plants had an average canopy area of 370.16 ± 86.91 cm² and 323.73 ± 79.04 cm², respectively. Maduro plants inoculated with *B. bassiana* had a canopy area of 402.38 ± 90.40 cm² on average (Figure 2F).

Fresh weight of IDS RZ F1 plants was higher than that of Maduro plants in both experiments (Figures 3A, B; Table 1). In Exp 2022, fresh weight of plants inoculated with the entomopathogenic fungi was significantly higher than that of control plants for both cultivars (IDS RZ F1 - A. *muscarius*: $P = 0.001$; IDS RZ F1 - *B. bassiana*: $P < 0.001$; IDS RZ F1 - C. *fumosorosea*: $P < 0.001$; Maduro - A. *muscarius*: $P < 0.001$; Maduro - *B. bassiana*: $P < 0.001$; Maduro - C. *fumosorosea*: $P < 0.001$) (Figure 3B). IDS RZ F1 plants inoculated with *A. muscarius*, *B. bassiana* and *C. fumosorosea* had a fresh weight of 50.28 ± 11.24 g, 50.96 ± 83.48 g and 66.18 ± 6.28 g on average, respectively, while IDS RZ F1 control plants weighted 31.56 ± 6.81 g on average. Maduro plants inoculated with *A. muscarius*, *B. bassiana* and *C. fumosorosea* weighted 30.69 ± 8.62 g, 34.51 ± 11.49 g and 40.90 ± 8.73 g on average, respectively, while Maduro control plants only weighted 18.98 ± 8.13 g (Figure 3B). Similarly, dry plant weight was significantly higher in inoculated plants compared to control plants (IDS RZ F1 - A. *muscarius*: $P < 0.001$; IDS RZ F1 - *B. bassiana*: $P < 0.001$; IDS RZ F1 - C. *fumosorosea*: $P < 0.001$; Maduro - A. *muscarius*: $P = 0.002$; Maduro - *B. bassiana*: $P < 0.001$; Maduro - C. *fumosorosea*: $P < 0.001$) (Figure 3D). In contrast to Exp 2022, an effect of fungal inoculation on plant weight was not observed in Exp 2021 (Figure 3C). However, both for fresh weight and dry weight, there was an interaction effect between cultivar and treatment in Exp 2021. This interaction effect was not observed in Exp 2022 (Table 1).

3.2 Endophytic colonization of the plants

At the end of both experiments, endophytic colonization by the three fungi was assessed by subjecting a sample from the fifth true leaf from all investigated plants to PCR analysis. The inoculated fungi could not be detected in any leaves of either inoculated or control plants four weeks after inoculation.

4 Discussion

In this study, we investigated the plant growth promoting capabilities of different species of entomopathogenic fungi and assessed whether plant responses were mediated by plant cultivar. Overall, entomopathogenic fungi had positive effects on plant growth parameters. However, effects were more pronounced in the experiment performed in 2022 compared to the experiment performed in 2021, possibly due to different climatic factors, although both experiments were set-up in the same way in the same period of the year (Figure S1, Supporting information). Similarly, previous studies have shown that entomopathogenic fungi like *B. bassiana* promote plant growth in diverse plant

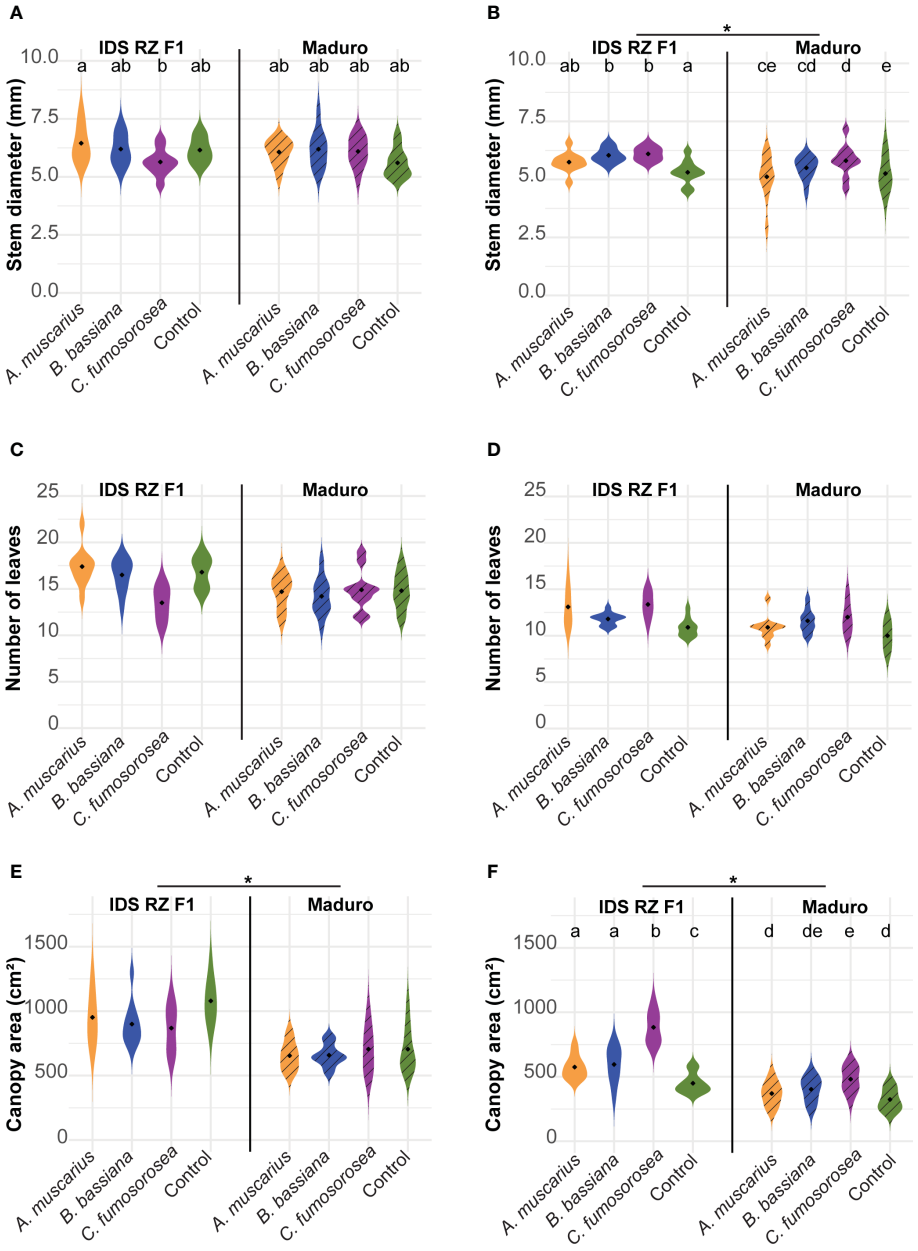

FIGURE 2
Stem diameter **(A, B)**, number of leaves **(C, D)**, canopy area **(E, F)** of *Capsicum annuum* cv. IDS RZ F1 and cv. Maduro, inoculated with *Akanthomyces muscarius* ARSEF 5128, *Beauveria bassiana* ARSEF 3097 or *Cordyceps fumosorosea* ARSEF 3682 compared to control plants four weeks after fungal inoculation (n = 10). The experiment was set up twice: in February-March 2021 **(A, C, E)** and in February-March 2022 **(B, D, F)**. Asterisks indicate a significant difference between the two cultivars (ANOVA, $P < 0.05$). Different letters indicate significant differences between treatments (Generalized linear model, $P < 0.05$). When no letters are given, no significant differences were observed.

species, including chive (Espinoza et al., 2019), cucumber (Shaalan et al., 2021), bean (Jaber and Enkerli, 2016), grapevine (Mantzoukas et al., 2021), maize (Tall and Meyling, 2018; Liu et al., 2022), red chili (Saragih et al., 2019), and wheat (Guzmán et al., 2021). By contrast, there are also studies that found no or sometimes negative effects of endophytic entomopathogenic fungi on plant growth (Vega, 2018; Moloinyane and Nchu, 2019). Our results also showed that plant growth promoting effects differ with fungal species. Specifically, we found that inoculation with *C. fumosorosea* resulted in the strongest growth promotion of sweet pepper, while effects of inoculation with *A. muscarius* and *B. bassiana* were less pronounced.

Although most growth variables were affected by fungal inoculation in the 2022 experiment, fungal inoculation had the largest effect on leaf area and consequently plant weight. Plants inoculated with the tested entomopathogenic fungi had larger leaves and a larger canopy area, which can have strong implications for crop yield. With a greater canopy area, photosynthesis can be enhanced, vegetative growth increased, and consequently the aging of the plant delayed (Worku et al., 2007; Jo and Shin,

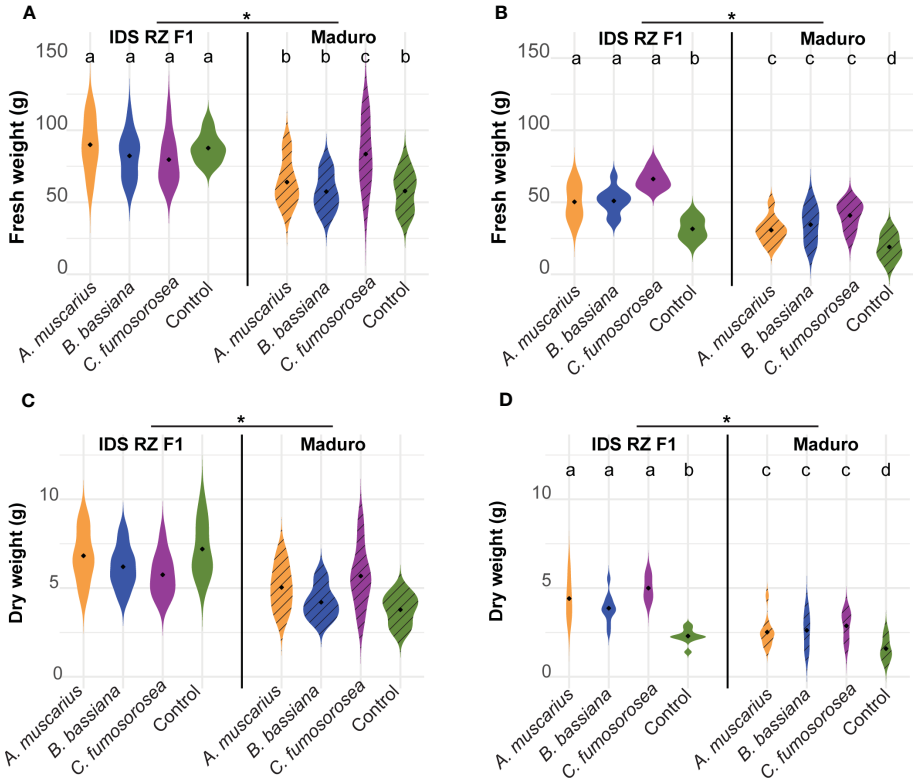

FIGURE 3
Fresh weight **(A, B)** and dry weight **(C, D)** of *Capsicum annuum* cv. IDS RZ F1 and cv. Maduro, inoculated with *Akanthomyces muscarius* ARSEF 5128, *Beauveria bassiana* ARSEF 3097 or *Cordyceps fumosorosea* ARSEF 3682 compared to control plants four weeks after fungal inoculation (n = 10). The experiment was set up twice: in February-March 2021 **(A, C)** and in February-March 2022 **(B, D)**. Asterisks indicate a significant difference between the two cultivars (ANOVA, $P < 0.05$). Different letters indicate significant differences between treatments (Generalized linear model, $P < 0.05$). When no letters are given, no significant differences were observed.

2020). Therefore, most studies on plant growth include leaf and/or canopy area as a major growth parameter, as plant weight is often too general as a parameter for plant development (Jo and Shin, 2020). It needs to be noted, however, that effects in our study were evaluated up to four weeks after fungal inoculation. While we specifically focused on vegetative growth in this study, further studies should be performed on how the observed growth promotion by fungal inoculation affects the growth of sweet pepper when the plants are balancing vegetative and generative growth.

Effects of fungal treatments resulted in similar trends in both cultivars. However, effects were more pronounced in the IDS RZ F1 cultivar, resulting in stronger significant differences between the treatments, while fungal treatments more often had a small to neutral effect on Maduro plant growth. Similarly, Canassa et al. (2020) found differences in plant growth between strawberry cultivars upon inoculation with entomopathogenic fungi. Fungal colonization of the internal parts of a plant is mediated by various biomolecules which drive dynamic changes in the expression of genes in the host plant and the fungus (Pieterse et al., 2014; Mattoo and Nonzom, 2021), and consequently can lead to strain- and cultivar-dependent differences. Furthermore, differences in plant colonization degree may affect plant responses (Jaber and Ownley, 2018). In our study, inoculated fungi could not be detected at the end of the experiment, suggesting that endophytic colonization was transient or that the fungi did not establish systematically in the plants, or at least not in the investigated leaf tissues (fifth leaf). Colonization of plant tissue by entomopathogenic fungi may be transient, with recovery of the fungi only in the first days after inoculation, especially when plants are grown in non-sterile soil, as was the case in this study (Posada et al., 2007; Gurulingappa et al., 2010; Allegrucci et al., 2017). Many factors may affect the degree to which entomopathogenic fungi colonize plant tissue, including inoculation method, environmental conditions and competing rhizosphere and endosphere microorganisms (Tefera and Vidal, 2009; Parsa et al., 2018; Rajab et al., 2020), but the exact mechanisms and forces behind endophytic colonization by entomopathogenic fungi still remain to be elucidated (Vega, 2018). Nevertheless, despite limited or even no endophytic colonization, beneficial effects of inoculation with entomopathogenic fungi have been observed, indicating that long term colonization or systemic colonization is not required to induce positive fungus-mediated effects (Parsa et al., 2018; Tall and Meyling, 2018). Further research should explore how and to which extent our plants were colonized by the fungal strains and how this affected plant responses. Regardless of fungal treatments, there were clear differences between both sweet pepper cultivars. In both experiments performed, Maduro plants were shorter, had

smaller leaves and weighed significantly less than IDS RZ F1 plants. Contrary to our results, Maduro is described as generally slightly bigger than IDS RZ F1 according to the cultivar description. On the other hand, IDS RZ F1 is selected to produce fruits somewhat earlier than Maduro, so it is possible that young IDS RZ F1 plants, as we have studied, grow slightly faster. Nevertheless, although IDS RZ F1 plants were bigger than Maduro, both had the same number of leaves, meaning that IDS RZ F1 has a more open growth, which makes harvesting, and general handling of the crop, easier.

Taking together that inoculation with entomopathogenic fungi has been shown to protect plants against pests and pathogens (Bamisile et al., 2018; Vega, 2018) and that our results clearly show that inoculation of sweet pepper with entomopathogenic fungi enhances plant growth, these fungi have the potential for multitarget effects in crops on both growth promotion and biocontrol. However, the underlying mechanisms remain to be unraveled. Enhanced plant growth might have been facilitated via improved acquisition of nutrients, phytohormone production, induced resistance, production of antibiotics and secondary metabolites, and/or production of siderophores (Vega, 2018; Baron and Rigobelo, 2022). For example, inoculation of potato with *Metarhizium brunneum* resulted in an increased leaf area and plant weight, which was correlated with an increased amount of nitrogen and phosphorous content, and an increased water use efficiency (Krell et al., 2018). Which scenario is at play for the fungi investigated in our study, remains to be unraveled. Further, more research is required on the secondary metabolites produced by these endophytic entomopathogenic fungi, which may possibly end up in the fruits, as some have been found to possibly be toxic to mammals (including humans), such as beauvericin (Hu et al., 2016; Mallebrera et al., 2018).

In conclusion, our results indicate that plant root inoculation with entomopathogenic fungi enhanced overall plant growth of sweet pepper, but effects depend on fungal strain and crop cultivar. Effects also differed between years, suggesting that environmental factors can influence the outcome of endophytic colonization by entomopathogenic fungi on plant growth. Strongest plant growth promoting effects were observed for cv IDS RZ F1 inoculated with *C. fumosorosea* ARSEF 3682, expressed by enhanced canopy area and increased plant weight. These results open possibilities for the implementation of plant inoculation with entomopathogenic fungi as plant growth promoters to support and stimulate sustainable agriculture.

Data availability statement

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

Author contributions

LW, HJ and BL designed the experiment, discussed the data, and revised the manuscript. LW and NRP performed the experiment. LW analyzed data, and prepared the first draft. All authors contributed to the article and approved the submitted version.

Funding

We would like to thank the Flemish Fund for Scientific Research (FWO; grant to LW (1S79919N)) and KU Leuven (grant to BL (C24E/19/052)) for financial support.

Acknowledgments

We are grateful to Rijk Zwaan (De Lier, the Netherlands) and Enza Zaden (Enkhuizen, the Netherlands) for providing sweet pepper seeds. We also would like to thank Ellen Vandenbussche and Noé Cuvelier for assistance with the experiments, and Hendrik Siongers and Clara Gambart for their technical assistance with the image-based tool for calculation of the canopy area.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1196765/full#supplementary-material

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TYPE Original Research
PUBLISHED 14 June 2023
DOI 10.3389/fpls.2023.1125630

OPEN ACCESS

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RECEIVED 16 December 2022
ACCEPTED 23 May 2023
PUBLISHED 14 June 2023

CITATION
Segaran G and Sathiavelu M (2023) Fungicidal and plant growth-promoting traits of *Lasiodiplodia pseudotheobromae*, an endophyte from *Andrographis paniculata*. *Front. Plant Sci.* 14:1125630. doi: 10.3389/fpls.2023.1125630

Fungicidal and plant growth-promoting traits of *Lasiodiplodia pseudotheobromae*, an endophyte from *Andrographis paniculata*

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Introdution: Fungal endophytes are widespread and dwell inside plant cells for at least part of their life without producing any symptoms of infection. Distinct host plants may have different fungal endophyte rates and community compositions. Despite this, the endophytic fungi connected with the host plant and their hostile behaviors, remain unknown.

Methods: The objective of the current research was to isolate and identify endophytic fungal species from the root of *Andrographis paniculata*. The effects of fungal isolate APR5 on the mycelial growth of phytopathogens and the production of plant-promoting traits were assessed.

Results and discussion: Endophytic fungal isolate APR5 showed higher inhibitory efficacy in dual and double plate assay against the tested phytopathogenic fungi. The scanning electron microscope analysis demonstrated that the phytopathogenic fungal hyphae were coiled by endophytes which makes them shrink and disintegrate. Further, an ethyl acetate crude extract effectively suppressed the mycelium growth of *Rhizoctonia solani* by 75 $\pm$ 0.1% in an agar well diffusion assay. The fungal isolate APR5 was identified as *Lasiodiplodia pseudotheobromae* using the nuclear ribosomal DNA internal transcribed spacer (ITS) region and qualitatively evaluated for their capacity to produce plant growth-promoting hormones. Gas chromatography-mass spectrometry was implemented to acquire a preliminary understanding of the secondary metabolic profile of ethyl acetate crude extract. 1-octadecene, erythritol, niacin, oleic acid, phenol, pantolactone, phenyl ethyl alcohol, *p*-cresol, and tbutyl hydroquinone are the metabolites analyzed in a crude extract of APR5 isolate and are reported to have antimicrobial properties.

KEYWORDS

agriculture, biocontrol agent, chemical pesticide, endophyte, food production, phytopathogenic fungi

1 Introduction

The global population has grown significantly from 1.6 billion in 1900 to 7.0 billion in 2011, in the past century. It is estimated that by the year 2050, there will be 9.7 billion people around the world, increasing the demand for water resources. Under this scenario, the production of food will need to expand by around 70% by 2050 and twice or triple by 2100, while aiming to reduce the impact on the environment (Poveda et al., 2021). Fungi are a prominent disease-causing agent on plants with a huge loss of up to 90% of agricultural production (Elamathi and Mathanraj, 2017). Soil-borne fungal pathogens reduce agricultural productivity and degrade the quality of food products. Such fungal infections with a wide host range spread diseases in a variety of commercially important crops (Dukare et al., 2020). These well-known soil-borne pathogens may be found in many types of soil. Due to their saprophytic nature, they may spend more time in the soil. This condition has been documented in at least 32 nations, with warm-climate countries being the hardest impacted (Karthika et al., 2020). Banana, cucumber, potato, tomato, and tobacco are the mainly affected crops by soil-borne pathogens all over the globe. The deadliest ailment to strike tomato plants worldwide, particularly in uplands, is Fusarium wilt. In the wilted plants with yellowed leaves, Fusarium wilt causes a 60–70% reduction in fruit output and infects 30–40% of the crop annually (Jinal and Amaresan, 2020; Karthika et al., 2020).

Macrophomina phaseolina causes seedling blight, charcoal, stem, and root rot and affects approximately 500 plant species from over 100 families all around the world. It affects commercially significant vegetables, cotton, sorghum, sunflower, and legumes and has a wide geographic spread in tropical and subtropical nations. When exposed to humans, *M. phaseolina* can infect immunosuppressed patients (Javed et al., 2021). When the temperature is high (30–35°C) and soil moisture is low (under 60%), it lowers farmer profitability by inducing major yield loss in sorghum and soybean. When the disease emerged at the pre-emergence stage, groundnut cultivars experienced 100% yield loss (Marquez et al., 2021). Due to its enduring nature, it can survive for up to 3 years in the shape of microsclerotia as resistant forms in infected plant detritus or dirt (Khan et al., 2021). *Rhizoctonia solani* is a major soil-borne fungus detected in both cultivated and non-cultured soils. It lives as sclerotia in the soil and does not produce asexual spores. The most prevalent infection induced by *R. solani* is seedling damping-off (Goudjal et al., 2014). Sclerotia are superficial, firm, and distinctively shaped dark brown to black masses, are the most obvious symptom of black scruf, and result in distorted and fractured tubers. The wide host range and overwintering characteristics of *R. solani* make them difficult to control using conventional biological and chemical methods (Rafiq et al., 2020).

To suppress the occurrence of soil pathogenic fungi, synthetic fungicides notably bavistin, benomyl, and thiram have traditionally been utilized (Dukare et al., 2020). These fungicides were transformed into poisonous compounds by the host plant tissue or by pathogens. In addition to fungicide resistance and increasing soil pollution, the widespread use of chemical fungicides has the potential to disrupt microbial ecosystems and weaken the ozone layer (Goudjal et al., 2014). About 10 to 40% of the nutrients from chemical fertilizers are ultimately absorbed by plants and the remainder are leached, their use would aid in reducing the loss of nutrients (Poveda et al., 2021). The rise in production demand, restrictions on agrochemicals usage, and the emergence of resistance towards the chemical products used led to the need for new and effective biocontrol agents (Elamathi and Mathanraj, 2017). Due to their non-polluting and eco-safe nature, biocontrol agents with plant growth-promoting traits can leads to chemical-free sustainable agriculture (Dukare et al., 2020).

Endophytic fungi are ubiquitous and stay intercellular or intracellular in plants for at least a portion of their lives without triggering infection symptoms (Nayak et al., 2016). Darnal, Germany discovers endophytes in 1904. Endophytic fungi similarly colonize plant tissues as plant pathogens and mycorrhizae, with a series of stages that include host recognition, fungal spore germination, epidermal penetration, and tissue colonization (Nayak et al., 2016). Mutualistic, symbiotic, communalistic, and trophobiotic are the various interaction types found between host plants and endophytes (Masi et al., 2019). The frequencies and community compositions of fungal endophytes may vary for different host plants (Piska et al., 2015). Endophytic fungi are identified to have mutualistic relationships with their hosts and mostly protect plants from tissue-invading pathogens or herbivores by producing secondary metabolites, phytohormones that encourage plant development, or by delivering nutrients to the host. They may also interact directly with their hosts through niche competition, hyperparasitism, by releasing poisonous substances and by inducing systemic resistance (Radu and Kqueen, 2002; Bila´nski and Kowalski, 2022). By secreting plant growth-promoting chemicals that might confer resistance to the host plant during favorable environmental circumstances, the endophytic fungi improve the growth response in infected host plants mostly through nutrient cycle (Piska et al., 2015). Endophytic microorganisms are a relatively unexplored community that is currently gaining attraction in medical and agricultural research. Different researchers worked on the endophytic fungi of various medicinal plants in and around India (Nayak et al., 2016; Roy et al., 2016).

Andrographis paniculata is an erect annual herb with a harsh flavor and belongs to the Acanthaceae family. Andrographis is a genus of little annual shrubs with 28 species primarily found in tropical Asia. In north-eastern India, the plant is known as Maha-tita, or "king of bitters" (Nayak, 2015). It is native to India and Sri Lanka and the plant is extensively cultivated in Asia. In China, Indonesia, Hong Kong, the Philippines, Malaysia, and Thailand, it is used as traditional herbal medicine. It is referred to as Hempedu Bumi in Malaysia. Flavonoids, diterpenes, lactones, aldehydes, alkanes, and ketones were found in this medicinal plant. Andrographolide and Kalmeghin are the bioactive chemical compounds found in their leaves (Firdous et al., 2020). In addition to its widespread usage as an immunostimulant, Kalmegh is stated to have anti-snake venom, antihepatotoxic, antimalarial, antibiotic, antihepatitic, anti-inflammatory, antipyretic and anti-thrombogenic effects (Nayak, 2015). It is recognized as

'Sirunangai' or 'Siriyanangai' in Tamil (Arunachalam and Gayathri, 2010). *A. paniculata* harbors endophytic bacteria with the capability to act as plant growth regulators and promoters (Masi et al., 2019). 14-Deoxyandrographolide, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide, and andrographolide are the labdane type diterpene lactones and a major bitter component of this plant (Rashid et al., 2018). Due to the existence of many bioactive metabolites, medicinal plants have a specific microbiome that can enhance the potential for interaction with microorganisms. Plant growth-promoting microbes stimulate plant growth by competing with microbial pathogens, activating plant defense responses, and secreting plant growth-promoting chemicals that include auxins, bacterial volatiles, and cytokinins (Sinha and Raghuwanshi, 2015). Research findings on medicinal plants and endophytes have revealed that the therapeutic properties of medicinal plants are not only due to the chemicals found in the plant but also to the endophytes that dwell within the plant (D'Souza and Hiremath, 2015). Selecting plants for investigating endophytic fauna for a specific goal usually involves several considerations. The objective of this experiment was to evaluate the hostile behavior of soil-borne pathogenic fungi and fungus endophytes from the plant *A. paniculata*. The present research will contribute to the investigation and application of endophytic fungus for enhanced plant disease management.

2 Materials and methods

2.1 Procurement of indicator microorganisms

Fusarium oxysporum, Macrophomina phaseolina, and *Rhizoctonia solani* are the diagnostic phytopathogens used for assessing the antifungal ability of endophytic fungus. The Department of Plant Pathology at Annamalai University in Chidambaram graciously provided with these soil-borne fungus phytopathogens. The maintenance and cultivation of fungal strains were carried out on a potato dextrose agar (PDA, HiMedia Laboratories, Mumbai, India).

2.2 Isolation of endophytic fungus from *Andrographis paniculata*

The root segments were carefully detached from the healthy *Andrographis paniculata* in Ranipet district, Tamilnadu, India (Latitude 12.9272; Longitude 79.36883). The plant parts were cleaned with distilled water to get rid of dirt and debris. Following 4% NaOCl solution for 3 minutes, 70% ethanol for 1 minute, and 70% ethanol for 30 seconds, the surface of the root was sterilized. Upon that, the root segments were washed three times with clean Milli-Q water. 100 µL of Milli-Q water from the final wash was spread over the fresh PDA plate to check the efficacy of surface sterilization (control plate). The surface-sterilized root segments were then cut into tiny sections of about 0.5 cm, placed on PDA plates, and incubated for 7 to 10 days at 27 ± 2°C until the fungal endophytes appeared. The fungal strains were purified using the single hyphal tip method and then plated on a PDA medium (Rakshith et al., 2013).

2.3 Antagonistic activity

2.3.1 Dual culture method

A dual culture experiment was conducted to evaluate the antagonistic activity of endophytic fungus against soil-borne phytopathogens. On the opposing plate, active pathogenic (3 days old culture of *F. oxysporum, M. phaseolina*, and *R. solani)* and endophytic fungus were put as 8 mm mycelial plugs with a 3 cm gap between them and 1 cm from the border. The control dish contains only the pathogenic fungi disc. The experiment was carried out in triplicates and incubated at 27 ± 2°C. When the pathogenic fungus completely covered the control dish, growth suppression was recorded (Rakshith et al., 2013).

The following equation was used to estimate the growth inhibition rate:

The percentage of inhibition (%) = [(RC–RT)/RC] ×100

Whereas RC denotes the radius of the control colony,

RT denotes the radius of the test colony.

2.3.2 Double plate technique

Endophytic fungus were grown on sealed petri plates to assess their volatile compound production. About 5 mm discs of test pathogens and endophytic fungi were each placed in the center of two separate bottom petri dishes. One of the plates (with the pathogen) was then flipped over to the other bottom containing endophyte to form a chamber. This experimental setup was sealed with parafilm and kept for 7 days at 27 ± 2°C. Endophytic fungus without pathogens at the bottom were used as a control. The percentage of inhibition was assessed following a week of monitoring (Chen et al., 2016).

2.3.4 Scanning electron microscopy analysis

Visualization of the morphologic changes in pathogenic fungus was done using scanning electron microscopy (SEM) analysis. To investigate changes in the hyphal morphology of test fungi caused by the antagonistic action of endophyte, 0.5 cm pieces of agar media from the edge of the inhibition zone were analyzed. The samples were prepared to view under SEM (EVO/18 Research , Carl Zeiss).

2.4 Molecular genomic identification of the endophytic fungus

Molecular identification was carried out by employing 18S rRNA sequencing. Using the NucleoSpin® Tissue Kit, the genomic DNA was extracted. Using the universal primers ITS-1F (5'-TCCGTAGGTGAACCTTGCGG-3') and ITS-4R (5'-TCCTCCGCTTATTGATATGC-3'), the genomic DNA was amplified. The PCR was conducted in a 20 µL reaction mixture that comprised 5 pM of forward and reverse primers, template DNA, 0.1 mg/mL BSA, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 1X PCR buffer (100 mM Tris HCl, pH-8.3; 500 mM KCl),

0.2 mM each dNTP (dATP, dGTP, dCTP, and dTTP) and 2.5 mM $MgCl_2$. The 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 40 s, extension at 72°C for 60 s, and final extension at 72°C for 5 minutes were the first step in the amplification process. A PCR thermal cycler was used to do the PCR amplification (GeneAmp PCR System 9700, Applied Biosystems). The PCR results were examined using a UV transilluminator and 1.2% agarose gel electrophoresis. Using BLAST search, the outcomes were matched to the National Center for Biotechnology Information (NCBI). A phylogenetic tree was created by the neighbor tree joining method (Jenila and Gnanadoss, 2018).

2.5 Analysis of the antifungal activity of a crude extract

2.5.1 Preparation of endophytic fungal extracts

The endophytic fungus (3 days old culture) were grown for 21 days under steady circumstances at 27 ± 2°C in a 500 mL conical flask (Borosil graduated narrow mouth flasks code 4980024) containing 300 mL potato dextrose broth. After the incubation period, Whatman No. 1 filter paper was deployed to separate the fungal mat from the culture filtrate. Multiple solvents with different polarities were used to extract the secondary metabolites from culture filtrates using a separating funnel (Borosil funnel code-6400). Petroleum ether (pet ether), dichloromethane (DCM), ethyl acetate (EA), and butanol were some of the solvents utilized. The chemical compounds were extracted from the fungal mycelium mat using methanol. The organic phase was obtained and condensed in a rotary evaporator (Model: RE100-Pro). The extracted metabolites were dried and stored for further analysis at -20°C.

2.5.2 Agar well diffusion assay

Phytopathogenic fungal discs of about 8 mm (*F. oxysporum, M. phaseolina*, and *R. solani)* were placed in the center of a fresh PDA plate, and different solvent extracts of various filtrate concentrations (25, 50, 75, and 100 µg/mL) were loaded into four wells made in equivalent distance. For antifungal tests, the desiccated crude preparations were reconstituted with dimethyl sulfoxide (DMSO). The control plates with pathogens were loaded with 10% DMSO solvent and incubated at 27 ± 2°C. The results from the control plate were compared to the proportion of mycelial growth inhibition in the test plate. Three replications of the assay were done for each treatment. Using the above-mentioned formula, the growth inhibition percentage of phytopathogens' radial mycelial growth was calculated (Wei et al., 2020).

2.5.3 Poisoned food technique

Poisoned food bioassay was used to evaluate the effectiveness of fungal crude extracts against phytopathogens. Molten PDA medium was mixed with fungus extracts (1000 µg, 500 µg, and 250 µg/mL DMSO), which are thought to be poisonously feeding for pathogens. Intoxicated PDA plates were inoculated with a pathogen's mycelia plug, which was then incubated at 27 ± 2°C for 7 days. By contrasting the radial expansion of the pathogen cultured in the test and control plates (DMSO), the impact of extracts on the growth of the pathogen was identified. The inhibition percentage formula was used to calculate the findings as a percent suppression of pathogen development (Gupta et al., 2022).

2.5.3 GC-MS analysis

GCMS was used to assess the crude extract of *L. pseudotheobromae* APR5. The investigation was done on an Agilent 7890B gas chromatography system and an Agilent MS 240 Ion Trap with HP-5MS capillary column (5 percent phenyl methyl polysiloxane, 30 m, 250 M, 0.25 M). The startup oven temperature was 50°C, which was set for 1 minute, proceeded by a 10°C min^{-1} ramp to 200°C, which was held for 1 minute, then a 5°C min^{-1} ramp to 325°C, which was held for 1 minute. A total of 1 liter was supplied, and the temperature was maintained at 280°C. The carrier gas was helium, and the ionizing electron energy was 70 eV. The extract was separated into tenths of a liter. The ions were found in the 50–1000 m/z range. The GC required 25 minutes to complete. The dried crude obtained was diluted with the same solvent and studied with GC-MS analysis (Veilumuthu et al., 2022).

2.5.4 Analysis of ethyl acetate crude extracts by Fourier transform infrared spectroscopy

The fungal crude extracts were FTIR analyzed using a Shimadzu FT-IR spectrophotometer (Model: IR Affinity). The functional groups contained in the chemical compounds were recorded in the range of 4000–400 cm-1. The infrared absorption spectrum is used to determine the chemical bonds in the molecule. The annotated spectrum indicates that the chemical bonds in the sample absorb a certain wavelength of light. For FTIR instrumentation examination, the dried crude extract of fungus was employed (Veilumuthu and Christopher, 2022).

2.6 Plant growth-promoting traits

For the screening of indole acetic acid (IAA) synthesis, the isolated endophyte was grown on Czapek broth medium for 7 days at 27 ± 2° C. After seven days, the samples were filtered, and the amount of IAA in the culture filtrate was measured by the addition of 1 mL of Salkowski reagent to 2 mL of the filtrate and incubated for 30 minutes in the dark (Bilal et al., 2018). To determine whether siderophores were present, 1 mL of the fungus culture's supernatant was combined with 0.4 mL of 2% liquid $FeCl_3$. The transition from yellow to brown confirms the presence of siderophore synthesis. To investigate the production of hydrocyanic acid (HCN), Whatman paper strips (dipped in the solution of 0.3% picric acid and 1.5% Na_2CO_3) were attached to the top lid of a petri dish, and fungus isolates were grown on a PDA medium. When the yellow color paper strip turns brown, it is considered to be positive. The presence of ammonia generation was detected by adding 2–3 droplets of Nessler's reagent to the culture supernatant of fungus grown in 10 mL of peptone. Pikovskaya's agar medium (PVK, Himedia) was supplemented with 0.1% zinc oxide and 2.5% tricalcium phosphate at pH 7.0 ± 0.2 to screen the ability of the fungal isolate to solubilize phosphate. The inoculation of fungal culture was done on a medium and after 24–48 h of incubation at 28°C, the

formation of a halo inhibitory zone around the fungal radial growth indicated a positive outcome (Chowdhary and Sharma, 2020).

2.7 Statistical analysis

To achieve three values for *in vitro* experiments, samples were analyzed in three replicates and the outcomes were measured. The GraphPad Prism Version 9.5.1 (733) software was used to perform the statistical analysis. All results were presented in terms of mean ± standard deviation (SD).

3 Results

3.1 Ethnomedicinal investigation of selected medicinal plants

In the present research, *Andrographis paniculata*, a medicinal plant was examined for its fungal endophytes. Being one of the bitterest herbs, it is highly valued in traditional medicine. Previous studies documented the anti-fungal and anti-typhoid properties of plant extracts. Consequently, the primary goal of this work was to identify a potent endophytic fungus with biocontrol and plant growth-promoting traits.

3.2 Isolation and identification of fungal endophytes from *Andrographis paniculata*

The endophytic fungus isolate APR5 was isolated from the healthy roots of *Andrographis paniculata*. The single hyphal tip approach was used to purify the endophytic fungal isolates, which were then plated on a PDA medium (Figure 1). No fungal or bacterial growth was observed on the control plates. The fungal isolate APR5 is a fast-growing white fungus that turns black after 72 h. The results of the ITS analysis showed that isolate APR5 was most similar to *L. pseudotheobromae* with >97% identity. The 18S rRNA sequence of *L. pseudotheobromae* isolate APR5 was deposited in Genbank and the accession number (OP999617) was received. The sequences from the nucleotide BLAST result were used to create the phylogenetic tree (Figure 2). To our knowledge, no studies have been conducted on the biocontrol ability and plant growth promoting traits of endophytic fungal isolate *L. pseudotheobromae*.

3.3 The inhibitory effects on phytopathogenic fungi

3.3.1 Antagonistic activity of endophytic fungus on test pathogens

The fungal isolate APR5 showed efficient antagonistic activity against *F. oxysporum*, *M. phaseolina*, and *R. solani* in a dual culture assay. The dual culture plates display the inoculation of endophytic fungus APR5 (on the right side) with the appropriate fungal pathogens (on the left side). *L. pseudotheobromae* grows and completely covers the colony of pathogen *F. oxysporum* through mycoparasitic activity in 3 days of incubation (Figure 3A). The endophyte stops growing when it gets in contact with the pathogens *M. phaseolina* and *R. solani*. Here, both the endophyte and pathogenic fungi compete for the substrate (Figures 3B, C). Among the tested three pathogenic fungi, *F. oxysporum* was highly inhibited with an inhibition percentage of 70 ± 0.15%, followed by *R. solani* (66 ± 0.1%) and *M. phaseolina* (54 ± 0.1%).

3.3.2 Volatile metabolites of endophytic fungus

The antagonistic effect of endophytic fungus APR5 was analyzed by performing a double plate assay. When compared to the control,

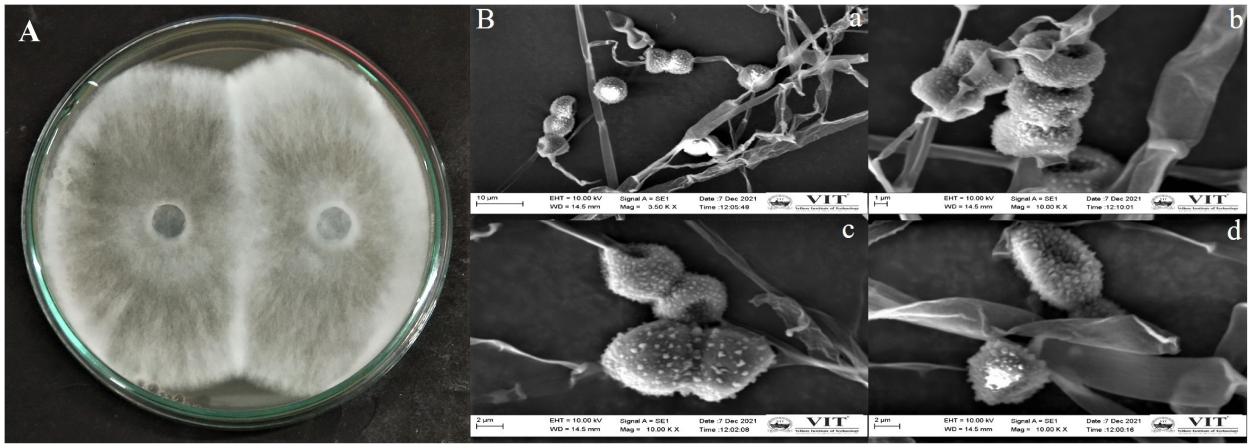


FIGURE 1
(A) Morphological appearance of endophytic fungus *Lasiodiplodia pseudotheobromae* APR5 isolated from *Andrographis paniculata* root; **(B)** The scanning electron micrographs of spores and hyphae of endophytic fungus *Lasiodiplodia pseudotheobromae* APR5 **(A)** (scale bar = 10 µm), **(B)** (scale bar = 1 µm), **(C)** (scale bar = 2 µm), **(D)** Morphological characteristics of a single spore (scale bar = 2 µm). The spores and hyphae were observed at 3500x and 10,000x.

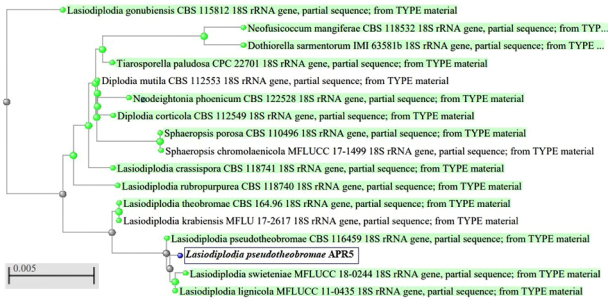


FIGURE 2
Phylogenetic tree of endophytic fungus *Lasiodiplodia pseudotheobromae* APR5 isolated from the root region of *Andrographis paniculata*.

the development of test phytopathogens was significantly slowed down by the VOCs produced by the fungal endophyte. The pathogenic radical growth in the experimental group was much less than that in the control group (Figure 4). The inhibition percentage for *F. oxysporum*, *M. phaseolina*, and *R. solani* was 65 ± 0.1%, 24 ± 0.05%, and 70 ± 0.1% on the seventh day respectively.

3.3.3 Analysis of antagonistic actions using SEM images

The phytopathogenic fungi from the dual culture plate were observed using SEM and it was discovered that the endophytes were responsible for the aberrant morphology of the fungus hyphae. The endophytes induced morphological anomalies in the pathogenic fungal hyphae, according to the SEM findings. The endophyte coiled around the hyphae of pathogenic fungi (Figures 5A–C). Shriveling and hyphal disintegration were the morphological alterations observed on the pathogen's hyphae. The hyphal breakages were observed on the fungal pathogens that are co-cultured with the potent endophyte (Figure 5D).

3.4 Antifungal bioassay of fungal crude extracts

The impacts of different crude extracts of *L. pseudotheobromae* APR5 on the mycelial growth of soil-borne pathogens (*F. oxysporum*, *M. phaseolina*, and *R. solani)* at different concentrations ranging from 100-25 µg/mL were examined. Significant antifungal activity was observed in the ethyl acetate crude extract against *R. solani* (Figure 6). In contrast to the control wells, which were covered with fungal hyphae, the hyphal growth was reduced toward the wells filled with ethyl acetate crude. When compared to the control plate, EA crude inhibited mycelial growth at the rate of 75 ± 0.1%. The organic fractions that were extracted with pet ether suppressed the mycelial growth of *F. oxysporum* with an inhibition percentage of 74 ± 0.05%. The inhibition percentage of all three concentrations of ethyl acetate crude extract was > 50%. The simple linear regression was analyzed using GraphPad Prism 9.5.1. The inhibition rate and log [concentration] value showed a significant linear association based on the outcomes of the toxicity test (R^2 = 0.9363, $p < 0.5$). With increasing pet ether extract concentrations, the hyphae's growth and branching patterns were disturbed, resulting in the aberrant bending of the pathogenic fungal colony. On the other hand, the controls showed normal hyphal development. Results are displayed as the percentage of inhibition in Table 1. These findings suggest that *A. paniculata* associated with *L. pseudotheobromae* APR5 have a range of remarkable disease-suppressing properties.

3.5 *In vitro* antifungal activity test

The agar dilution technique was used to evaluate the antifungal activity of EA crude extracts, which showed the highest inhibition of 75 ± 0.1% in the agar diffusion assay. We evaluated various concentrations of fungal crude extracts using the food poisoning method to assess the fungicidal activity. The effectiveness of fungal endophytes in combating phytopathogens was confirmed by the results of the poisoned food approach. The most prominent bioassay for evaluating the efficacy of endophytic fungus against a broad range of diseases is the poisoned food technique (Gupta et al., 2022). The outcomes are shown as the percentage inhibition in radial growth (PIRG) values of pathogens cultured on a PDA medium poisoned with ethyl acetate crude extracts of endophyte. The concentrations of each crude extract that was examined ranged from 0–1000 ppm. The petri dishes were incubated for seven days at room temperature. By monitoring the growth of fungal colonies on all four plates (1,000 ppm, 500 ppm, 250 ppm, and control DMSO), the treatment's effectiveness was determined (cm) (Figure

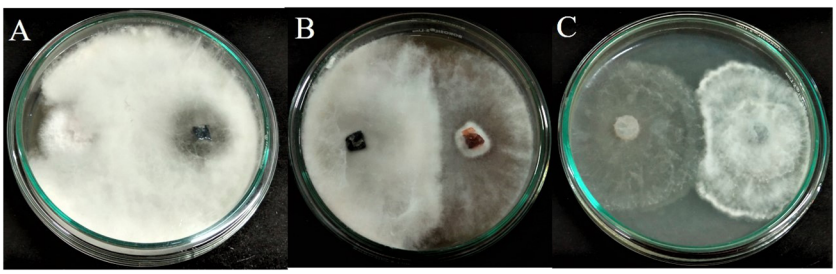


FIGURE 3
Antagonistic activity of endophytic fungus *Lasiodiplodia pseudotheobromae* APR5 towards tested soilborne phytopathogens **(A)** *Fusarium oxysporum*, **(B)** *Macrophomina phaseolina* and **(C)** *Rhizoctonia solani*.

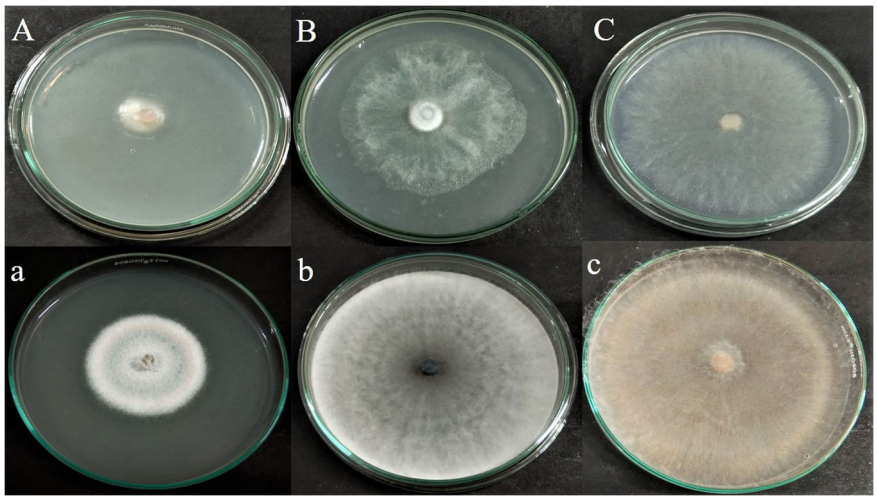


FIGURE 4
Antagonistic effect of volatile organic compound produced by *Lasiodiplodia pseudotheobromae* APR5, against tested soilborne phytopathogens **(A)** *Fusarium oxysporum*, **(B)** *Macrophomina phaseolina* and **(C)** *Rhizoctonia solani*; (a),(b),(c) are their corresponding control plates.

7). The secondary metabolites present in EA crude extract inhibited the mycelial growth of phytopathogens by reductions in hyphal diameter (Table 2). At 1000 ppm, an inhibition percentage of 35 ± 0.05% was observed in the mycelial growth of *F. oxysporum* growth.

3.6 Analysis of crude extracts by GC-MS

The GC-MS investigation was carried out to identify chemical compounds present in the EA crude extract of *L. pseudotheobromae* APR5, which showed the highest inhibitory effect of 75 ± 0.1% towards *R. solani*. By comparing the mass spectra with the MS spectral database, chemical compounds were identified based on the data of molecular formula, molecular mass, structures, and retention time. The peak area reflected a quantitative percentage of the expected chemical in ethyl acetate crude extract (Figure 8). Phenol, erythritol, phenylethyl alcohol, niacin, *t*-butylhydroquinone, 1-octadecene, octadecanoic acid, oleic acid, and *p*-cresol were the chemical compounds with antimicrobial activity identified from the selected crude extract. Table 3 illustrates a few chemical compounds from ethyl acetate crude with significant biological activity.

3.7 FT-IR Analysis

FT-IR spectrum of this core exhibited a broad intense peak at 3533.59 cm^{-1} corresponding to the phenolic OH stretching frequency and then the C-H band of alkanes concerning 2985.81 cm^{-1}. The presence of the sharp intense bands suggests the adsorption of the capping layer of the nanoparticles corresponds to C = N bond, C–O

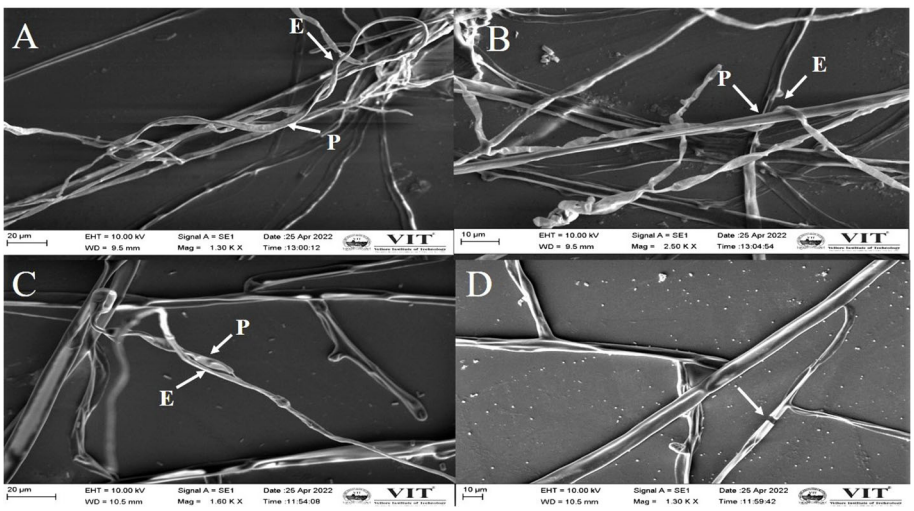


FIGURE 5
Scanning electron microscopy image demonstrating the morphological changes in the hyphae of *M. phaseolina* **(A)**, **(B)** and *R. solani* **(C, D)**.

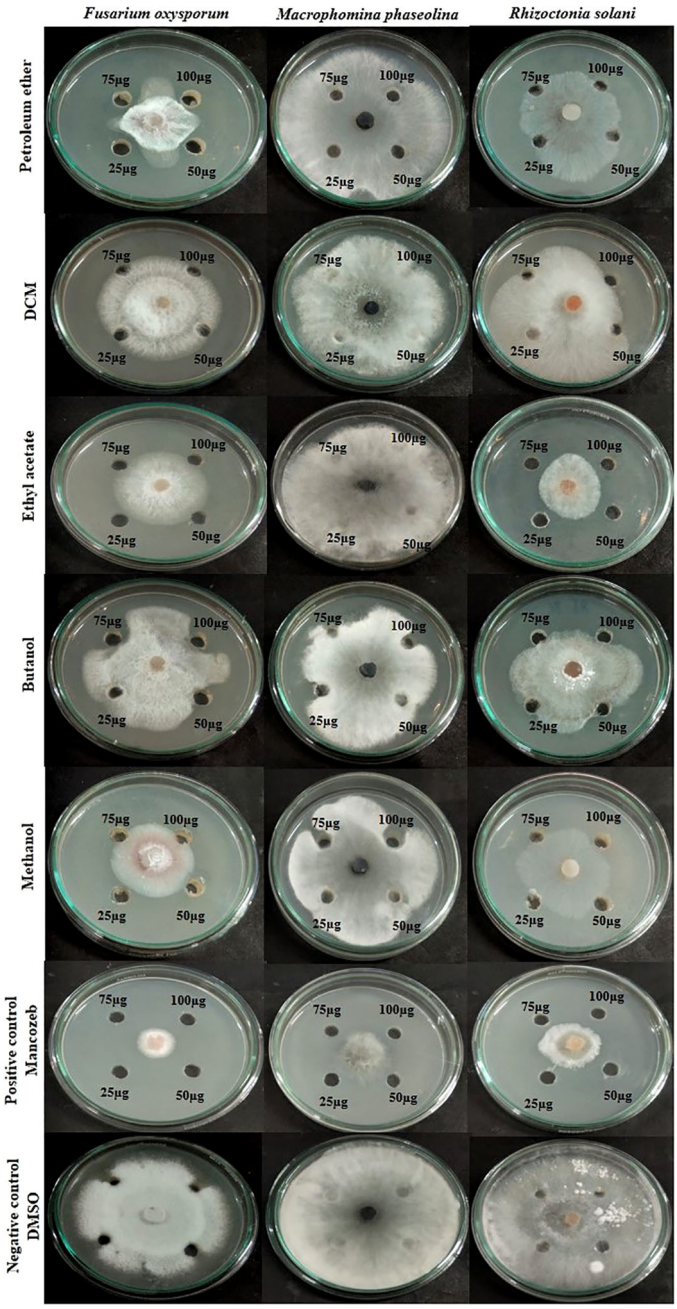


FIGURE 6
Inhibition of pathogenic fungal mycelial growth induced by different fungal crudes obtained from *Lasiodiplodia pseudotheobromae*.

bond stretch of ether groups, and N = H bond located at the stretching frequency of 1735.93 cm^{-1}, 1043.49 cm^{-1}, and 1234.44 cm^{-1} respectively. The FTIR spectrum was displayed in Figure 9.

3.8 Plant growth-promoting traits

The endophyte *L. pseudotheobromae* APR5 was observed to produce IAA which is indicated by the appearance of dark pink color (Figure 10A). However, the isolate did not produce HCN. A difference in color intensity (Figure 10B) between the test and control samples revealed that the isolate APR5 was a siderophore producer. The lack of brown color development after the addition of Nessler's reagent confirmed that the ammonia production outcome was not positive. The isolate APR5 was not a phosphate solubilizer, as evidenced by the results of the phosphate solubilization experiment (Table 4).

4 Discussion

Endophytic fungi offer a lot of promising applications in farming and food production. In recent times, the advancement

TABLE 1 Antifungal activity of extracts obtained from *Lasiodiplodia pseudotheobromae* isolate of *Andrographis paniculata*.

Pathogen / Extract	*Fusarium oxysporum*				*Macrophomina phaseolina*				*Rhizoctonia solani*			
	Concentration µg/mL				Concentration µg/mL				Concentration µg/mL			
	25	50	75	100	25	50	75	100	25	50	75	100
Pet ether	69 ± 0.05	72 ± 0.11	73 ± 0.05	74 ± 0.05	6 ± 0.05	6 ± 0.05	14 ± 0.05	19 ± 0.15	34 ± 0.15	35 ± 0.1	50 ± 0.05	65 ± 0.1
DCM	27 ± 0.07	28 ± 0.1	31 ± 0.05	33 ± 0.1	19 ± 0.1	21 ± 0.1	26 ± 0.1	24 ± 0.1	–	37 ± 0.05	45 ± 0.05	61 ± 0.05
Ethyl acetate	55 ± 0.12	56 ± 0.12	57 ± 0.05	58 ± 0.05	–	7.1 ± 0.1	5 ± 0.05	19 ± 0.1	62 ± 0.15	63 ± 0.1	70 ± 0.1	75 ± 0.1
Butanol	33 ± 0.1	34 ± 0.05	52 ± 0.05	53 ± 0.1	50 ± 0.05	33 ± 0.1	36 ± 0.05	48 ± 0.1	36 ± 0.11	38 ± 0.2	59 ± 0.05	62 ± 0.11
Methanol	54 ± 0.05	55 ± 0.05	66 ± 0.1	69 ± 0.1	63 ± 0.05	62 ± 0.1	26 ± 0.05	24 ± 0.1	47 ± 0.05	51 ± 0.15	58 ± 0.1	64 ± 0.15
Standard	83 ± 0.1	84 ± 0.05	85 ± 0.05	87 ± 0.05	74 ± 0.1	75 ± 0.05	82 ± 0.05	84 ± 0.05	71 ± 0.05	75 ± 0.05	73 ± 0.1	82 ± 0.05

* Values are expressed as inhibition percentage Mean ± SD, n = 3.
'-' denotes no antifungal activity.

of new genetic and bioinformatics approaches has enabled the identification of fungal endophytes species with the potential to stimulate the growth of their host plants, due to a range of various processes. The isolation of novel endophytes with significant potential for application in agriculture will be facilitated by studies on microbial diversity in novel plant species as well as in various geographical settings and conditions (Poveda et al., 2021). Without exhibiting any disease symptoms in the hosts, endophytic fungi live inside host plant tissue. Their attachment may be obligatory or facultative and they engage in complicated interactions that include antagonistic behavior and mutualism. The growth of endophytes is severely constrained by plants, but they use a variety of strategies to gradually adapt to their habitats (Ikram et al., 2022). Few investigations have documented the existence of biocontrol agents with the capacity to promote plant growth while simultaneously acting as antagonists against a variety of fungi diseases. Biological control microorganisms have been perceived as a beneficial and ecologically secure alternative to synthetic fungicides for controlling soil-borne diseases (Khan et al., 2021). Several authorized biocontrol agents from the genera *Agrobacterium, Bacillus, Candida, Coniothyrium, Gliocladium, Pseudomonas, Streptomyces*, and *Trichoderma* are widely commercialized (Khan et al., 2021). Therefore, the purpose of this research was to investigate the potential of endophytic fungi to suppress fungal pathogens with a wide host range along with stimulating plant productivity and growth. When used in the field, a biocontrol agent with broad-spectrum antifungal properties has greater prospects than those that are active,

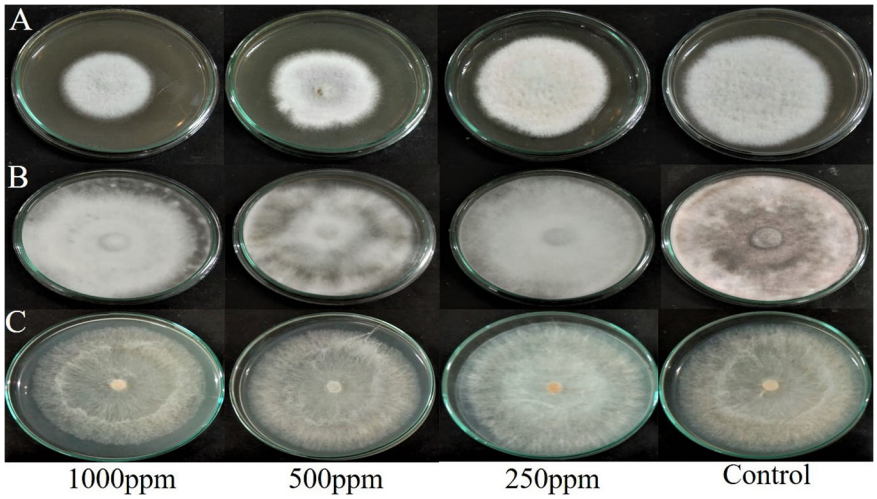


FIGURE 7
Effect of ethyl acetate crude extract from *Lasiodiplodia pseudotheobromae* on mycelial growth of phytopathogens **(A)** *Fusarium oxysporum*, **(B)** *Macrophomina phaseolina*, and **(C)** *Rhizoctonia solani* at different concentrations (1000 ppm, 500 ppm, 250 ppm, and control DMSO) after 7 days of incubation.

TABLE 2 Mycelial growth and inhibition percentage of test phytopathogenic fungi at 1000ppm.

Phytopathogens	Mycelia growth (cm)	Mycelia growth inhibition (%)
Fusarium oxysporum	4.8	35 ± 0.05
Macrophomina phaseolina	8.5	4 ± 0.07
Rhizoctonia solani	7.6	15 ± 0.07

*Values are expressed as inhibition percentage Mean ± SD, n = 3.

particularly against one or two microorganisms (Ali et al., 2020b). Endophytic fungal species from the genera *Curvularia, Chaetomium, Piriformospora, Fusarium, Epicoccum, Trichoderma*, and *Penicillium* are well recognized for increasing the plant host's resistance towards biotic and abiotic stresses (Rajani et al., 2021; Khan and Javaid, 2022). The synthesis of bioactive compounds, direct competition for nutrients and space with the pathogen, or activation of induced systemic resistance are plausible mechanisms by which *Aspergillus terreus* confers resistance to the host against *Colletotrichum gloeosporioides* (Gupta et al., 2022). VOCs and n-VOCs generated by *Fusarium solani* F4-1007 (endophyte of *Solenostemma arghel*) had the strongest antifungal efficacy, inhibiting *Cochliobolus spicifer* colony formation by 37.27% and 37.1%, respectively. *Penicillium oxalicum* and *Sarocladium kiliense* were the endophytes isolated from the medicinal plant *Aloe dhufarensis* had strong antifungal properties against the pathogenic *Fusarium* sp. and during the VOCs analysis, they revealed the presence of amide, fatty acids, 1,2-diols, fatty acid methyl esters and furfuryl alcohol (Abdel-motaal et al., 2022). In addition to mycoparasitism, VOCs are very crucial for the endophyte *Trichoderma* to combat pathogenic fungi. The development of *Fusarium oxysporum*-CFO, *Sclerotinia sclerotiorum*-TSS and *Sclerotium rolfsii*-CSR were considerably suppressed by endophytic *Trichoderma* sp. in a double-plate experiment (Rajani et al., 2021). *Trichoderma* spp. has drawn a lot of interest for its use in the treatment of *S. rolfsii* due to their exceptional capacity for root colonization, destruction of sclerotia, and generation of antifungal metabolites. Inducing plant defense reactions, producing enzymes that break down cell walls, mycoparasitism, antibiosis, and competition for resources and niches are some of the mechanisms adopted to suppress the development of fungal pathogens (Ali et al., 2020a).

Endophytes associated with medicinal plants have antagonistic behavior toward phytopathogens that cause illness and can produce secondary metabolites that are antioxidant, antimicrobial, and insecticidal (Abdel-motaal et al., 2022). The antibiosis action of strain *Talaromyces* sp. DYM25 prevented the development of *Fusarium equiseti*. The bioactive persistence of filtered broth against *F. equiseti* was initially tested, demonstrating its potential as a bio-control agent across a variety of circumstances including the presence of metal ions, high temperature, an alkaline environment, and UV radiation. In the pot experiment findings, *F. equiseti* induced cucumber wilt, which could be prevented by utilizing the fermentation broth of *Talaromyces* sp. DYM25 (52.9%) (Luo et al., 2021). The diameter of the inhibitory zone clearly showed that the endophytes *Pleosporales* sp., *Phoma* sp., *Cytospora pruinosa*, *Thielavia basicola*, and *Fusarium lateritium* showed the greatest antibiosis towards *Hymenoscyphus fraxineus*. Cytoplasmic extrusions, spiral twists, the formation of torulose hyphae, and excessive lateral branching are the morphophysiological deformations of *H. fraxineus* hyphae, developed under endophyte pressure. The majority of horticulture and crops are the target for the endophyte-based biocontrol techniques that are now being explored. The pathogen *Cronartium ribicola*, which causes the debilitating illness white pine blister rust, was efficiently inhibited by fungal endophytes of *Pinus monticola* (Bila´nski and Kowalski, 2022). *Colletotrichum siamense* isolated from *Piper nigrum* leaves and *Paecilomyces variotii* from *Caralluma acutangula* demonstrated antifungal potential against the widespread

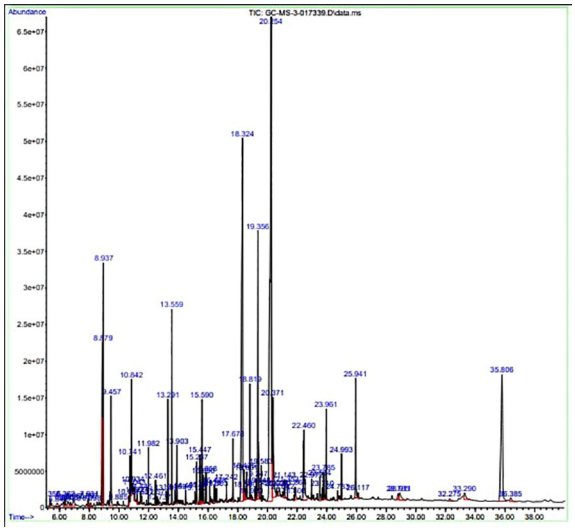
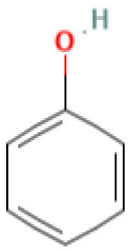
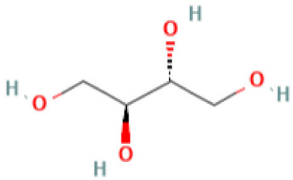
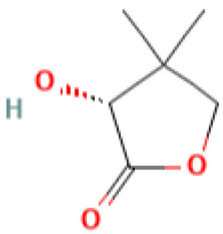


FIGURE 8
Gas chromatography-mass spectrometry profile of ethyl acetate crude extract from *Lasiodiplodia pseudotheobromae* APR5.

pathogen (Poveda et al., 2021). *Phomopsis* sp., an endophytic fungus has attracted a lot of interest in the finding of new biochemically and physiologically effective metabolites and has direct usage in medicine and agricultural biotechnology. Pyrocidines A and B were the antibiotics recently found from the endophyte *Acremonium zeae* of maize and showed considerable antifungal activity against *Fusarium verticillioides* and *Aspergillus flavus* (Yu et al., 2009). 3b-Hydroxy-ergosta-5-ene,3-oxo-ergosta-4,6, 8 and 22-tetraene, 3b, 5a-dihydroxy-6b-acetoxy-ergosta-7,22-diene,and 3b, 5a-dihydroxy-6b-phenylacetyloxy-ergosta-7,22-diene are the antimicrobial steroids from *Colletotrichum* sp., an endophyte of *Artemisia annua*, displayed fungistatic activities towards pathogenic fungi such as *Helminthosporium sativum, Phytophthora capisici, Rhizoctonia cerealis, Gaeumannomyces graminis* var. *tritici*, and *Phytophthora capisici* in the crops. The finding of effective medications or insecticides from endophytes is challenging because most steroid chemicals derived from endophytes have moderate antimicrobial activity. Pestalachloride A and B, two novel antibiotics isolated from endophytic *Pestalotiopsis adusta*, exhibit considerable antifungal efficacy against three plant diseases causing fungal pathogens *Gibberella zeae, Verticillium arboretum* and *Fusarium culmorum*. A group of phenolic acids from *Phoma* sp., of the Guinea plant, inhibits the mycelial growth of *Ralstonia solanacearum* and *Sclerotinia sclerotiorum* (Yu et al., 2009). However, a rising number of publications suggest that the application of endophyte can be reliably used to safeguard forests and ornamental trees (Bila´nski and Kowalski, 2022)

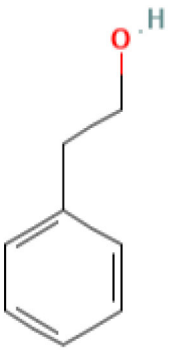
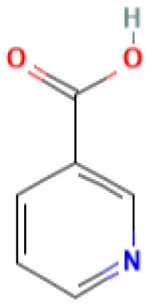
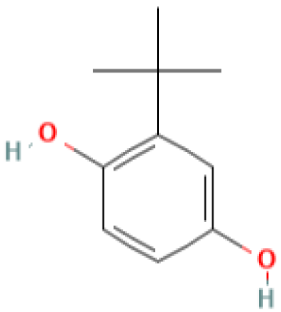
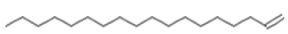
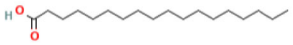
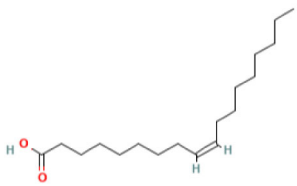
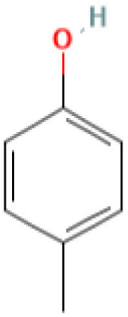
Lasiodiplodia pseudotheobromae is a cryptic species that were previously identified as *Lasiodiplodia theobromae* (Alves et al., 2008). In tropical and subtropical areas, *Lasiodiplodia* species are widespread and exist in a range of monocotyledonous, dicotyledonous, and gymnosperm. Lasiodiplodia is a member of the Ascomycota phylum, Dothideomycetes class, Botryosphaeriales order, and Botryosphaeriaceae family, which is composed of 110 species and 17 fungal genera. Members of this family, including the species in the genus, infect a wide spectrum of hosts or live as saprophytes or endophytes inside living tissues (Coutinho et al., 2017). The species has been discovered in Africa, Europe, and Latin America, where it has been found in fruit trees and forests. Similar to *L. theobromae, L. pseudotheobromae* also appears to have a worldwide distribution and a diverse host range. *L. pseudotheobromae* F2 obtained from undamaged *Illigera rhodantha* (Hernandiaceae) flowers exhibited antibacterial activity. Lasiodiplines E from the fungal isolate was effective towards clinical strains such as *Veillonella parvula, Bacteroides vulgates*, *Streptococcus* sp., and *Peptostreptococcus* sp. By modifying bacterial cells and limiting their proliferation, ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64, an endophytic fungus from the leaf of *Ocimum sanctum* was active

TABLE 3 List of chemical compounds in the ethyl acetate crude extract of *Lasiodiplodia pseudotheobromae* APR5.

Name of the compound	RT	Molecular weight	Molecular Formula	Area %	Structure	Biological activity	Reference
Phenol	6.362	94.11	C_6H_5OH	0.19		Antimicrobial	(Sabbineni, 2016)
Erythritol	6.723	122.12	$C_4H_{10}O_4$	0.12		Antimicrobial	(Shimizu et al., 2022)
Pantolactone	8.098	130.14	$C_6H_{10}O_3$	0.14		Antiplasmodial	(Baldé et al., 2021)

(Continued)

TABLE 3 Continued

Name of the compound	RT	Molecular weight	Molecular Formula	Area %	Structure	Biological activity	Reference
Phenylethyl alcohol	8.879	122.16	$C_8H_{10}O$	4.21		Antimicrobial	(Lilley and Brewer, 1953)
Niacin	10.934	123.11	$C_6H_5NO_2$	0.13		Antimicrobial Antioxidant, Anti-inflammatory, Anticarcinogenic, Antitubercular	(Naglah et al., 2015)
t-Butylhydroquinone	15.120	166.22	$C_{10}H_{14}O_2$	0.18		Antimicrobial	(Ooi et al., 2013)
1-Octadecene	15.590	252.5	$C_{18}H_{36}$	1.19		Antimicrobial	(Hameedha et al., 2014)
Octadecanoic acid	20.254	284.5	$C_{18}H_{36}O_2$	25.94		Antimicrobial	(Kima et al., 2016)
Oleic Acid	22.460	282.5	$C_{18}H_{34}O_2$	2.95		Antimicrobial	(Dilika et al., 2000)
p-Cresol	23.785	108.14	C_7H_8O	0.39		Antimicrobial	(Harrison et al., 2021)

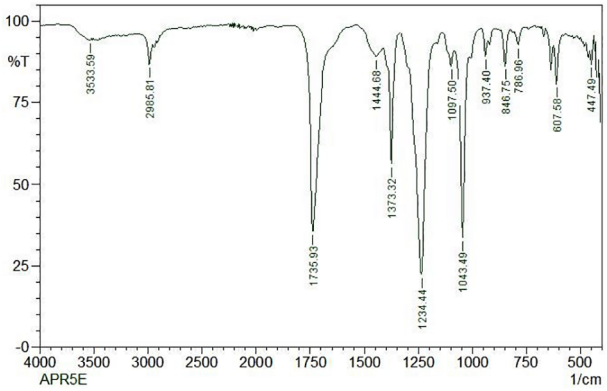


FIGURE 9
FTIR analysis of ethyl acetate crude extract from *Lasiodiplodia pseudotheobromae* APR5.

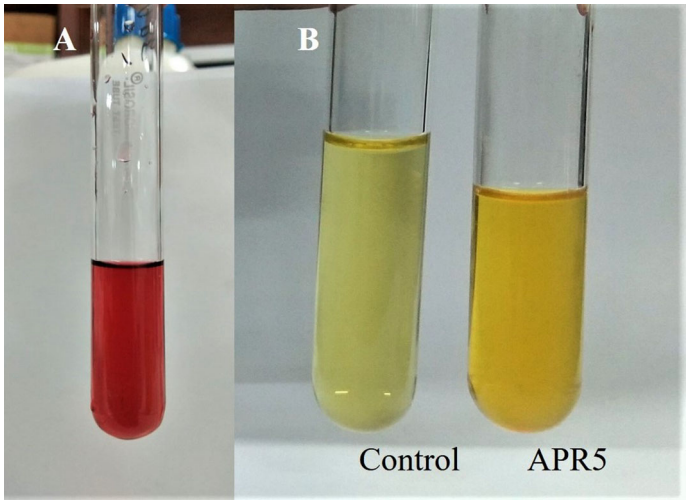


FIGURE 10
Growth-promoting characteristics of *Lasiodiplodia pseudotheobromae* APR5 **(A)** The production of IAA and **(B)** Siderophore.

against Methicillin-resistant *Staphylococcus aureus*. The growth of both Gram-positive and Gram-negative bacteria was significantly suppressed (Jalil and Ibrahim, 2022).

Indole-3-acetic acid (IAA) is a kind of auxin that was associated with plant growth. For the development and growth of shoots and roots, indole acetic acid (IAA) is a crucial chemical substance. Plant growth-promoting compounds like indole acetic acid (IAA) and gibberellins were secreted by endophytic and soil fungi. IAA was more effectively produced by Trichoderma isolate obtained from the rhizosphere region (Syamsia et al., 2015). *Talaromyces* sp. from *Caltha appendiculata* tubers generated an IAA of 7.60 ± 0.32 mg/L on a PDB medium supplied with L-tryptophan (Wang et al., 2022). IAA produced by microorganisms enhances the root surface area and thus improves the uptake of nutrients and water (Ali et al., 2020b). IAA was produced by *Penicillium roqueforti* (CGF 1) in yeast, malt, glucose, and sucrose at concentrations of 36.9 g/mL, 36.0 g/mL, and 35.7 g/mL respectively. IAA levels in *Trichoderma reesei* isolated from *Solanum surattense* were from 40-52 g/mL in sucrose, 39.5 g/mL in yeast and glucose, and 38.0 g/mL in malt extract (Ikram et al., 2022). *Alternaria alternata (Solanum nigrum), Aspergillus awamori (Withenia somnifera), Aspergillus niger (Camellia sinensis), Colletotrichum fructicola (Coffea arabica), Colletotrichum siamense*

TABLE 4 Growth-promoting traits of *Lasiodiplodia pseudotheobromae* APR5 isolated from *A. paniculata*.

Plant growth-promoting traits	Result
IAA production	+++
HCN production	–
Siderophore production	+
Ammonia production	–
Phosphate solubilization	–

'+' indicate positive, '-' indicates no production.

(Piper nigrum), Epicoccum nigrum (Caralluma acutangula), Fusarium tricinctum (Solanum nigrum) and *Penicillium crustosum (Teucrium polium)* are the IAA producing fungal endophytes. Furthermore, *Aspergillus terreus* obtained from paprika plants is capable of producing IAA in tomato plants, which promotes its growth and inhibits the bacterial speck disease brought on by *Ralstonia solanacearum*, *Pseudomonas syringae* pathovar (pv.) tomato, and *Colletotrichum acutatum. Trichoderma harzianum, T. asperellum* and *Paecilomyces formosus* enhance seedling growth, length of shoot and plant biomass in *Capsicum chinense*, whereas *Beauveria brongniartii* from *Carica papaya* improves the diameter of the fruit (Poveda et al., 2021).

Epicoccum nigrum isolated from the host plant *Pistacia vera* generates siderophores in the *in vitro* condition. The endophytic fungus *Beauveria brongniartii* can solubilize phosphate and also generates IAA and siderophores on *Capsicum chinense* and *Carica papaya* (Poveda et al., 2021). Plant growth-promoting endophytes actively invade plant tissues and enhances the host plants' growth and crop yield. The biochemical and physiological metabolism depend heavily on iron. Since the oxidation of ferrous iron and elemental Fe to insoluble ferric iron, cannot support microbial development and the free iron content in the environment is extremely low with the range of 10^{-7} mol (Ikram et al., 2022). The amount of dissolved ferrous iron in calcareous soils is between 10^{-10} to 10^{-9} M which is two to three orders of magnitude less than the amount needed by living things (10^{-7} to 10^{-5} M). The siderophore-mediated iron absorption system used by a few microbes has evolved as a result of environmental constraints and biological necessities. The insoluble ferric iron present in the environment is transported into the cell with the help of siderophores. Various microorganisms synthesize siderophores and combat plant diseases due to this, the bioavailability of iron for pathogens is diminished (Poveda et al., 2021). Therefore, further research into the application of biological control in the management of vegetable diseases will be valuable (Luo et al., 2021). Our study is the first report to reveal *L. pseudotheobromae* as the fungal endophyte from the medicinal plant *A. paniculata*. In addition to providing the foundation for future research and development of new biopesticides from a fungal source. The current investigation established the existence of antifungal inhibitors in crude extracts of the endophytic fungus isolated from *A. paniculata*. To better understand the potential and processes of these natural inhibitors, more research needs to be done to define the bioactive components of the extracts.

5 Conclusion

Endophytes can enhance the host plants' development and resistance to adverse environmental circumstances. Endophytic fungi associated with *A. paniculata* have not been studied in terms of plant-protecting biocontrol agents. Understanding the colonization and function of endophytic fungi found in various regions of medicinal plants is the purpose of this work. For the first time, inhibitors were discovered in crude extracts of endophytic fungi derived from *A. paniculata*, laying the groundwork for future research. To acquire a better knowledge of the capability and actions of natural inhibitors, more research into the bioactive compounds of the extracts should be explored. According to the findings of the present study, the compounds present in the extracts can be used in medicinal applications to safeguard eukaryotic models and plants. However, further research is required to examine all the expenses and advantages of concealing fungal endophytes in a variety of environmental situations to expand the usage and proficiency of endophytes in agriculture.

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

The authors confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation. All authors contributed to the article and approved the submitted version.

Funding

The authors are thankful to the management of VIT for providing the "VIT SEED GRANT" (SG ID: SG20220100) for carrying out this research work.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 23 August 2023
DOI 10.3389/fpls.2023.1206691

OPEN ACCESS

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RECEIVED 16 April 2023
ACCEPTED 21 July 2023
PUBLISHED 23 August 2023

CITATION
Yuan T, Qazi IH, Li J, Yang P, Yang H, Zhang X, Liu W and Liu J (2023) Analysis of changes in bacterial diversity in healthy and bacterial wilt mulberry samples using metagenomic sequencing and culture-dependent approaches. *Front. Plant Sci.* 14:1206691. doi: 10.3389/fpls.2023.1206691

Analysis of changes in bacterial diversity in healthy and bacterial wilt mulberry samples using metagenomic sequencing and culture-dependent approaches

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Introduction: Mulberry bacterial wilt is a serious destructive soil-borne disease caused by a complex and diverse group of pathogenic bacteria. Given that the bacterial wilt has been reported to cause a serious damage to the yield and quality of mulberry, therefore, elucidation of its main pathogenic groups is essential in improving our understanding of this disease and for the development of its potential control measures.

Methods: In this study, combined metagenomic sequencing and culture-dependent approaches were used to investigate the microbiome of healthy and bacterial wilt mulberry samples.

Results: The results showed that the healthy samples had higher bacterial diversity compared to the diseased samples. Meanwhile, the proportion of opportunistic pathogenic and drug-resistant bacterial flora represented by *Acinetobacter* in the diseased samples was increased, while the proportion of beneficial bacterial flora represented by *Proteobacteria* was decreased. *Ralstonia solanacearum* species complex (RSSC), *Enterobacter cloacae* complex (ECC), *Klebsiella pneumoniae*, *K. quasipneumoniae*, *K. michiganensis*, *K. oxytoca*, and *P. ananatis* emerged as the main pathogens of the mulberry bacterial wilt.

Discussion: In conclusion, this study provides a valuable reference for further focused research on the bacterial wilt of mulberry and other plants.

KEYWORDS

bacterial wilt, drug-resistant bacteria, *Enterobacter cloacae* complex, *Klebsiella*, mulberry, opportunistic pathogens, RSSC

Introduction

Mulberry is a perennial dicotyledonous tree or shrub (Dai et al., 2020) that is widely cultivated throughout subtropical and temperate regions, and has a significant economic value (Xie et al., 2020). Mulberry leaves are exclusively used as a food source for the domesticated silkworm *Bombyx mori* L. (Ji et al., 2008; Chan et al., 2016). Besides its use as silkworm forage, mulberry is now used as a raw material in animal feed (Jiang et al., 2022), medicine (Meng et al., 2020) and food industry (Maqsood et al., 2022). However, the occurrence of mulberry diseases has seriously affected the healthy and stable development of the sericulture industry (Dong et al., 2021). For instance, mulberry bacterial wilt is a destructive disease that seriously affects the yield and quality of mulberry (Dong et al., 2021). Mulberry bacterial wilt was first reported in 1969 in Shunde City, Guangdong province of China, and has spread to most mulberry planting areas in Guangdong (Lai et al., 1979). Mulberry bacterial wilt is still prevalent in the main sericulture-producing areas of Guangdong, Guangxi and other places in China (Dai et al., 2016), and has been reported in many other mulberry planting areas in the country.

Mulberry bacterial wilt is a vascular disease which is difficult to diagnose with the naked eye at the initial stage of infection. However, in the middle stage of the disease, the leaves lose moisture and then curl or wilt, turning black or brown. In the late stage of the disease, the leaves of the whole plant are withered until they fall off, the xylem turns brown streaked or dark brown, and white pus-like bacteria overflow from the cross-section of the diseased root (Wang et al., 2008; Zhu et al., 2010; Zhou et al., 2021; Luo et al., 2022; Yuan et al., 2023a).

The pathogen of mulberry bacterial wilt has complex and diverse characteristics. Lai et al. (1979) isolated and identified the pathogen of mulberry bacterial wilt for the first time. Initially, *Pseudomonas solanacearum* was considered as a pathogen causing mulberry bacterial wilt, which was later renamed as *Ralstonia solanacearum*, and now classified as *R. pseudosolanacearum*. Wang et al. (2008) reported for the first time that the mulberry wilt was also caused by *Enterobacter cloacae* complex (*ECC*). Subsequently, Zhu et al. (2010) isolated *E. mori* from mulberry wilt disease samples. Zhou et al. (2021) isolated *E. roggenkampii* strain KQ-01 from the bacterial wilt-resistant mulberry cultivar YS283, which can cause mulberry wilt. Luo et al. (2022) isolated *Klebsiella michiganensis* AKKL-001 from mulberry bacterial disease samples, which can also cause mulberry wilt. Recently, *Pantoea ananatis* strain LCFJ-001 was isolated from mulberry bacterial wilt disease samples and was reported to cause mulberry wilt (Yuan et al., 2023a).

Currently reported pathogens of mulberry bacterial wilt can be divided into four categories: *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea* (Supplementary Figure 1). The gradual increase in sericulture production and exchange activities in the recent times has also led to an increased occurrence of bacterial wilt in mulberry fields in China, leading to significant challenges to the healthy development of the sericulture industry in the country. As this complex disease is caused by a number of pathogens, it still remains to be known which pathogen is the main pathogen, making its prevention and control difficult. It has been reported that the occurrence of plant diseases is related to changes in their crop microbiome, and that the study of changes in their microbiome can further reveal their pathogenesis (Li et al., 2022). Therefore, in order to further understand the basis of pathogenesis and provide a valuable reference for prevention and control, this study was carried out to explore the changes in mulberry microbiome in bacterial wilt and healthy samples of mulberry. In the present study, we collected (2017 to 2022) 35 mulberry bacterial wilt disease samples from Guangdong, Guangxi, and other regions of China. The diseased mulberry samples were isolated and tested for pathogenicity of pathogenic bacteria. At the same time, due to the limitations of traditional culture-dependent method, we also used the metagenomic sequencing to further explore the main pathogenic groups in the diseased and healthy mulberry samples.

Materials and methods

Metagenomic sequencing of mulberry samples

Collection of mulberry samples

A survey of mulberry fields where mulberry wilt was prevalent in Liucheng (109.24°, 24.65°) and Rong'an (109.35°, 25.15°) counties of Guangxi, China was conducted (see Supplementary Figures 2A–I for description). The mulberry samples with typical disease symptoms in the field were processed for laboratory verification.

Metagenomic sequencing of mulberry samples

The pH values of the diseased (wilted) plants and rhizosphere soil of typical mulberry in Liucheng and Rong'an were tested. Eight samples were collected (Supplementary Table 1) and sent to the Science Corporation of Gene Co., Ltd. for metagenomic sequencing to analyze the types, and abundance of pathogens in the samples.

Extraction of genomic DNA

The genome DNA was extracted from samples using the Ezup Column Bacteria genomic DNA purification kit (Sangon Biotech (Shanghai) Co., Ltd., China). DNA purity and concentration were measured by gel electrophoresis and *NanoDrop* 2000 (Thermo Scientific) spectrophotometer (Yuan et al., 2023b).

Amplification of the target region

The *16S rRNA gene* consists of nine hypervariable regions flanked by regions of more conserved sequence. To maximize the effective length of PE 250 sequencing reads of Illumina HiSeq2500, the region encompassing the V3 and V4 hypervariable regions of the *16S rRNA* gene was targeted for sequencing. The V3-V4 hypervariable region was amplified using a specific primer with the barcode (Supplementary Table 2). All PCRs were carried out in 40 μL reactions with 20 μL of 2×Taq MasterMix, 0.5 μM forwards and reverse primers, and approximately 10 ng of template DNA. Temperature cycling consisted of denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 20 s., and finally, 72°C for 7 min. The purity and concentration of all amplicons were characterized by gel electrophoresis and Qubit@ 2.0 Fluorometer (Thermo Scientific). The amplicons with bright main strips and the right length were chosen for the subsequent experiments (Yuan et al., 2023b).

Library preparation and metagenomic sequencing

PCR products with different barcodes were mixed in equidensity ratios. Then, a mixture of PCR products was used to prepare pair-end sequencing libraries. The libraries were generated following the Illumina HiSeq 2500 standard protocol (Illumina, Inc., San Diego, CA). Paired-end reads (250 bp) were generated on the Illumina HiSeq2500 platform. Three replicates of each sample were used for metagenomic sequencing (Yuan et al., 2023b). The metagenomic sequencing data have been uploaded to the NCBI (National Center for Biotechnology Information) with accession number PRJNA911049. Finally, based on taxonomy, the abundance of each bacterial genus was counted, and Origin 2019b software (OriginLab Corporation, Northampton, MA, USA) was used to make bacterial abundance maps of different samples and the Shannon, Chao-1 and Simpson values of each sample were calculated.

Analysis of mulberry samples using culture-dependent approach

Collection of mulberry samples

During 2019 to 2022, a total of 35 samples of diseased plants were collected from Guangxi, Guangdong and Hainan in China (Supplementary Table 3). *M. atropurpurea* varieties Lun40 and Kangqing10 were used as the healthy group (Supplementary Table 3), and 20 copies of each variety were collected in mulberry field of the South China Agricultural University, Guangzhou, Guangdong, China (113.35°, 23.17°).

Isolation of bacteria from mulberry samples

The experimental design is depicted in Supplementary Figure 3. Firstly, the collected diseased or healthy roots were rinsed under the faucet, and the surface stains were washed with soapy water and the samples were wiped with a clean gauze. The roots were cut into small sections of three centimeters in length using clean scissors. Then, the sections were soaked in 75% ethanol for 1 min, rinsed with sterile water three times, soaked in 0.1% mercuric chloride for 5 min, and rinsed with sterile water five times. Then, the surface-sterilized small section was placed in a sterile glass petri dish, the xylem in the center was removed with sterile tweezers and scissors, cut into pieces and ground in a sterile mortar. The ground xylem was placed in 10 mL of sterile saline and in a shaker at 28°C and 140 r/min for 10 minutes to form a liquid containing xylem bacteria. The liquid was then removed and diluted eight times according to the 10-fold dilution method (Yuan et al., 2023a).

A total of 0.1 mL of each gradient was spread evenly on Lysogeny Borth (LB) agar plates (Guangdong Huankai Co., Ltd., China) and nutrient agar plates (Guangdong Huankai Co., Ltd., China). Then, the plates were placed in a biochemical incubator at 28°C for two days for cultivation. Finally, single colonies were picked from LB agar and nutrient agar media and drawn on new nutrient agar plates, and each colony was purified for seven generations (Yuan et al., 2023a).

Classification of bacteria

Classification of the bacteria was based on analysis of *16S rRNA gene* using universal primer 27F/1492R (Bredow et al., 2015). All strains were inoculated in nutrient broth medium (Guangdong Huan Kai Co., Ltd., China) and placed in a shaker at 28°C and 140 r/min for 12 h. Bacterial genomic DNA was extracted using the Ezup Column Bacteria Genomic DNA purification kit (Sangon Biotech (Shanghai) Co., Ltd., China). DNA from all purified isolates was used for PCR amplification of the *16S rRNA gene*, which was performed in a 25 μL volume under the following conditions: one cycle of 98°C for 4 min, followed by 30 cycles of 98°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and final an extension at 72°C for 10 minutes. The PCR-amplified products were transferred to a laboratory in Shanghai, China, at Sangon Biotechnology Co. Ltd. in Shanghai, China, and then sequenced by the Sanger method (Yuan et al., 2023a).

The generated sequences were aligned using BioEdit software version 7.0 and then subjected to analysis by the Basic Local Alignment Search Tool (BLAST) search program of the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the sequence homology with closely related organisms (Altschul et al., 1997). Microorganisms with high homology (97-100%) were selected as the closest matches. All bacterial isolates were assigned to the genus level separately based on information from the closest microorganisms. In addition, the NCBI taxonomic database was used to classify all bacterial strains at the phylum, class, order, and family levels (Yuan et al., 2023b). All bacterial *16S rDNA* sequences generated in this study have been submitted to the NCBI. The accession numbers OP990608-OP990981 are bacterial *16S rDNA* sequences derived from healthy samples; OP989957-OP990607 are bacterial *16S rDNA* sequences derived from diseased samples. Finally, based on the *16S rRNA gene* bacterial identification results, the abundance of each bacterial genus was counted, and the bacterial abundance maps of different samples were made using Origin 2019b software (OriginLab Corporation, Northampton, MA, USA) and the Shannon, Chao-1, and Simpson values of healthy and diseased samples were calculated.

16S rRNA gene phylogenetic tree construction

The *16S rDNA* sequences of typical strains of *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea* were downloaded from the List of Prokaryotic names with Standing in Nomenclature (https://lpsn.dsmz.de/). The *16S rDNA* of six strains of *Ralstonia*, 30 strains of *Enterobacter*, 12 strains of *Klebsiella*, and 12 strains of *Pantoea* were selected for construction of phylogenetic trees (Supplementary Table 4). At the same time, the *16S rDNA* sequences of strains identified in our laboratory (Supplementary Table 4) and the *16S rDNA* sequences downloaded from the NCBI were used as references. The sequences were compared using MUSCLEv.3.8.31 software. The phylogenetic trees were constructed using the maximum likelihood tree with MEGA-X software, and the bootstrap value was set to 1000 (Yuan et al., 2023a).

Cultivation of mulberry branches

Healthy 15-year-old healthy *M. atropurpurea* cultivar Lun40 (susceptible to bacterial wilt) obtained from the South China Agricultural University mulberry field (Guangzhou, Guangdong, China), (113.35°, 23.17°) was selected as plant material for this study. The samples were collected in December 2021. Firstly, old branches of Lun40 with a diameter of 0.5-0.75 centimeter were selected and cut into stem segments of 10-12 centimeters in length and containing three lateral shoots. The stems were washed with soapy water to remove surface dust and soaked in 0.5% sodium hypochlorite solution for five hours. The stems were then inserted into the sterile MS liquid medium and placed in an artificial climate incubator at 25°C, 12 h/d light, and 85% humidity. The culture was incubated for 25 days until the lateral shoots sprouted and exhibited 2-3 leaves. During this period, the sterile MS liquid medium was changed every day (Yuan et al., 2023b).

Pathogenicity test

To investigate the pathogenicity of *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea*, the following procedures were adopted: 1) the pure cultures of all the bacteria (Supplementary Table 4) were placed in nutrient broth medium overnight. The overnight cultured bacteria solution was adjusted to OD600 nm=0.1 with sterile MS liquid medium. 2) After cultivation, the Lun40 mulberry branch with 2~3 leaves was placed into the diluted bacterial solution. The sterile MS liquid medium was set as the healthy group. 3) The treated Lun40 mulberry branches were cultured for 12 days in an artificial climate incubator at 28°C, 12 h/d light, and 85% humidity, and the disease incidence in plants was observed. 4) Morbidity rate= (A-B)/C×100%. A: The total number of diseased mulberry branches in the experimental group; B: The total number of diseased mulberry branches in the control group; C: The total number of mulberry branches (Yuan et al., 2023b).

Analysis of variance (ANOVA) was performed using Excel software. Each set of experiments for each pathogen species was tested using six healthy mulberry branches. With the sterile MS liquid medium as a control, each group had three replicates (Yuan et al., 2023b).

Data statistics

Data analysis was performed using a one-way analysis of variance (Levene's test was used to evaluate the equality of variance before analysis), and the least significant difference test was used to determine the significant difference between the means as a *post hoc* analysis. $P<0.05$ was considered significant. Excel 2016 software (Microsoft, Redmond, WA, USA) and Origin 2019b 64Bit were used to analyze and map the data.

Results

Analysis of metagenomic sequencing quality

The description of sequencing data of the bacterial *16S rDNA* V3-V4 regions collected from mulberry xylem and rhizosphere soil are shown in Table 1. Briefly, the GC was greater than 53%, Q20 was greater than 96%, and Q30 was greater than 94%. This indicates a low sequencing error rate and high quality and reliability of the data.

Analysis of diversity of bacterial community in mulberries based on metagenomic sequencing

As shown in Table 2, the bacterial community OTU numbers, Shannon index, Chao-1 index and Simpson index of the diseased rhizosphere soil (QKB04 and QKB08) and xylem (QKB03 and QKB07) were lower compared to the healthy rhizosphere soil (KB06 and QKB10) and xylem (QKB05 and QKB09). From these, the bacterial diversity of the rhizosphere soil and xylem of the diseased mulberry was lower compared to the healthy mulberry.

TABLE 1 Description of metagenomic sequencing data.

Sample	Reads(#)	Base(nt)	GC(%)	Q20(%)	Q30(%)
QKB04**	167,870	41,967,500	53.74	96.60;88.78	94.03;82.91
QKB06*	183,782	45,945,500	54.6	96.82;89.16	94.30;83.23
QKB08**	167,210	41,802,500	54.68	96.81;89.51	94.33;83.81
QKB10*	188,724	47,181,000	54.04	96.81;89.58	94.36;83.96
QKB03**	156,488	39,122,000	55.03	96.72;89.52	94.18;83.93
QKB05*	120,666	30,166,500	54.12	96.81;89.74	94.39;84.16
QKB07**	125,124	31,281,000	53.49	96.80;88.99	94.32;83.20
QKB09*	136,356	34,089,000	53.73	96.90;89.79	94.53;84.26

"*": healthy group; "**" diseased group; Reads(#): The total number of reads for sequencing; Bases (nt): the number of bases for sequencing = the total number of reads for sequencing×150 (150 is the length of the sequencing read); Q20 (%): the proportion of bases with a sequencing quality value greater than 20 (error rate less than 1%) in R1 and R2 sequencing reads; Q30 (%): the proportion of bases with sequencing quality more significant than 30 (error rate less than 0.1%) in R1 and R2 sequencing reads; GC (%): GC proportion.

TABLE 2 The number of OTUs and diversity index of the read sequence (Tags) bacterial community of the sequenced branch samples.

Sample	Shannon	Chao-1	Simpson	OTU	Tags
QKB04**	6.90^{c}	5554.90^{b}	0.87^{b}	1364^{b}	5676^{a}
QKB06*	7.39^{d}	15270.62^{e}	0.93^{c}	5543^{f}	30833^{c}
QKB08**	6.77^{c}	12231.04^{d}	0.89^{b}	4329^{e}	26340^{b}
QKB10*	7.42^{d}	15988.35^{e}	0.94^{c}	6531^{g}	39377^{d}
QKB03**	5.36^{a}	6968.12^{b}	0.81^{a}	2750^{c}	24642^{b}
QKB05*	5.84^{b}	7704.39^{c}	0.90^{bc}	2860^{c}	27899^{b}
QKB07**	5.48^{a}	3178.83^{a}	0.88^{b}	862^{a}	5387^{a}
QKB09*	5.23^{a}	6928.89^{b}	0.88^{b}	3303^{d}	39219^{c}

"*": indicates the healthy group; "**" indicates the diseased group. Different superscript letters in the vertical column indicate significant differences between means by one-way analysis of variance (ANOVA) and least significant difference (LSD) test (P<0.05).

Analysis of bacterial community composition of mulberries based on metagenomic sequencing

The phylum-level abundance distribution of bacterial populations in the mulberry rhizosphere soil was associated with 17 phyla (Figure 1A). From these, Proteobacteria had the highest abundance in diseased and healthy samples, followed by Actinomycetes and unclassified bacteria. At the genus level, the taxonomic sequence of the mulberry rhizosphere soil was associated with 49 genera (Figure 1B). The abundance distribution of the dominant flora are shown in Table 3. *Pseudomonas* accounted for the largest proportion, followed by *Mycobacteria*, *Erwinia* and *Ralstonia*, respectively. The *Pseudomonas* disease samples showed a significant downward trend ($P<0.05$), whereas the *Erwinia* disease samples showed a significant upward trend ($P<0.05$). Interestingly, there was no significant difference in the abundance of *Ralstonia* between healthy and diseased samples (P<0.05).

The phylum-level abundance distribution of bacterial populations in mulberry xylem was associated with seven phyla (Figure 1A). Proteus was the first dominant bacterial group in both healthy and diseased mulberry samples and its abundance accounted for more than 90% in both healthy and diseased groups. Many sequences in xylem could not be classified (7.8% richness), indicating the diversity of the xylem bacteria. At the genus level, the bacteria in the mulberry xylem part were related to 23 genera (Figure 1B). The abundance distribution of the dominant bacterial taxa is shown in Table 3. *Pseudomonas* was found to be the most abundant, followed by *Erwinia* and *Ralstonia*, respectively. Interestingly, the abundance of *Pseudomonas* was lower in the diseased group compared to the healthy mulberry group, whereas abundance of *Erwinia* and *Ralstonia* showed a reverse trend.

Bacterial composition and diversity in mulberries based on a culture-dependent approach

A total of 1052 strains of the xylem bacteria were isolated from all samples. From these, 389 strains were from the healthy mulberry samples (CKS) (Supplementary Table 5), 663 strains were from the diseased (bacterial wilt) mulberry samples (MBWS) (Supplementary Table 6). Based on the results of *16S rRNA* gene, CKS culturable strains were divided into 58 genera, distributed in 4 phyla, 6 classes, 18 orders and 28 families (Supplementary Table 5). The Shannon, Simpson and Pielou values were 3.03, 0.90 and 0.75, respectively (Table 4). The culturable strains of MBWS were divided into 69 genera, distributed in 4 phyla, 9 classes, 17 orders and 31 families (Supplementary Table 6). The values of Shannon, Simpson and Pielou were 3.17, 0.92 and 0.75, respectively (Table 4). This finding indicated that the diversity of the xylem bacteria in the MBWS samples was slightly higher compared to the CKS samples ($P>0.05$).

All isolates belonged to Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria phyla. From these, Proteobacteria was found to the dominant phylum in the bacterial community of mulberry xylem (Figure 2A). The most abundant Proteobacteria (CKS 70.95%, MBWS 88.98%) mainly contained the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria-like bacteria. Firmicutes was the second most dominant phylum (CKS 19.53%, MBWS 4.67%) and contained only bacilli, consisting of *Bacillus* (Figure 2A). *Actinomycetes* was the third most dominant bacterial phylum (CKS 8.99%, MBWS 2.71%) and was represented by *Microbacteria* (Figure 2A). At the genus level (Figure 2B), *Pseudomonas*, *Enterobacter*, and *Acinetobacter* were found to be the main groups of the xylem bacteria.

Analysis of bacterial community of mulberries based on culture-dependent approach

Unique and shared bacterial genera between healthy and diseased mulberry groups are shown in the Venn diagram (Figure 3). The number of shared attributes across all groupings was 33 (Figure 3). In addition, the number of unique genera in the MBWS was higher than the number of unique and shared genera in the CKS group. Genera including *Enterobacter*, *Pseudomonas*, *Acinetobacter*, *Delftia*, *Pantoea*, *Stenotrophomonas*, *Rhizobium*, *Bacillus*, *Agrobacterium*, *Kosakonia*, and *Microbacterium*, with an average segregation rate of >1% in MBWS and CKS, were the 11 core genera of mulberry xylem bacteria (Figure 2B).

The separation frequencies of *Achromobacter*, *Acinetobacter*, *Brenneria*, *Brucella*, *Delftia*, *Escherichia*, *Herbaspirillum*, *Klebsiella*, *Ochrobactrum*, *Pantoea*, *Ralstonia*, *Rhizobium* and *Stenotrophomonas* in the CKS were significantly lower (P<0.05) compared to the MBWS group. Additionally, *Herbaspirillum*, *Brenneria*, *Klebsiella* and *Ralstonia* were isolated only in the diseased samples (Figure 4A).

The isolation frequencies of *Agrobacterium*, *Agrococcus*, *Atlantibacter*, *Microbacterium*, *Bacillus*, *Lysinibacillus*, *Oceanobacillus*, *Paenibacillus*, *Enterobacter*, *Kosakonia*, *Pseudomonas*, *Staphylococcus* and *Streptomyces* were significantly higher (P<0.05) in the CKS compared to the MBWS group. Additionally, *Agrococcus*, *Paenibacillus*, and *Streptomyces* were not isolated in the MBWS group (Figure 4B).

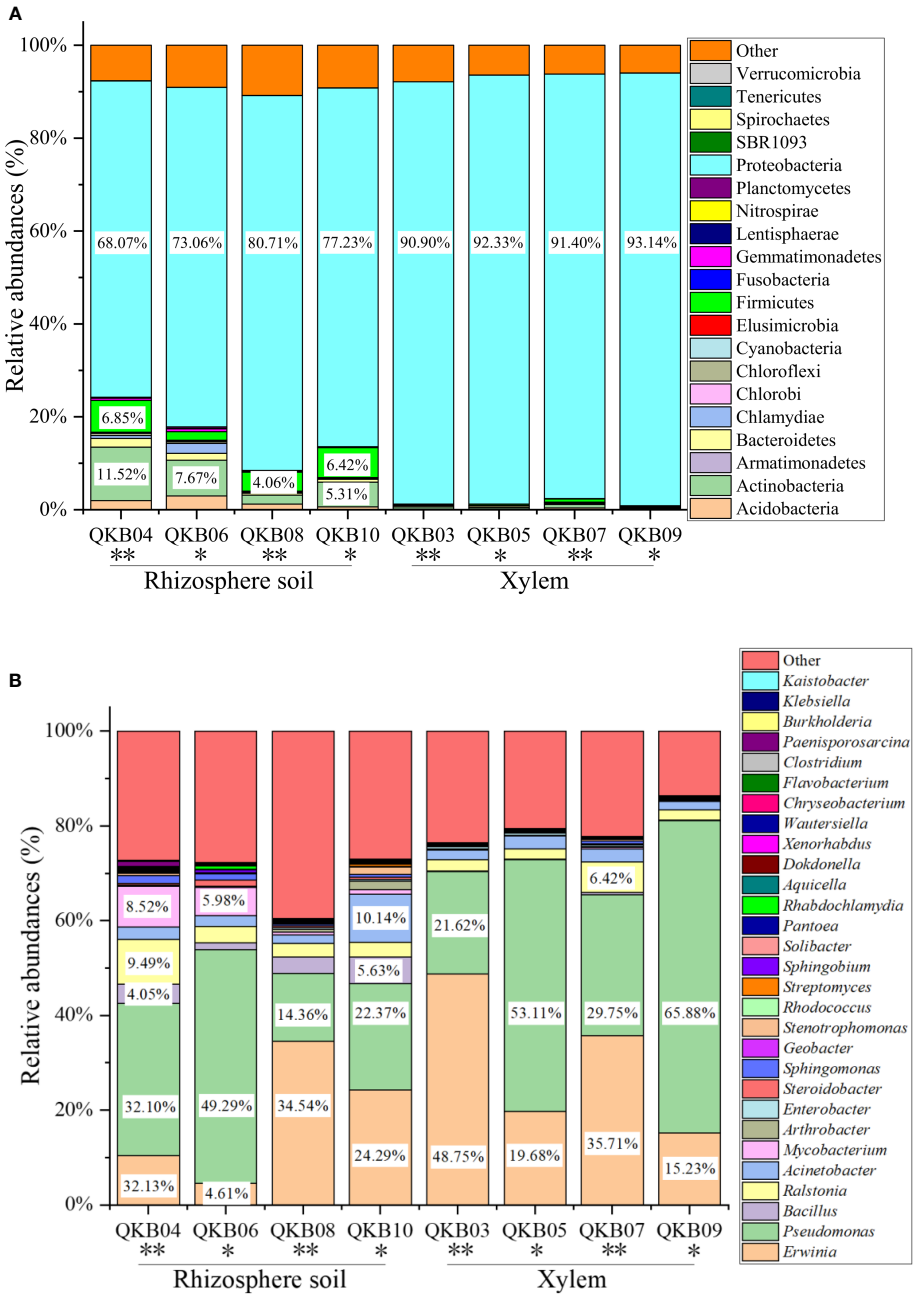

FIGURE 1
Abundance distribution of bacterial populations based on metagenomic sequencing at the phylum **(A)** and genus **(B)** levels. QKB04: Liucheng (109.24°, 24.65°) diseased sample rhizosphere soil; QKB06: Liucheng (109.24°, 24.65°) healthy sample rhizosphere soil; QKB08: Rong'an (109.35°, 25.15°) diseased sample rhizosphere soil; QKB10: Rong'an (109.35°, 25.15°) healthy sample rhizosphere soil; QKB03: Liucheng (109.24°, 24.65°) diseased sample xylem; QKB05: Liucheng (109.24°, 24.65°) healthy sample xylem; QKB07: Rong'an (109.35°, 25.15°) diseased sample xylem; QKB09: Rong'an (109.35°, 25.15°) diseased sample xylem; "*": indicates the healthy group; "**" indicates the diseased group.

10.3389/fpls.2023.1206691

TABLE 3 Richness of important bacterial genera in different samples based on metagenomic sequencing data.

Species	QKB04**	QKB06*	QKB08**	QKB10*	QKB03**	QKB05*	QKB07**	QKB09*
Acinetobacter	2.66%	2.15%	1.78%	10.15%	2.05%	2.73%	2.88%	1.73%
Arthrobacter	0.15%	0.14%	0.55%	1.79%	0.075%	0.045%	0.11%	0.018%
Bacillus	4.05%	1.38%	3.38%	5.63%	0.14%	0.16%	0.43%	0.13%
Enterobacter	0.12%	0.12%	0.43%	0.45%	0.42%	0.44%	0.37%	0.19%
Erwinia	10.37%	4.62%	34.54%	24.30%	48.76%	19.69%	35.71%	15.23%
Klebsiella	0.017%	0.15%	0.030%	0.086%	0.040%	0.097%	0.019%	0.14%
Mycobacterium	8.52%	5.98%	0.68%	0.90%	0.19%	0.12%	0.32%	0.11%
Paenisporosarcina	1.12%	0.019%	0.042%	0.053%	0.0035%	0.016%	0.037%	0.0025%
Pantoea	0.14%	0.039%	0.072%	0.070%	0.11%	0.15%	0%	0.079%
Pseudomonas	32.13%	49.29%	14.37%	22.37%	21.62%	53.11%	29.76%	65.88%
Ralstonia	9.49%	3.54%	2.86%	3.17%	2.34%	2.15%	6.42%	2.15%
Sphingomonas	1.71%	1.27%	0.28%	0.51%	0.15%	0.21%	0.59%	0.12%
Stenotrophomonas	0.45%	0.15%	0.11%	1.53%	0.13%	0.11%	0.30%	0.13%
Steroidobacter	0.29%	1.34%	0.36%	0.48%	0.11%	0.10%	0.22%	0.090%

"*": indicates the Healthy group; "**" indicates the Diseased group.

The distribution of four main types of pathogenic bacteria in mulberries

In order to explore the main group of pathogenic bacteria causing bacterial wilt of mulberry, the distribution of *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea* in 35 diseased samples was analyzed (Figure 5). From these diseased samples, *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea* were isolated from 6 (17.14%), 30 (85.71%), 12 (34.28%) and 12 (34.28%) diseased samples, respectively. From the 30 diseased samples in which *Enterobacter* was isolated, *Klebsiella*, *Pantoea*, and *Ralstonia* were isolated from 10, 9, and 5 samples, respectively.

TABLE 4 Profiles of bacterial community diversity in the biomass of diseased and healthy mulberry samples based on culture-dependent approach.

	M. atropurpurea	
	MBWS**	CKS*
Number of isolates	663[b]	389[a]
Number of genera	69[b]	58[a]
Shannon-Weaver (H')	3.17[a]	3.03[a]
Simpson's index (D)	0.92[a]	0.90[a]
Pielou's evenness (E)	0.75[a]	0.75[a]

"*"Healthy group; "**" Diseased group; MBWS: mulberry bacterial wilt sample. CKS: Healthy samples (healthy mulberry samples). Different letters in the same row indicate significant difference between means by one-way analysis of variance (ANOVA) and least significant difference (LSD) test ($P<0.05$).

Phylogenetic analysis of four main types of pathogenic bacteria

Classification was based on *16S rDNA* sequences of *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea* (Figure 6). *Ralstonia* was mainly concentrated in the RSSC and *R. pickettii* (Figure 6A). There were two main groups of *Enterobacter*: the ECC (*E. kobei*, *E. chengduensis*, *E. chuandaensis*, *E. hormaechei*, *E. cloacae*, *E. sichuanensis*, *E. roggenkampii*, E. *ludwigii*, and *E. cancerogenerus*.) and *E. lignolyticus* (Figure 6B). *Klebsiella* species were mainly divided into *K. michiganensis* and *K. oxytoca* (Figure 6C). *Pantoea* species were mainly clustered into two groups i.e., *P. dispersa* and *P. anthophila* (Figure 6D).

Pathogenicity test of four main types of pathogenic bacteria

To further understand the role of *Ralstonia*, *Enterobacter*, *Klebsiella* and *Pantoea* in mulberry wilt, the pathogenicity test was conducted. As shown in Table 5, it was found that the average pathogenicity rate of *Ralstonia* derived from the MBWS was found to be 60.13%. The pathogenicity rate of *Ralstonia* with *16S rRNA* accumulated in the RSSC (*R. solanacearum* species complex) clade was higher than 43.33%, while the pathogenicity rate of *Ralstonia* aggregated in *R. pickettii* was 0% (Figure 6A). The average pathogenicity rate of *Enterobacter* derived from the MBWS was found to be 44.89%. From these, the main pathogenic group was concentrated in the ECC (*E. cloacae complex*) (Figure 6B), with greatly varying pathogenicity rates between them. The average

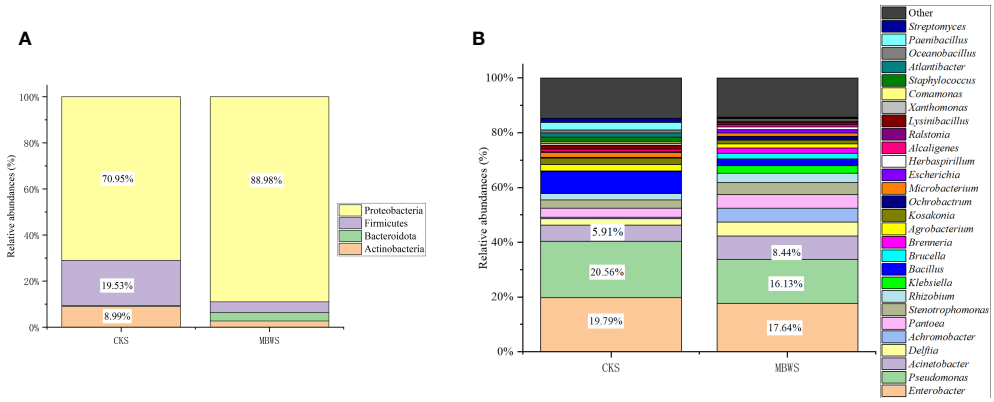

FIGURE 2
Relative abundance (%) of cultivable bacteria in different communities isolated from healthy and diseased mulberry at the phylum **(A)** and genus **(B)** levels. MBWS, mulberry bacterial wilt sample; CKS, Healthy samples (healthy mulberry samples).

pathogenicity rate of *Klebsiella* derived from the MBWS was found to be 44.76%. From these, the main pathogenic groups were *K. michiganensis*, *K. quasipneumoniae*, *K. oxytoca* and *K. pneumoniae* (Figure 6C), with greatly varying pathogenicity rates between them. The average pathogenicity rate of *Pantoea* derived from the MBWS was found to be 6.79%. From these, *P. ananatis* strain LCFJ-001 had the highest pathogenicity rate of 38.33%, while the others showed 0% pathogenicity rate (Figure 6D).

Discussion

Plant bacterial wilt is generally considered a highly destructive xylem disease caused by the *R. solanacearum* complex (RSSC). However, the advancement of bacterial wilt research shows that, in addition to other pathogens, the *E. cloacae* complex (ECC) can also cause bacterial wilt in African marigoldx (Jeevan et al., 2022), ginger (Cosmas et al., 2016) and mulberry plants (Wang et al., 2008; Zhu et al., 2011). Although the pathogenic bacteria of mulberry bacterial wilt are said to be complex and diverse, they mainly include *Ralstonia* (Pan et al., 2013), *Enterobacter* (Wang et al., 2008; Wang et al., 2010; Zhu et al., 2010; Zhu et al., 2011; Zhou et al., 2021), *Klebsiella* (Luo et al., 2022) and *Pantoea* (Yuan et al., 2023a). In order to better elucidate the interaction between the microbiome and mulberry, we used combined metagenomic sequencing and a culture-dependent approaches to explore the composition and diversity of bacterial communities in mulberry bacterial wilt samples.

We found 19 phyla and 112 genera in the diseased and healthy mulberry rhizosphere soil and xylem using Illumina HiSeq2500 sequencing. In contrast, four phyla and 97 genera were isolated and characterized using a culture-dependent approach. This discrepancy in the result infers that this phenomenon maybe linked to the inherent limitation of the culture-dependent method, as it is not entirely possible to isolate all xylem bacteria due to the limitation of

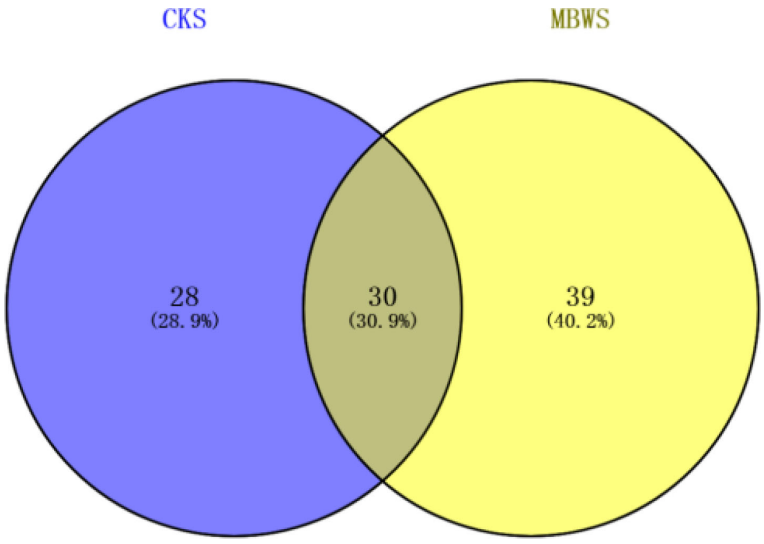

FIGURE 3
Bacterial Venn diagrams of healthy and diseased mulberry samples. MBWS, mulberry bacterial wilt sample; CKS, healthy mulberry samples.

the medium. On the contrary, it has been said that the metagenomic sequencing method can compensate for this limitation of the culture-dependent method (Zhang et al., 2020). Based on the results of metagenomic sequencing and Shannon, Chao-1, Simpson, and OTU, it was observed that the number and diversity of microbial flora in rhizosphere soil and xylem of healthy mulberry were higher than those of the diseased mulberry samples. Suhaimi et al. (2017) and Tao et al. (2022) also found that the diseased samples had a lower microbial diversity compared to the healthy samples. However, Kaushal et al. (2020) found contrasting result and reported higher OTU richness and diversity in the symptomatic roots. It is generally believed that a low microbial diversity in microbial communities favors pathogen invasion (Locey and Lennon, 2016). This argument is supported by finding of our recent report in which we found that the diversity of endophytes in highly resistant or moderately resistant varieties of mulberry bacterial wilt was significantly higher compared to the weakly resistant or susceptible varieties (Yuan et al., 2023b). This evidence also supports finding of the present study and demonstrate a potential link between diversity of microbial species and susceptibility.

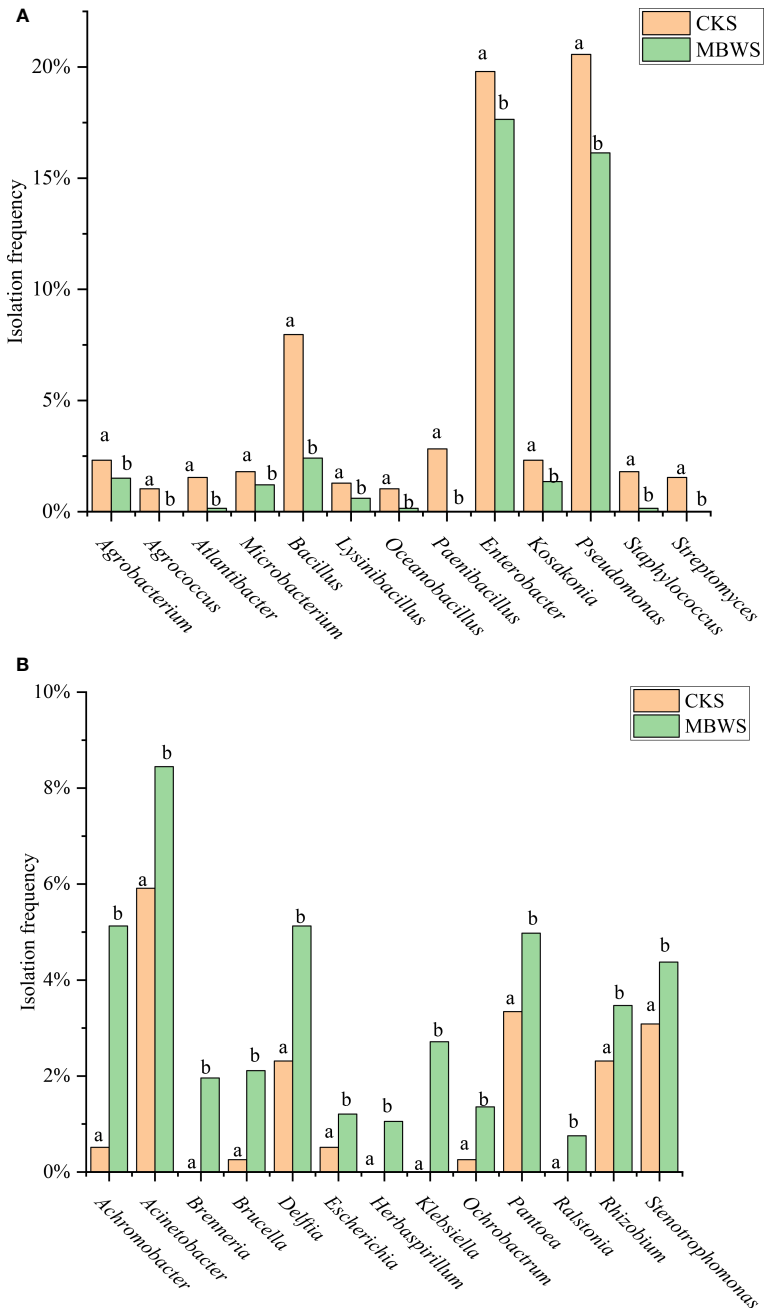

FIGURE 4
Isolation rates of abundant bacteria isolated from healthy and diseased mulberry. **(A)** Bacterial species and classification rates greater than CK in MBWS; **(B)** Bacterial species and classification rates greater than MBWS in CK; MBWS: mulberry bacterial wilt sample. CKS: Healthy samples (healthy mulberry samples). Bars with different letters indicate a significant difference between means by one-way analysis of variance (ANOVA) and least significant difference (LSD) tests ($P < 0.05$).

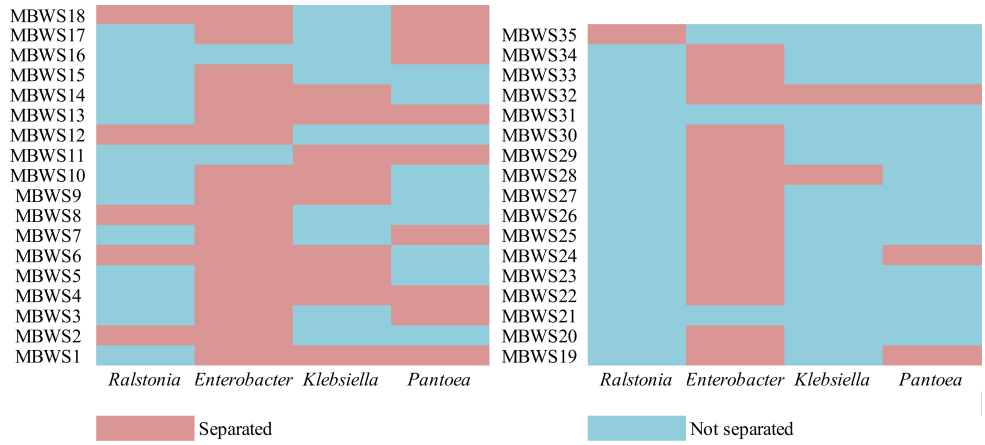

FIGURE 5
Isolation of *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea* in different mulberry bacterial wilt samples.

Interestingly, both metagenomic sequencing and culture-dependent approaches revealed that Proteobacteria was a main phylum in both diseased and healthy mulberry rhizosphere soils and xylem, followed by Firmicutes and Actinobacteria. Proteobacteria, Firmicutes, and Actinobacteria were essential components of bacteria in healthy and diseased mulberry xylem. This finding is in line with the evidence reported by Yuan et al. (2023b); Xu et al. (2019) and Ou et al. (2019). The culture-dependent method also revealed that Proteobacteria and Bacteroidetes had greater (P<0.05) abundance in the diseased mulberry xylem compared to the healthy samples. In contrast, Actinobacteria and Firmicutes had greater (P<0.05) abundance in the healthy xylem compared to the diseased samples. Interestingly, Kaushal et al. (2020) have reported that the abundance of Proteobacteria and Actinobacteria showed a similar trend in the banana Mchare cultivar. Suhaimi et al. (2017) have also reported that the healthy samples had higher richness of Proteobacteria than the diseased samples.

At the subordination level, metagenomic sequencing revealed that *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Acinetobacter* were the dominant genera, accounting for more than 1% of the eight samples tested. On the other hand, the culture-dependent approach revealed that *Enterobacter*, *Pseudomonas*, *Acinetobacter*, *Delftia*, *Pantoea*, *Stenotrophomonas*, *Rhizobium*, *Bacillus*, *Agrobacterium*, *Kosakonia*, and *Microbacterium* accounted for more than 1% of the microbial populations in healthy and diseased mulberry xylem. Overall, *Pseudomonas* and *Acinetobacter* were found to be the main constituent groups of the mulberry microbiome. This finding is supported by similar evidence reported by previous studies of Xu et al. (2019) and Ou et al. (2019), who reported that *Pseudomonas* was indeed an essential endophytic flora of mulberry. This evidence is further reinforced by reports of Suhaimi et al. (2017) and Kaushal et al. (2020) who also showed that *Pseudomonas* was an essential component of the banana bacterial flora. In our previous study, we found that *Pseudomonas* was one of the component of the endophytic flora in mulberry, but had no obvious control effect on the bacterial wilt of mulberry trees caused by *E. roggenkampii* strain KQ-01 (Yuan et al., 2023b). *Acinetobacter* was found to be an endophyte in mulberry and its proportion was significantly higher in mulberry varieties susceptible to bacterial wilt compared to the resistant varieties. In addition, the control rate of *Acinetobacter* against bacteria wilt caused by *E. roggenkampii* strain KQ-01 was higher than 80% (Yuan et al., 2023b). Seemingly, these results are contrasting and highlight that the precise roles played by *Pseudomonas* and *Acinetobacter* in plants need to be elucidated in future focused research.

Intriguingly, both metagenomic sequencing and culture-dependent methods employed in the present study showed that the proportion of *Pseudomonas* in the rhizosphere soil and xylem of healthy mulberry was higher compared to the diseased mulberry samples. Similarly, Suhaimi et al. (2017) showed that the abundance of *Pseudomonas* in the healthy banana samples was higher compared to the diseased samples. Using metagenomic sequencing, we found that the proportion of *Erwinia* bacteria in the rhizosphere soil and xylem of the diseased mulberry was higher compared to the healthy mulberry samples. However, this result was not supported by finding of the culture-dependent method. This finding is reinforced by evidence of our previous study in which a similar phenomenon was observed in mulberry samples (Yuan et al., 2023b).

In addition, the culture-dependent method revealed that the abundance of many opportunistic pathogens and drug-resistant bacteria was significantly higher in the xylem of the diseased samples compared to their healthy counterparts. Infections in humans have been reported mostly with opportunistic pathogens, including *Achromobacter* (Menetrey et al., 2021), *Acinetobacter* (Amorim and Nascimento, 2017), *Brucella* (Roop et al., 2021), *Delftia* (Deb et al., 2020), *Escherichia* (Bhatt et al., 2019), *Herbaspirillum* (Bloise et al., 2021), *Klebsiella* (Rodríguez–Medina et al., 2019), *Ochrobactrum* (Bratschi et al., 2020), *Pantoea* (Cobo et al., 2021), *Ralstonia* (Ryan and Adley, 2014) and *Stenotrophomonas* (Menetrey et al., 2021). Moreover, *Acinetobacter* (Shin et al., 2020), *Escherichia* (Tang et al., 2022), *Klebsiella* (Dong et al., 2022), *Pantoea* (Yoshimura et al., 2022) and

Stenotrophomonas (Ferreira et al., 2020) have been shown to have multidrug resistance.

In the present study, *Herbaspirillum*, *Klebsiella*, and *Ralstonia* were not isolated in the healthy mulberry xylem, indicating that these bacteria might have invaded after infection. Similar results were obtained by Hu et al. (2020), who found an increase in the relative abundance of *Ralstonia*, *Stenotrophomonas* and *Achromobacter* in the infected samples compared to the healthy samples. Although we suspect that the overuse of agricultural antibiotics and untreated farmyard manure exacerbates this situation, the precise underlying basis of this phenomenon remains to be explored. In addition, *Brenneria* was only isolated in the diseased mulberry samples but not in the healthy xylem. *Brenneria* has been reported to be a pathogen of woody plants that can cause cankers in plants including walnut (Poret–Peterson et al., 2019), oak (Denman et al., 2012), willow (Maes et al., 2009), alder

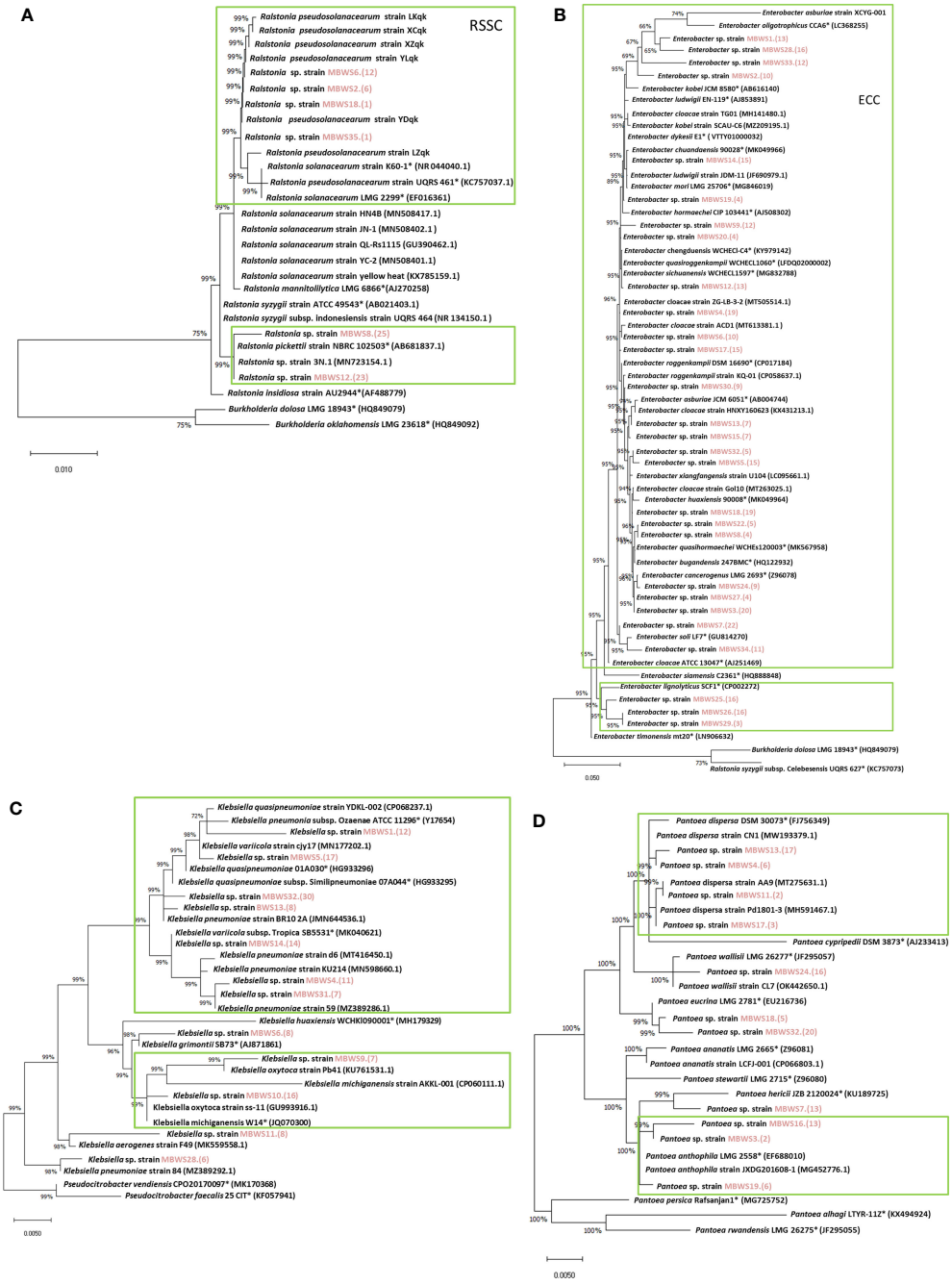

FIGURE 6
Phylogenetic trees of *Ralstonia* **(A)**, *Enterobacter* **(B)**, *Klebsiella* **(C)**, and *Pantoea* **(D)** based on *16S rRNA* genes. "*" indicates the representative species; the red marks are the isolates of this study.

(Maes et al., 2009) and poplar (Li et al., 2015). Currently, *Brenneria* is rarely reported in mulberry, and whether this pathogen is emerging as a new pathogen of mulberry still needs further investigation.

The culture-dependent method showed that many bacteria that have been reported to promote plant growth or control bacterial wilt were present in the mulberry samples. These included: *Agrobacterium* (Soares et al., 2020), *Microbacterium* (Singh and

TABLE 5 Pathogenicity tests of *Ralstonia*, *Enterobacter*, *Klebsiella*, and *Pantoea*.

Ralstonia		Enterobacter		Klebsiella		Pantoea	
Strain Name	Morbidity %	Strain Name	Morbidity %	Strain Name	Morbidity %	Strain Name	Morbidity %
LKqk	100±0	XCYG-001	36.66±1.66	AKKL-001	85±2.88	LCFJ-001	38.33±1.66
LZqk	63.33±1.67	KQ-01	100±0	YDKL-002	55±2.88	MBWS1.(11)	0±0
XCqk	100±0	MBWS1.(13)	38.33±1.67	MBWS1.(12)	61.66±1.66	MBWS3.(2)	33.33±1.66
XZqk	100±0	MBWS2.(10)	83.33±1.67	MBWS4.(11)	41.67±1.67	MBWS4.(6)	0±0
YDqk	63.33±1.67	MBWS3.(20)	71.67±1.67	MBWS5.(17)	36.67±3.33	MBWS7.(13)	0±0
YLqk	43.33±1.67	MBWS4.(19)	71.67±1.67	MBWS6.(8)	0±0	MBWS11.(2)	0±0
MBWS2.(6)	45±2.88	MBWS5.(15)	36.67±1.67	MBWS9.(7)	70±2.88	MBWS13.(17)	0±0
MBWS6.(12)	0±0	MBWS6.(10)	100±0	MBWS10.(16)	41.66±1.66	MBWS16.(13)	0±0
MBWS8.(25)	0±0	MBWS7.(22)	16.67±1.67	MBWS11.(8)	0±0	MBWS17.(3)	0±0
MBWS12.(23)	63.33±1.67	MBWS8.(4)	0±0	MBWS13.(8)	61.67±1.6	MBWS18.(5)	0±0
MBWS18.(1)	80±2.88	MBWS9.(12)	36.67±1.67	MBWS14.(14)	70±2.88	MBWS19.(6)	16.66±1.66
MBWS35.(1)	63.33±1.67	MBWS10.(15)	68.33±4.41	MBWS28.(6)	0±0	MBWS24.(16)	0±0
		MBWS12.(13)	0±0	MBWS31.(7)	58.33±1.67	MBWS32.(20)	0±0
		MBWS13.(7)	73.33±1.67	MBWS32.(30)	45±2.88		
		MBWS14.(15)	36.67±1.67				
		MBWS15.(7)	0±0				
		MBWS17.(15)	35±2.88				
		MBWS18.(19)	55±2.88				
		MBWS19.(4)	0±0				
		MBWS20.(4)	53.33±1.67				
		MBWS22.(5)	0±0				
		MBWS23.(1)	100±0				
		MBWS24.(9)	51.67±1.67				
		MBWS25.(16)	0±0				
		MBWS26.(16)	0±0				
		MBWS27.(4)	100±0				
		MBWS28.(16)	100±0				
		MBWS29.(3)	0±0				
		MBWS30.(9)	100±0				
		MBWS32.(5)	16.67±1.67				
		MBWS33.(12)	55±2.88				
		MBWS34.(11)	0±0				
Morbidity mean	60.13%[a]	Morbidity mean	44.89%[b]	Morbidity mean	44.76%[b]	Morbidity mean	6.79%[c]

Different letters in the same row indicate significant difference between means by one-way analysis of variance (ANOVA) and least significant difference (LSD) test (P<0.05). Values represent the mean. Error bars indicate ± standard deviation.

Singh, 2019), *Bacillus* (Im et al., 2020), *Lysinibacillus* (Lelapalli et al., 2021), *Oceanobacillus* (Alhindi and Albdaiwi, 2022), *Paenibacillus* (Abdallah et al., 2019), Enterobacter (Anand et al., 2021), *Kosakonia* (Brock et al., 2018), *Pseudomonas* (Zhuo et al., 2022) and *Streptomyces* (Olanrewaju and Babalola, 2019). In agreement with our finding, Hu et al. (2020) also reported similar results. They found that the relative abundance of *Pseudomonas*, *Bacillus*, and *Falsibacillus*, which are generally considered beneficial to plants, was significantly higher in the healthy mulberry samples compared to the diseased samples. This group of bacteria can be considered as a bank of beneficial microbial flora of mulberry. Interestingly, in the present study, the abundance of these bacteria was lower in the diseased samples compared to the healthy mulberry samples.

We further revealed that *Enterobacter* was the most widely distributed among the four types of pathogenic bacteria, accounting for 85.71%, followed by *Klebsiella* and *Pantoea*, which accounted for 34.28%. In contrast, *Ralstonia* accounted for the lowest (17.14%) proportion. This result indicated that *Enterobacter* might be the primary pathogen group causing bacterial wilt of mulberry, however, further focused research is needed to reinforce this evidence and gain more insights in this domain. Based on the *16S rDNA* sequence and its pathogenicity, *Ralstonia* was mainly clustered into two clades, the RSSC and *R. pickettii*. The pathogenicity of *Ralstonia* clustered in the same clade as the RSSC was greater than 45%, while clustered in the other clade, *R. pickettii* showed no pathogenicity. Meanwhile, *Enterobacter* was mainly clustered into the *ECC* and *E. lignolyticus*. A total of 73.91% of *Enterobacter* bacteria clustering in the *ECC* showed pathogenicity. *E. lignolyticus* clustered in one clade and showed no pathogenicity. *Klebsiella* was mainly clustered into two clades centered on *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, and *K. michiganensis*, and both showed pathogenicity. *Pantoea* mainly clustered into two clades centered on *P. dispersa* and *P. anthophila* and did not show strong pathogenicity. However, *P. ananatis* strain LCFJ-001 (CP066803.1) which was discovered earlier (Yuan et al., 2023a) by our laboratory was shown to be pathogenic, with a pathogenicity rate of 38.33%. The RSSC, ECC, *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. michiganensis*, and *P. ananatis* were found to be the main components of the pathogenic bacteria of mulberry bacterial wilt.

During the RSSC infection, the Sol system can be regulated to produce an acylated homoserine lactone (AHL) quorum signaling factor, which is ubiquitous in various gram-negative bacteria, but it is poorly studied in the RSSC (Flavier et al., 1997). When AHL reaches a critical concentration, it diffuses into the cell to bind transcriptional regulators and activates other virulence regulators (Baltenneck et al., 2021). Density-dependent signaling systems centered on AHL are standard in gram-negative bacteria and have been reported in *Enterobacter* (Shastry et al., 2018), *Klebsiella* (Hosny and Fadel, 2021), and *Pantoea* (Jiang et al., 2015). It remains to be explored if there is a possibility that the RSSC can secrete enough AHL through the Sol regulation system to cooperate with other pathogenic bacteria and to infect together.

Conclusion

The llumina HiSeq2500 sequencing and traditional culture medium approaches employed in the present study revealed that the bacterial diversity of healthy mulberry was higher compared to the diseased mulberry. The phyla Proteobacteria, Firmicutes and Actinobacteria constituted an important component of bacteria in the healthy and diseased mulberry. In addition, the abundance of many opportunistic pathogens and drug-resistant bacteria was significantly higher in the diseased samples compared to the healthy counterparts. It was found that the RSSC, ECC, *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. michiganensis*, and *P. ananatis* were the main components of the pathogenic bacteria of mulberry wilt. From these, the ECC was found to be the most widely distributed in the diseased samples. This study provides reference data for further focused research on the bacterial wilt of mulberry and other plants.

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/, PRJNA911049; OP990608-OP990981;OP989957-OP990607.

Author contributions

TY wrote the initial draft of manuscript, conceived experiment design, performed experiments, data analysis and implementation. IHQ participated in data analysis, interpretation of the results and revised and edited the draft. JHL, HY, PY, XZ, WL, and YQ collected materials, and assisted in the experiment and data analysis. JPL provided experimental platform and support, project supervision, and funding. All authors contributed to the article and approved the submitted version.

Funding

The research was financially supported by the China Agriculture Research System (CARS-18-ZJ0304).

Acknowledgments

We thank all lab mates who supported during this project.

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.2023.1206691/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 04 October 2023
DOI 10.3389/fpls.2023.1247342

OPEN ACCESS

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RECEIVED 25 June 2023
ACCEPTED 15 September 2023
PUBLISHED 04 October 2023

CITATION
Wang Y, Cao J-L, Hashem A, Abd_Allah EF and Wu Q-S (2023) *Serendipita indica* mitigates drought-triggered oxidative burst in trifoliate orange by stimulating antioxidant defense systems.
Front. Plant Sci. 14:1247342.
doi: 10.3389/fpls.2023.1247342

Serendipita indica mitigates drought-triggered oxidative burst in trifoliate orange by stimulating antioxidant defense systems

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Soil drought is detrimental to plant growth worldwide, particularly by triggering reactive oxygen species (ROS) burst. *Serendipita indica* (*Si*), a culturable root-associated endophytic fungus, can assist host plants in dealing with abiotic stresses; however, it is unknown whether and how *Si* impacts the drought tolerance of citrus plants. To unravel the effects and roles of *Si* on drought-stressed plants, trifoliate orange (*Poncirus trifoliata* L. Raf.; a citrus rootstock) seedlings were inoculated with *Si* and exposed to soil drought, and growth, gas exchange, ROS levels, antioxidant defense systems, and expression of genes encoding antioxidant enzymes and fatty acid desaturases in leaves were measured. Soil drought suppressed plant biomass, whereas *Si* inoculation significantly increased plant biomass (10.29%-22.47%) and shoot/root ratio (21.78%-24.68%) under ample water and drought conditions, accompanied by improved net photosynthetic rate (105.71%), water use efficiency (115.29%), chlorophyll index (55.34%), and nitrogen balance index (63.84%) by *Si* inoculation under soil drought. Soil drought triggered an increase in leaf hydrogen peroxide and superoxide anion levels, while *Si* inoculation significantly reduced these ROS levels under soil drought, resulting in lower membrane lipid peroxidation with respect to malondialdehyde changes. Furthermore, *Si*-inoculated seedlings under soil drought had distinctly higher levels of ascorbate and glutathione, as well as catalase, peroxidase, and glutathione peroxidase activities, compared with no-*Si*-inoculated seedlings. *Si* inoculation increased the expression of leaf *PtFAD2*, *PtFAD6*, *PtΔ9*, *PtΔ15*, *PtFe-SOD*, *PtCu/Zn-SOD*, *PtPOD*, and *PtCAT1* genes under both ample water and soil drought conditions. Overall, *Si*-inoculated trifoliate orange plants maintained a low oxidative burst in leaves under drought, which was associated with stimulation of antioxidant defense systems. Therefore, *Si* has great potential as a biostimulant in enhancing drought tolerance in plants, particularly citrus.

KEYWORDS

antioxidation, citrus, endophytic fungus, gas exchange, oxidative damage

Introduction

Drought stress (DS) is a frequent environmental factor that has a detrimental impact on plant physiological activities and morphological performance, such as lowering leaf gas exchange, slowing plant growth, and overproduction of reactive oxygen species (ROS) (Ahluwalia et al., 2021). ROS are highly reactive and toxic by-products of photosynthesis and photorespiration processes in plants, and the excess ROS causes oxidative damage to various macromolecules, thereby limiting plant growth and development (Ilyas et al., 2021; Tyagi et al., 2022a). Superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are the two most prevalent ROS induced by DS in plants (Miller et al., 2010). Plants also possess antioxidant defense systems to scavenge ROS, where antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and others, and non-enzymatic antioxidants include ascorbic acid (AsA), glutathione (GSH), carotenoids, and tocopherols (Mukarram et al., 2021). As a result, uncovering changes in antioxidant defense systems could clarify the drought-resistant potential of plants.

Citrus is the most widely grown fruit crop in the world (Addi et al., 2022). Because of its poor root hairs, trifoliate orange (*Poncirus trifoliata* L. Raf.), a common citrus rootstock, relies heavily on extraradical hyphae of arbuscular mycorrhizae in roots for water and nutrient uptake from the soil (Ortas, 2012). Symbiotic associations between arbuscular mycorrhizal fungi (AMF) and plants are prevalent, with AMF providing water and mineral nutrients to the host and the host providing carbohydrates to the fungal partner (Prasad et al., 2008; Tyagi et al., 2022b). Earlier studies have demonstrated that AMF could enhance drought tolerance in citrus, and the underlying mechanism is associated with mycorrhizal improvement of root structure and physiological activities, as well as stressed gene expression activation (Marulanda et al., 2007; Yaghoubian et al., 2014; Cheng et al., 2021; Liu et al., 2022; Wang et al., 2023). However, the application of AMF in the citrus field is limited because it cannot be cultured *in vitro* on a large scale without host plants. As a result, selecting an effective culturable endophytic fungus with functions similar to AMF has become a pressing problem in citriculture.

Serendipita indica (formerly *Piriformospora indica*) (*Si*) is a culturable endophytic fungus that can colonize a variety of host roots, including citrus (Varma et al., 2012; Yang et al., 2021a). *Si* possesses AMF-like characteristics (Mensah et al., 2020) and was isolated from an Indian desert (Verma et al., 1998), suggesting that it may be drought-tolerant. Earlier studies had reported significant increases in biomass and sustained growth in barley (*Hordeum vulgare*) and Arabidopsis (*Arabidopsis thaliana*) after inoculation with *Si* under drought (Sherameti et al., 2008; Ghaffari et al., 2019). Proteomics demonstrated that the colonization of *Si* raised photosynthesis-related protein levels in drought-stressed host plants (Ghaffari et al., 2019). *Si* colonization in cabbage (*Brassica campestris*) decreased leaf malondialdehyde (MDA) levels under DS, and several antioxidant enzyme activities were upregulated within 24 h (Sun et al., 2010). After *Si* inoculation, wheat (*Triticum aestivum*), eggplant (*Solanum melongena*), and walnut (*Juglans regia*) decreased ROS levels and elevated CAT and POD activities in leaves (Yaghoubian et al., 2014; Swetha and Padmavathi, 2020; Liu et al., 2021). *Si* inoculation also changes the expression of stressed genes under DS. *Si* inoculation, for example, boosted the expression of four drought-associated genes in leaves of drought-stressed cabbage, namely, *DREB2A, CBL1, ANAC072*, and *RD29A* (Sun et al., 2010). However, *Si* inoculation in wheat inhibited CAT activity under drought conditions, achieving a significant level at −0.5 MPa (Hosseini et al., 2017). In maize, CAT and ascorbate peroxidase (APX) activities were also decreased under DS by *Si* (Hosseini et al., 2018). These conflicting results show that *Si* is variable in modulating antioxidant defense systems in host plants and more research needs to be investigated, especially as the molecular mechanism lags behind physiological advances.

Citrus plants, particularly trifoliate orange, have been demonstrated to be a host plant for *Si*, and inoculation with *Si* promoted their growth behavior through increasing auxin levels and nutrient acquisition (Yang et al., 2021a; Liu et al., 2023). However, it is unknown whether and how *Si* impacts the drought tolerance of trifoliate orange in terms of antioxidant defense systems. This study was carried out to investigate the effects of *Si* inoculation on growth, leaf gas exchange, ROS levels, antioxidant enzyme activities, antioxidant levels, and the expression of genes encoding antioxidant enzymes and fatty acid desaturases under DS. Such study can evaluate the potential of *Si* as a biostimulant for drought tolerance in citrus.

Materials and methods

Plant culture and experimental design

Four-leaf-old trifoliate orange seedlings grown in autoclaved sands were chosen. *Si* was inoculated at the time of transplanting. *Si* was provided by Prof. Z.-H. Tian (Yangtze University), which was kept in our laboratory. The proliferation of this fungus was performed *in vitro* as per the protocol of Yang et al. (2021a), achieving a spore suspension of 5.0×10^8 CFU/mL and a mycelial solution of 0.018 g/mL.

Three seedlings were planted in a plastic pot that had been pre-filled with an autoclaved mixture consisting of soil and river sands mixed in a 4: 1 ratio by volume to obtain a relative low Olsen-P level (9.73 mg/kg). At the time of transplanting, 12.5 mL of spore suspension and 14.5 mL of mycelial solution were inoculated around roots of potted seedlings as the inoculation treatment (+*Si*). In contrast, the uninoculated treatment (−*Si*) also received the same volume but autoclaved spore suspension and mycelium solution (Rong et al., 2022). The treated seedlings were subjected to the controlled environments described by Cao et al. (2023). The weighing method was used to keep the soil moisture of these potted plants at 75% of the maximum water holding capacity (MWHC) in the field (well-watered, WW). The condition lasted for 7 weeks. Subsequently, the soil moisture regime was altered for half of the plants to 55% of the MWHC in the field (DS) for 9 weeks, while the soil moisture regime remained unchanged for the remaining plants.

Thus, this study consisted of two factors: *Si* inoculation treatments (+*Si* and − *Si*) and two soil moistures (WW and DS). There were four treatments, each with six replications, with a total of 72 seedlings and 24 pots.

Determination of growth and root fungal colonization frequency

After 9 weeks of drought exposure, the treated plants were harvested and weighed promptly. The Epson Root Scanner (V700) and WinRHIZO software (2007b) were used to quantify root surface area and volume. Then, 1-cm root segments were selected and stained for *Si* colonization in the roots using the method of Phillips and Hayman (1970). In addition, root segments were cut into thin slices of longitudinal sections using a double-sided blade. Subsequently, a drop of 0.05% trypan blue was introduced to observe the fungal colonization. Root fungal colonization was examined under a microscope, and root fungal colonization frequency was estimated as the percentage of *Si* -colonized root segment number to total detected root segment number.

Determination of leaf physiological variables

On a sunny day (9:00 a.m.) before harvest, leaf gas exchange parameters, including net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (Gs), were measured on the fourth leaf below the tip of trifoliate orange seedlings using a Li-6400 portable photosynthesizer (Li-COR, USA). The photosynthesizer was preheated for 20 min before used. After calibrating and zeroing the photosynthesizer, the leaf area, ambient water vapor pressure, and CO_2 concentrations were set at 6.5 cm^2, 1.01 kPa, and 400 μmol/m^2/s, respectively. During measurement, the data were recorded after stabilization. Water use efficiency (WUE) was defined as the Pn/Tr ratio.

A portable plant polyphenol-chlorophyll meter (Dualex Scientific+, Orsay, France) was used to measure nitrogen balance index (Nbi) and chlorophyll index (Chi) in leaves.

The concentration of leaf H_2O_2 was determined according to the KI colorimetric method reported by Velikova et al. (2000). Leaf $O_2^{\cdot-}$ levels were assayed using the protocol outlined by Zou et al. (2015). Leaf MDA concentrations were measured according to the thiobarbituric acid method described by Sudhakar et al. (2001).

Leaf CAT activity was determined colorimetrically at 240 nm according to the method described by He et al. (2020). The absorbance of reaction solutions changed by 0.01 at 240 nm in 1 min as a unit (U) of CAT. Leaf POD activity was assayed using the guaiacol method described by Chance and Maehly (1955), where the absorbance of reaction solutions changed by 0.1 at 470 nm in 1 min as a U of POD. Leaf APX activity was determined as per the protocol outlined by Wu (2018), where the reaction solution consisted of 50 mM potassium phosphate buffer (the enzyme extraction solution), 6 mM AsA, and supernatants. The absorbance of reaction solutions changed by 0.01 at 290 nm in 1 min as a U of APX. Leaf glutathione reductase (GR) activity was analyzed according to the method of Chen and Wang (2002), where the reaction mixture consisted of 1 mM NADPH, 0.1 M tricine-NaOH buffer (the enzyme extraction solution), supernatants, and 5 mM oxidized GSH. The absorbance of reaction solutions changed by 0.01 at 340 nm in 1 min as a U of GR.

Fresh leaf samples (0.30 g) were ground into a homogenate in 5 mL of 5% trichloroacetic acid solution and centrifuged at 15,000×*g* for 15 min. The supernatant was used for the assay of AsA and GSH, and the procedure for the assay had been described in detail by Li et al. (2022).

Determination of the expression of genes encoding antioxidant enzymes and fatty acid desaturases

Total RNA of leaves was extracted using the TaKaRa MiniBEST Plant RNA Extraction Kit. Following detection of total RNA concentration and purity, total RNA was reverse transcribed into cDNA based on the PrimeScript™ RT reagent Kit with the gDNA Eraser kit. Each treatment's cDNA obtained was employed as the template for RT-PCR amplification. According to the findings of Wu et al. (2019a), five antioxidant enzyme genes and four fatty acid saturase genes were chosen and thus designed for their primers in qRT-PCR (Supplementary Table S1). The internal reference gene in this investigation was *β-actin*. The SYBR Green PCR Master Mix and Real-time PCR Detection System (BIO-RAD, Hercules, USA) were used for real-time PCR. There were three biological replicates for each determination. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate expression of genes. Relative expression of genes was normalized with the uninoculation treatment under WW conditions.

Statistical analysis

The two-way analysis of variance under the condition of SAS software (v8.1) was used to compare the variance of the experimental data, and Duncan's multiple-range test was performed to assess significant ($p< 0.05$) differences across treatments.

Results

Effects of DS on root fungal colonization frequency

Fungal colonization was found in roots of *Si*-inoculated seedlings, but not in no-*Si*-inoculated seedlings, with more transparent pear-shaped chlamydospores in *Si*-inoculated roots under DS (Figures 1A, B) than under WW (Figures 1C, D). Root fungal colonization frequency was 30.1% under WW conditions and 61.9% under DS conditions, respectively (Table 1). *Si* inoculation and DS treatment interacted ($p< 0.01$) to affect root fungal colonization frequency.

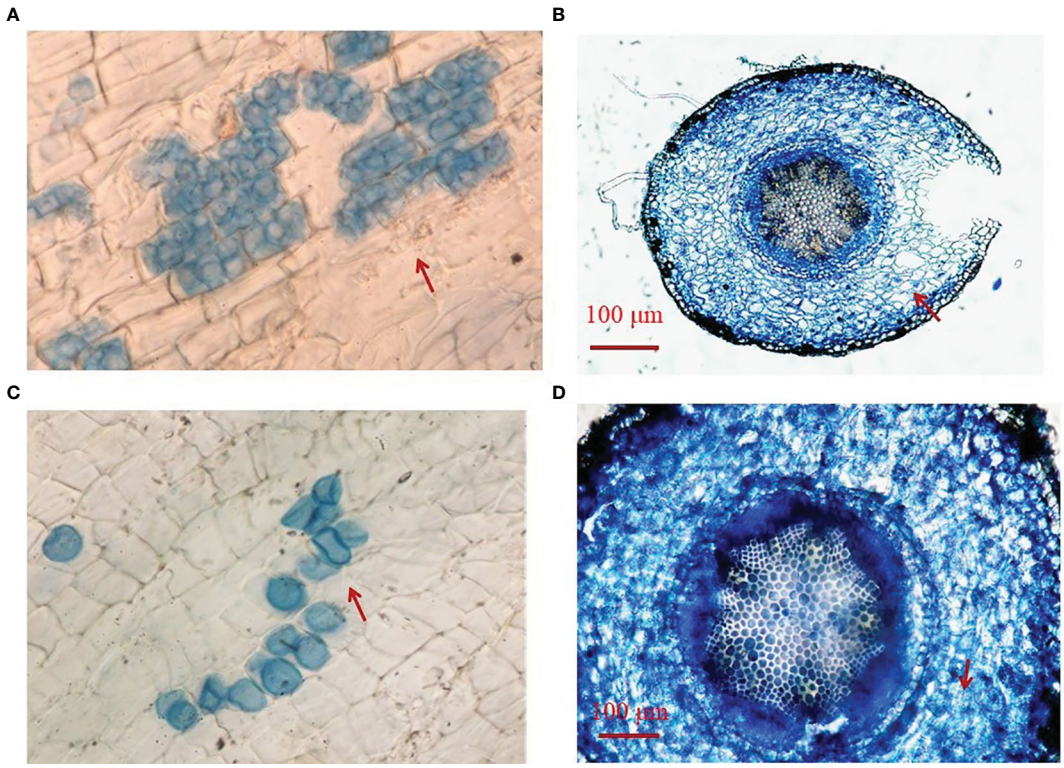

FIGURE 1
Root colonization of *Serendipita indica* (*Si*) in trifoliate orange seedlings under drought stress **(A, B)** and well-watered **(C, D)** conditions. The red arrow indicates transparent pear-shaped chlamydospores.

Effects of Si inoculation on plant growth variables under DS

DS and Si inoculation significantly impacted plant growth behavior of trifoliate orange seedlings (Supplementary Figure S1). DS treatment inhibited plant biomass, root surface area, and root volume of *Si*-inoculated plants by 23.74%, 15.29%, and 12.50%, respectively, compared with WW treatment (Table 1). Leaf number, biomass, root surface, and root volume of no-*Si*-inoculated plants were also decreased under DS versus WW by 8.38%, 17.94%, 21.71%, and 14.06%, respectively. *Si* inoculation significantly increased shoot/root ratio, leaf number, biomass, root surface area, and root volume under WW conditions by 24.68%, 17.80%, 22.47%, 26.93%, and 12.50%, respectively, and under DS conditions by 21.78%, 19.82%, 10.29%, 13.82%, 17.31%, and 14.55%, respectively, compared with no-*Si* inoculation. The interaction of DS treatment and *Si* inoculation significantly affected biomass.

Effects of Si inoculation on leaf gas exchange under DS

Leaf Pn, Gs, and Tr in *Si*-inoculated seedlings was inhibited under DS versus WW by 42.89%, 43.63%, and 43.40%, respectively, and leaf Pn and WUE in no-*Si*-inoculated seedlings were also suppressed by 40.86% and 64.20%, respectively (Figures 2A–D). Compared with no-*Si* inoculation, *Si* inoculation profoundly raised leaf Pn, Tr, and Gs under WW conditions by 113.01%, 179.16%, and 165.67%, respectively, and it also raised leaf Pn and WUE by 105.71% and 115.29% under DS conditions, respectively. DS and *Si* inoculation interactively ($p< 0.01$) affected Tr, Gs, and WUE (Table 2).

Effects of Si inoculation on leaf chlorophyll index and nitrogen balance index under DS

Compared with WW treatment, soil drought significantly reduced leaf Chi of *Si*-inoculated seedlings and Nbi of no-*Si*-inoculated seedlings by 8.50% and 18.78%, respectively, coupled with an 8.78% significant increase in Nbi of *Si*-inoculated seedlings (Figures 3A, B). *Si* inoculation raised leaf Chi and Nbi by 58.33% and 22.34% under WW conditions and 55.34% and 63.84% under DS conditions, respectively, compared with no-*Si* treatment. A significant ($p< 0.01$) interaction appeared in Nbi (Table 2).

Effects of Si inoculation on leaf ROS levels under DS

Leaf H_2O_2 and O_2^- levels were significantly raised under DS versus WW conditions: 17.21% and 29.26% higher in *Si*-inoculated seedlings and 20.21% and 69.66% higher in no-*Si*-inoculated seedlings, respectively (Figures 4A, B). Compared with no-*Si*

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TABLE 1 Changes in root fungal colonization frequency and plant growth of trifoliate orange seedlings inoculated with *Serendipita indica* (*Si*) under well-watered (WW) and drought stress (DS) conditions.

Treatments	Root fungal colonization frequency (%)	Plant height (cm)	Leaf number (num./plant)	Biomass (g FW/plant)	Root surface area (cm^2)	Root volume (cm^3)	Shoot/root ratio
WW-*Si*	0c	12.98 ± 0.44b	16.72 ± 0.68bc	4.85 ± 0.27b	54.35 ± 2.77b	0.64 ± 0.06b	0.52 ± 0.04b
WW+*Si*	30.1 ± 4.3b	15.83 ± 3.49a	18.44 ± 1.53a	5.94 ± 0.36a	63.76 ± 3.31a	0.72 ± 0.06a	0.65 ± 0.06a
DS-*Si*	0c	12.26 ± 0.31b	15.28 ± 0.98c	3.98 ± 0.17c	42.55 ± 3.90c	0.55 ± 0.03c	0.53 ± 0.04b
DS+*Si*	61.9 ± 5.9a	14.69 ± 2.46ab	18.00 ± 1.55ab	4.53 ± 0.26b	54.01 ± 1.30b	0.63 ± 0.05b	0.64 ± 0.06a
Significance							
DS	**	NS	NS	**	**	**	NS
Si	**	**	**	**	**	**	**
Interaction	**	NS	NS	*	NS	NS	NS

Data (means ± SD, n = 6) followed by different letters in the same column indicate significant ($p< 0.05$) differences. NS, not significant at $p< 0.05$; *, $p< 0.05$; **, $p< 0.01$.

treatment, *Si* inoculation had a significantly inhibitory effect on leaf H_2O_2 and $O_2^{\cdot-}$ levels, with 8.88% and 21.54% lower under WW and 11.15% and 40.22% lower under DS, respectively. A significant ($p< 0.01$) interaction appeared in $O_2^{\cdot-}$ levels (Table 2).

Effects of Si inoculation on leaf MDA levels under DS

Leaf MDA levels were significantly increased by 16.95% in no-*Si*-inoculated seedlings, but not *Si*-inoculated seedlings, under DS versus WW (Figure 5). Compared to no-*Si* treatment, *Si* inoculation significantly reduced leaf MDA levels by 15.13% under WW and 17.12% under DS, respectively.

Effects of Si inoculation on leaf antioxidant levels under DS

Leaf AsA and GSH levels were significantly decreased under DS versus WW conditions by 45.89% and 7.13% in *Si*-inoculated seedlings and 15.04% and 9.42% in no-*Si*-inoculated seedlings, respectively (Figures 6A, B). However, *Si* inoculation significantly raised leaf AsA and GSH levels by 85.66% and 11.50% under WW conditions and 18.24% and 14.31% under DS conditions, respectively, compared with no-*Si* inoculation treatment.

Effects of Si inoculation on leaf antioxidant enzyme activities under DS

Compared with WW treatment, DS treatment significantly decreased leaf GR and APX activities by 12.09% and 24.26% in *Si*-inoculated seedlings, while it distinctly raised leaf POD activities by 34.07% in *Si*-inoculated seedlings, along with a significant decrease in leaf APX and CAT levels by 16.26% and 23.90% in no-*Si*-inoculated seedlings (Figures 7A–D). *Si* inoculation significantly increased leaf GR, APX, POD, and CAT activities under WW by 28.98%, 82.34%, 26.84%, and 11.88%, respectively, compared with no-*Si* inoculation. Under DS, *Si* inoculation significantly raised leaf POD, APX, and CAT activities by 87.36%, 64.92%, and 38.43%, respectively, compared with no-*Si* inoculation. A significant ($p< 0.01$) interaction appeared in POD and APX activities (Table 2).

Effects of Si inoculation on leaf antioxidant enzyme genes expression under DS

Compared with WW treatment, DS treatment triggered upregulation of *PtCu/Zn-SOD* and *PtCAT1* gene expression in leaves of *Si*-inoculated seedlings by 1.07- and 0.94-fold, respectively, but it also suppressed the expression of *PtMn-SOD*, *PtFe-SOD*, and *PtPOD* genes in leaves of no-*Si*-inoculated seedlings by 0.77-, 0.53-, and 0.07-fold, respectively (Figure 8). The expression of *PtMn-SOD*, *PtCu/Zn-SOD*, *PtPOD*, and *PtCAT1* genes in leaves of no-*Si* inoculated seedlings was upregulated under DS versus WW conditions by 0.79-, 0.25-, 3.24-, and 1.65-fold, respectively, accompanied by the downregulated expression of *PtFe-SOD* genes. *Si* inoculation induced the upregulated expression of *PtMn-SOD*, *PtFe-SOD*, *PtCu/Zn-SOD*, *PtPOD*, and *PtCAT1* genes under WW conditions by 8.38-, 5.46-, 2.12-, 5.28-, and 1.56-fold, respectively, compared with no-*Si* inoculation. Under DS conditions, *Si* inoculation upregulated the expression of *PtMn-SOD*, *PtFe-SOD*, *PtCu/Zn-SOD*, *PtPOD*, and *PtCAT1* genes by 0.19-, 3.43-, 4.19-, 0.38-, 0.87-fold, respectively. There was a significant interaction between DS treatment and *Si* inoculation on the expression of leaf *PtMn-SOD*, *PtFe-SOD*, *PtCu/Zn-SOD*, and *PtPOD* genes (Table 2).

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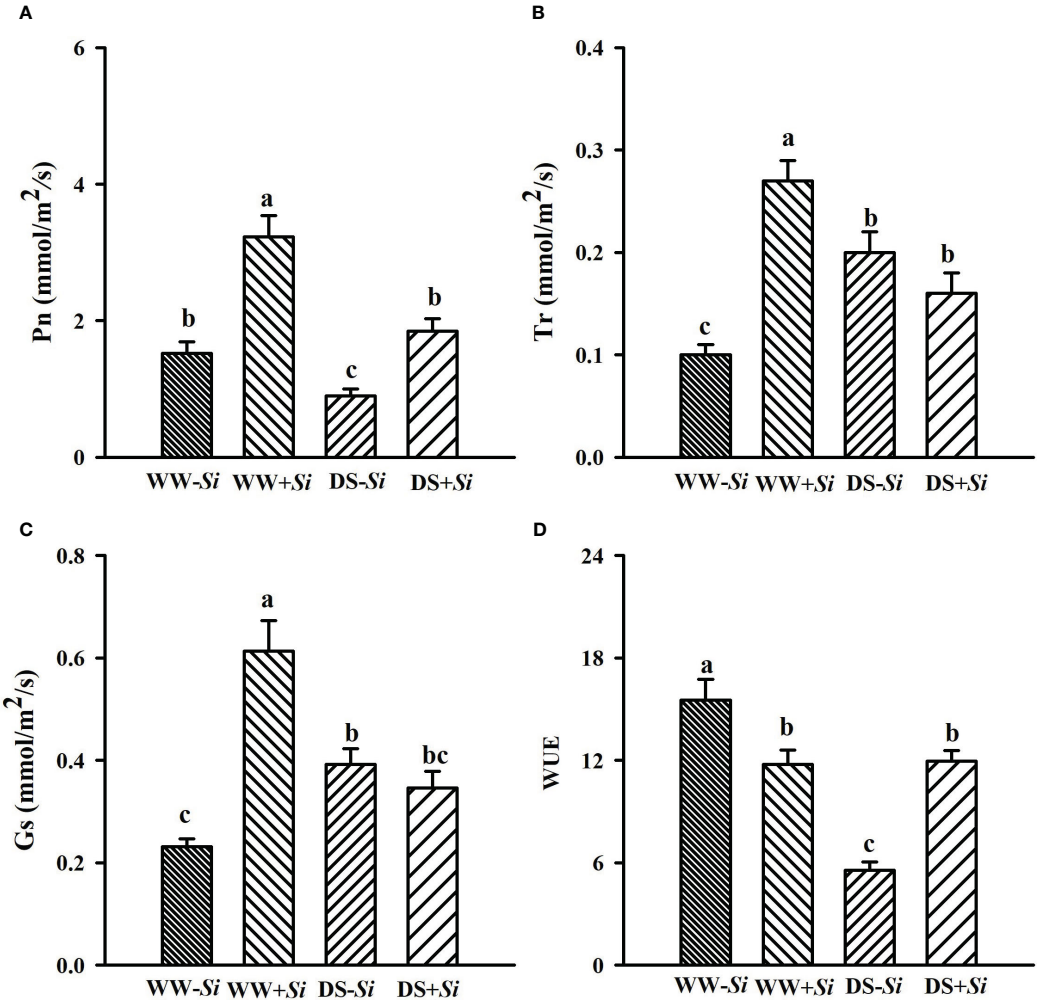

FIGURE 2
Effects of *Serendipita indica* (*Si*) on leaf net photosynthetic rate (Pn) **(A)**, transpiration rate (Tr) **(B)**, stomatal conductance (Gs) **(C)**, and water use efficiency (WUE) **(D)** of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 4) followed by different letters upon the bars indicate significant (p< 0.05) differences.

TABLE 2 Significance of variables in trifoliate orange seedlings inoculated with *Serendipita indica* (*Si*) under drought stress (DS) conditions.

Variables	DS	*Si*	Interaction	Variables	DS	*Si*	Interaction
Chi	*	**	NS	POD	*	**	**
Nbi	NS	**	**	GR	NS	*	NS
Pn	**	**	NS	APX	**	**	**
Tr	NS	**	**	*PtMn-SOD*	**	**	**
WUE	**	**	**	*PtFe-SOD*	**	**	**
Gs	NS	**	**	*PtCu/Zn-SOD*	**	**	**
H_2O_2	**	**	NS	*PtCAT1*	**	**	NS
O_2^-	**	**	**	*PtPOD*	**	**	**
MDA	**	**	NS	*PtFAD2*	**	**	NS
AsA	**	**	**	*PtFAD6*	**	**	**
GSH	**	**	NS	*PtΔ9*	**	**	**
CAT	**	**	NS	*PtΔ15*	**	**	**

NS, not significant at p< 0.05; *, p< 0.05; **, p< 0.01.

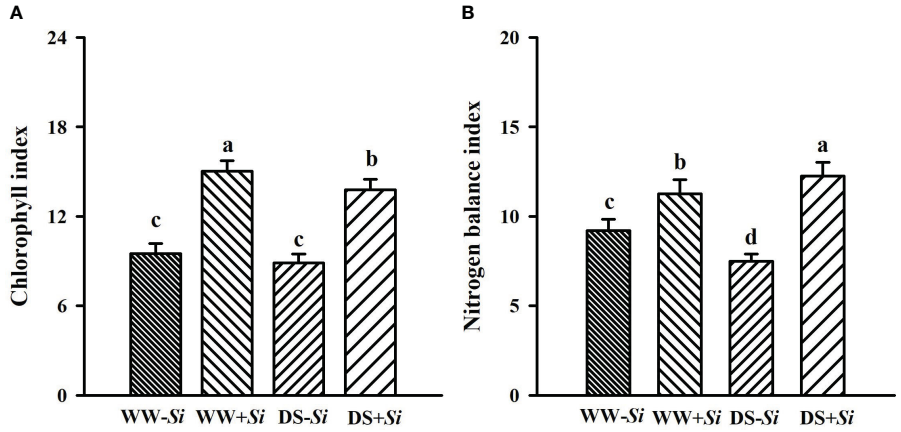

FIGURE 3
Effects of *Serendipita indica* (*Si*) on leaf chlorophyll index **(A)** and nitrogen balance index **(B)** in leaves of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 4) followed by different letters upon the bars indicate significant (p< 0.05) differences.

Effects of Si inoculation on leaf fatty acid desaturase genes expression under DS

The expression of *PtFAD2* gene in leaves of *Si*-inoculated plants was downregulated by 0.10-fold under DS versus WW conditions, accompanied by 0.35- and 0.86-fold upregulation of *PtFAD6* and *PtΔ15*, respectively (Figure 9). In leaves of no-*Si*-inoculated plants, the expression of *PtΔ9* gene was upregulated by 1.94- fold under DS versus WW conditions. Compared with no-*Si*-inoculated treatment, *Si* inoculation significantly raised the expression of leaf *PtFAD2*, *PtFAD6*, *PtΔ9*, and *PtΔ15* genes by 3.70-, 3.65-, 3.30-, and 1.18-fold under WW conditions, respectively, and by 4.94-, 8.52-, 0.63-, and 1.86-fold under DS conditions, respectively. DS treatment and *Si* inoculation interacted significantly to affect the expression of *PtFAD6*, *PtΔ9*, and *PtΔ15* genes (Table 2).

Discussion

In this study, root colonization frequency of *Si* in trifoliate orange seedlings was significantly increased under DS versus WW conditions, which is consistent with *Si*-colonized white clover under DS (Rong et al., 2022). *Si* was isolated from arid zones and is therefore well adapted to drought (Boorboori and Zhang, 2022). It has been shown that *Si* preferentially colonized the root-hair zone, and the colonization frequency of *Si* increased with root senescence (e.g., under drought conditions) (Schäfer and Kogel, 2009). Nevertheless, a decrease in root *Si* colonization was observed in wheat plants under DS versus WW conditions (Yaghoubian et al., 2014). In *Eleusin coracana* plants, DS also induced the decrease in root *Si* colonization (Tyagi et al., 2017). In maize, root *Si* colonization was not distinctly affected by DS (Xu et al., 2017). This suggests that the response of root *Si* colonization to DS is variable.

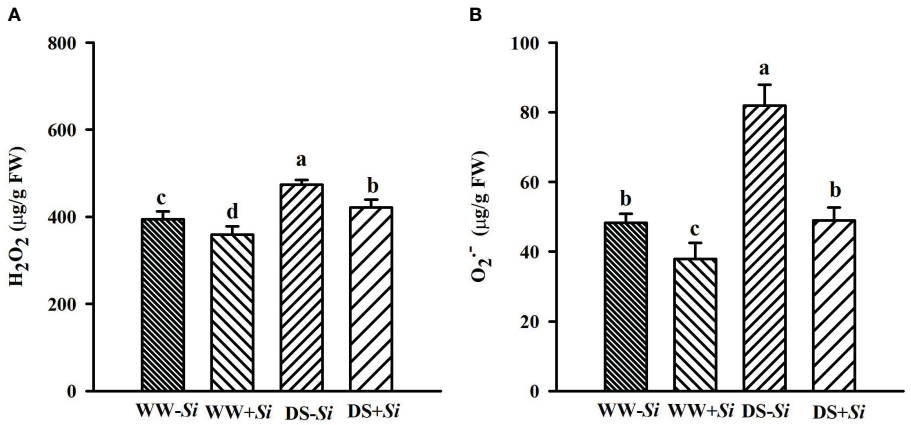

FIGURE 4
Effects of *Serendipita indica* (*Si*) on leaf hydrogen peroxide (H_2O_2) **(A)** and superoxide anion radical ($O_2^{\cdot-}$) **(B)** concentrations in trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 4) followed by different letters upon the bars indicate significant (p< 0.05) differences.

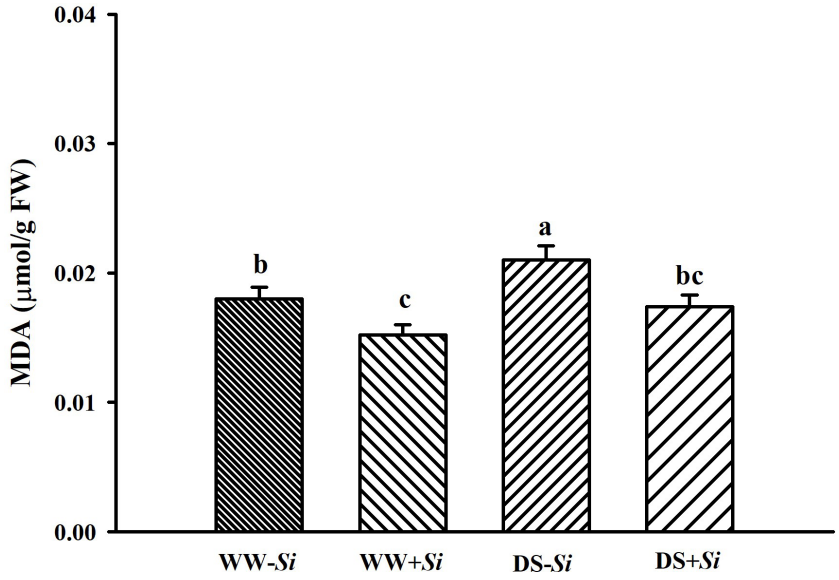

FIGURE 5
Effects of *Serendipita indica* (*Si*) on leaf malondialdehyde (MDA) concentrations in trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 4) followed by different letters upon the bars indicate significant (p< 0.05) differences.

Plants change root architecture in response to DS, with reduced lateral root density and allocating more nutrients to old roots (Lynch, 2018). Soil drought can strongly inhibit crop growth (Wahab et al., 2022). The present study also observed a decrease in plant growth variables under DS versus WW conditions, regardless of *Si* inoculation or not. However, *Si*-inoculated trifoliate orange seedlings represented greater plant growth performance and root surface area and volume, regardless of WW and DS. Similar results were reported in barley and wheat inoculated with *Si* under DS conditions (Hosseini et al., 2017; Ghaffari et al., 2019). Such changes may be linked to the fact that *Si* could promote the auxin and cytokinin synthesis of host plants (Liu et al., 2023; Rong et al., 2023).

Leaf gas exchange is closely linked to growth responses (Wu et al., 2019b). In the present study, DS treatment significantly reduced Pn, WUE, and Nbi in leaves of no-*Si*-inoculated seedlings, and Pn, Tr, Gs, Chi, and Nbi in *Si*-inoculated seedlings, compared with WW treatment. Interestingly, DS significantly raised Tr and Gs in no-*Si*-inoculated seedlings compared with WW treatment. This may be explained by the fact that prolonged DS irreversibly damages leaf tissues of no-*Si*-inoculated plants, thus accelerating Tr and Gs and leaving them in a more drought state (Yang et al., 2021b). In addition, *Si* application considerably raised Pn, Gs, Tr, Chi, and Nbi in WW-treated seedlings and Pn, Chi, Nbi, and WUE in DS-treated seedlings, compared with no-*Si*-inoculated treatment. This showed a significant improvement of WUE in *Si*-

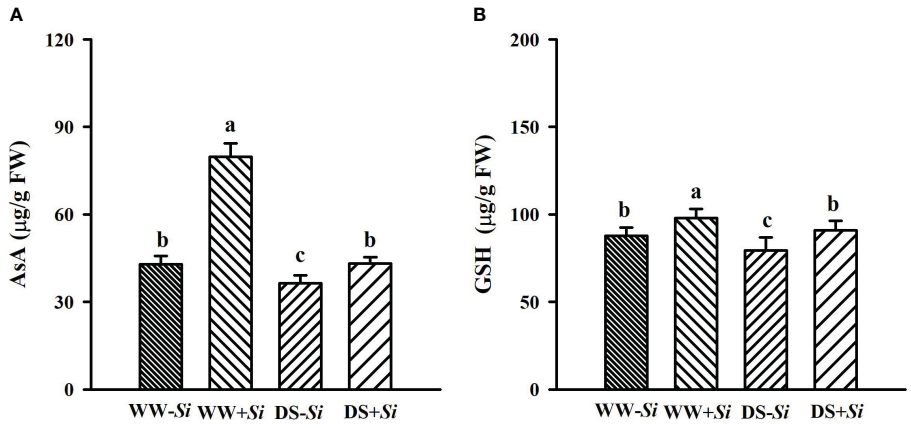

FIGURE 6
Effects of *Serendipita indica* (*Si*) on ascorbic acid (AsA) **(A)** and glutathione (GSH) **(B)** concentrations in leaves of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 4) followed by different letters upon the bars indicate significant (p< 0.05) differences.

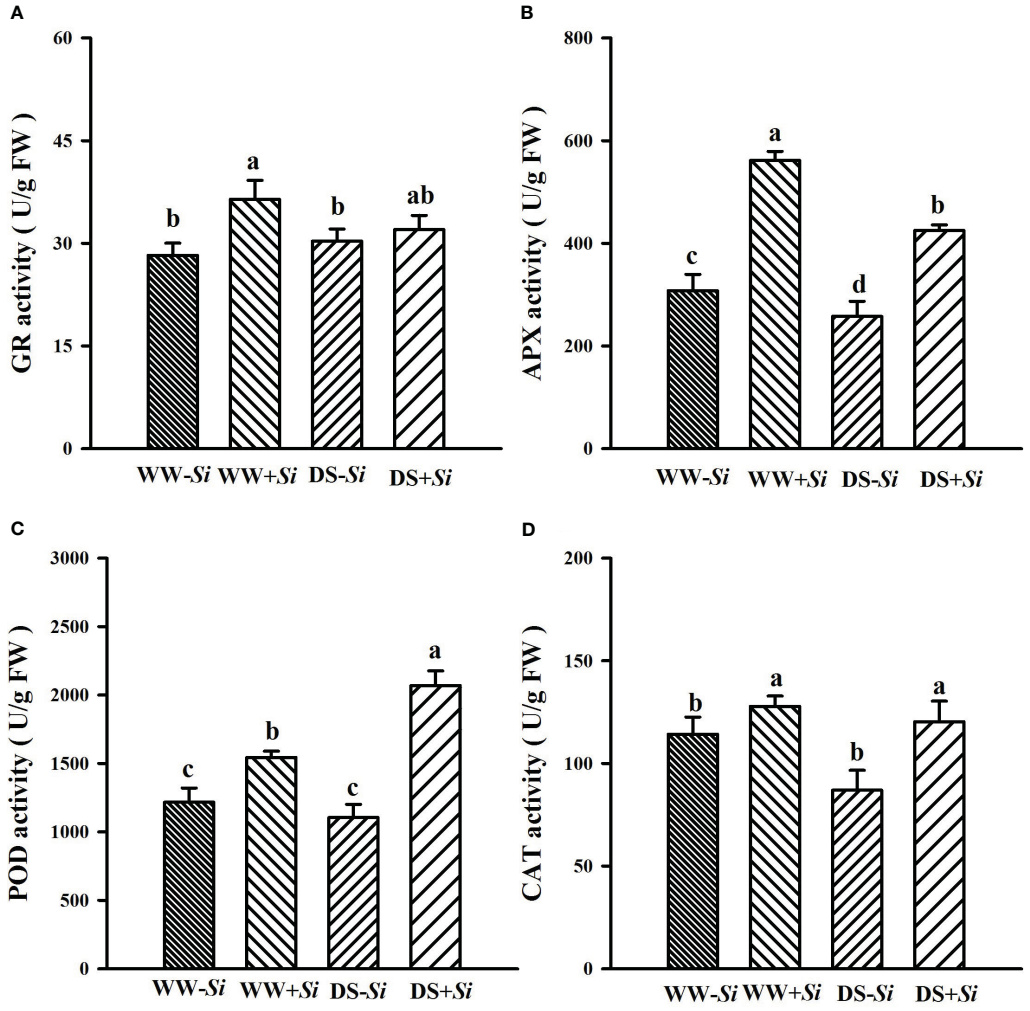

FIGURE 7
Effects of *Serendipita indica* (*Si*) on glutathione reductase (GR) **(A)**, ascorbate peroxidase (APX) **(B)**, peroxidase (POD) **(C)**, and catalase (CAT) **(D)** in leaves of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 4) followed by different letters upon the bars indicate significant (p< 0.05) differences.

inoculated seedlings only under DS conditions, which was related to the involvement of mycelium of *Si* in water uptake. On the other hand, the *Si* inoculation also enhanced Pn by promoting chlorophyll formation, accompanied by an enhancement of Nbi. Under DS, *Si*-inoculated rice plants also exhibited similar results (Saddique et al., 2018). In *Eleusine coracana* plants, *Si* inoculation distinctly raised chlorophyll levels under DS (Tyagi et al., 2017). These increases under both *Si* inoculation and DS conditions are associated with *Si*-promoted P uptake and photosystem II efficiency (Tariq et al., 2017). Li et al. (2021) also observed the raised Chi level in *Ipomoea batatas* plants after *Si* inoculation. Proteomics analysis showed that *Si* inoculation on barley led to significant upregulation of various photosynthesis-related protein levels under DS, including photosystem complex proteins and photorespiratory enzymes (Ghaffari et al., 2019).

In the present study, ROS levels were induced to increase, and MDA was elevated in *Si*- and no-*Si*-inoculated trifoliate orange seedlings under DS versus WW conditions, indicating that the drought triggered oxidative damage in trifoliate orange seedlings. Furthermore, inoculation with *Si* was able to significantly reduce leaf H_2O_2 and O_2^- levels as well as MDA concentrations, accompanied by a higher decrease under DS conditions than under WW conditions. Similar result was reported in maize plants under DS after *Si* inoculation (Kaboosi et al., 2023). MDA is a by-product of membrane lipid damage under DS (Pavlović et al., 2018). Sun et al. (2010) found that MDA levels in leaves of *Si*-colonized *B. campestris* plants in response to DS were delayed, coupled with the upregulation of antioxidant enzyme activities within 24 h. *Si* inoculation under DS also triggered a decrease in leaf ROS and MDA contents in *Triticum aestivum* and *Solanum melongena* (Yaghoubian et al., 2014; Swetha and Padmavathi, 2020). Therefore, *Si*-inoculated plants recorded lower oxidative burst and oxidative damage under drought, showing their enhanced drought tolerance. Nevertheless, *Si* colonization in walnut plants dramatically decreased MDA levels in leaves, but not roots under DS, suggesting a tissue dependency (Liu et al., 2021).

In plants, the AsA-GSH cycle, mediated by GR and APX, is associated with H_2O_2 scavenging (Irshad et al., 2021). Our study

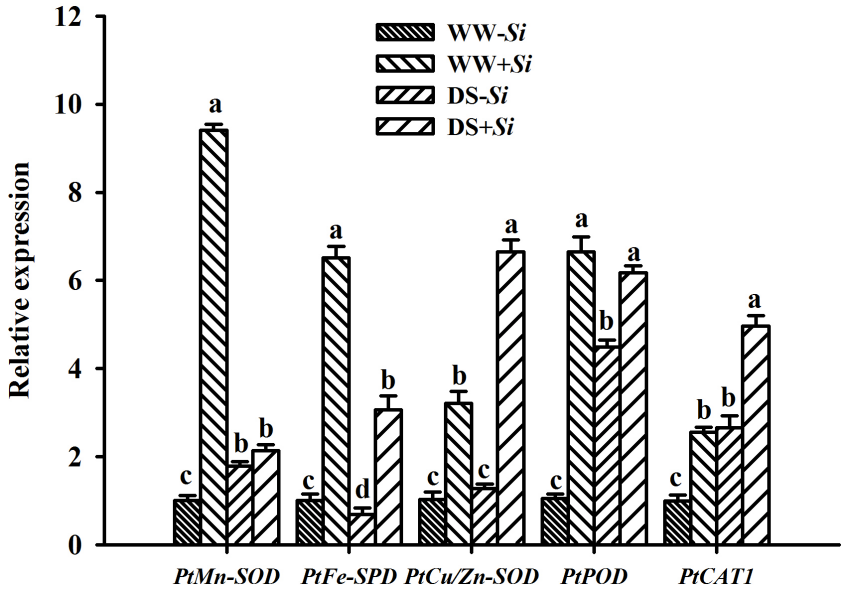

FIGURE 8
Effects of *Serendipita indica (Si)* on relative expression of five antioxidant enzyme genes in leaves of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 3) followed by different letters upon the bars indicate significant (p< 0.05) differences.

indicated that drought treatment markedly reduced leaf AsA and GSH levels, whereas inoculation with *Si* significantly increased leaf AsA and GSH levels, regardless of soil moisture regimes. Meanwhile, *Si*-inoculated seedlings also maintained a high APX activity under drought and a higher APX and GR activity under WW than no-*Si*-inoculated seedlings. This means that *Si*-inoculated plants have a more efficient AsA-GSH cycle to scavenge ROS under DS, which is in agreement with the results obtained by Rong et al. (2022) inoculating *Si* on white clover under DS. In *A. thaliana*, *Si* inoculation responded to DS by enhancing the AsA-GSH cycle pathway in plants (Sun et al., 2010). Under salt stress conditions, *Si* also provided tomato plants with a superior AsA-GSH cycle to

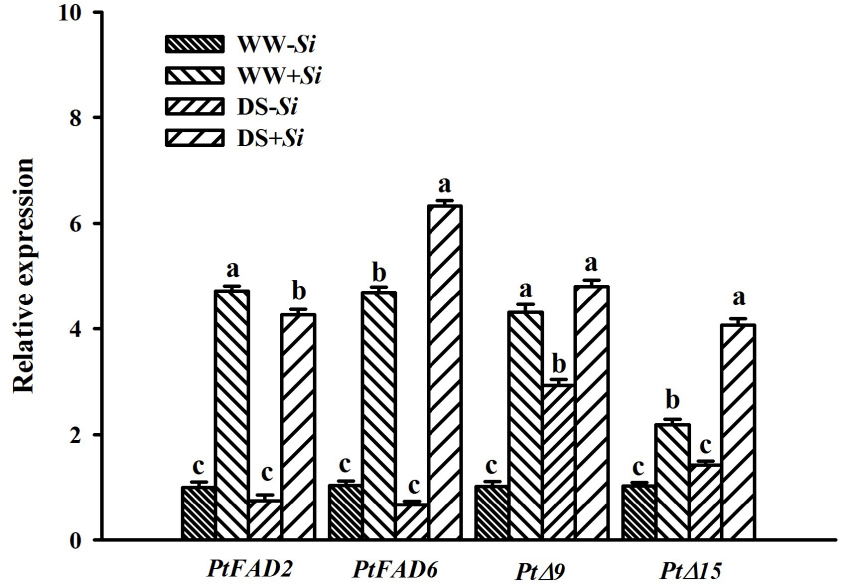

FIGURE 9
Effects of *Serendipita indica (Si)* on relative expression of four fatty acid desaturase genes in leaves of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, n = 3) followed by different letters upon the bars indicate significant (p< 0.05) differences.

eliminate ROS (Ghorbani et al., 2018), suggesting that *Si* plays an important role in modulating the AsA-GSH cycle under adversity.

This study also represented enhanced CAT and POD activities after *Si* inoculation under WW and DS. It has been demonstrated that *Si* colonization raised antioxidant enzyme activities in host plants including CAT and POD (Lin et al., 2019). Under drought, *Si* inoculation also enhanced the CAT activity of *I. batatas* (Li et al., 2021). Wheat inoculated with *Si* exhibited lower levels of lipid peroxidation as well as higher CAT and APX activities under DS (Yaghoubian et al., 2014). *Si* inoculation, on the other hand, reduced CAT activity of drought-stressed wheat and APX activity of drought-stressed wheat and maize (Hosseini et al., 2017; Hosseini et al., 2018). This indicated that *Si* effects on antioxidant enzyme activities are variable. Alternatively, *Si* inoculation activates drought-escape mechanisms in host plants, thereby doing not require enhanced antioxidant enzyme activities in response to drought (Jangir et al., 2021). In follow-up studies, we should explore how the *Si* activates the signaling pathway of antioxidant enzyme system in host plants subjected to DS.

Inoculation with *Si* also altered the expression of genes encoding antioxidant enzymes and fatty acid desaturases under DS, with increased expression in leaf *PtFe-SOD*, *PtCu/Zn-SOD*, *PtPOD*, *PtCAT1*, *PtFAD2*, *PtFAD6*, *PtΔ9*, and *PtΔ15* genes. Similarly, inoculation of *Rhizophagus irregularis* upregulated leaf *PpGR*, *PpMn-SOD*, and *PpCu/Zn-SOD* expression of *Robinia pseudoacacia* plants under 200 mM NaCl conditions, but not 100 mM NaCl (Chen et al., 2020). Wu et al. (2019a) also reported that *Funneliformis mosseae* inoculation upregulated root *PtFAD2*, *PtFAD6*, and *PtΔ9* gene expression in trifoliate orange under DS. In field citrus, *Si* inoculation also upregulated the expression of *CsPOD*, *CsCAT1*, and *CsFAD6* in leaves (Li et al., 2022). This suggests that even in the absence of abiotic stress, *Si* can activate the expression of antioxidant defense genes in host plants. In leaves of *B. campestris* and maize, *Si* inoculation also upregulated the expression of stressed genes (*DREB2A*, *CBL1*, *ANAC072*, and *RD29A*) under soil drought (Sun et al., 2010; Xu et al., 2017). In *Gerbera jamesonii* seedlings, *Si* inoculation also triggered the upregulated expression of *NHX2* and *SOS1* under salt stress (Chen et al., 2022). Sun et al. (2010) proposed that the Ca^{2+} sensing regulatory protein could activate *Si* to induce drought-responsive gene expression. However, whether this case occurred in this study remains to be verified.

Conclusions

In summary, *Si* inoculation alleviated the inhibitory effect of soil drought on growth, Pn, WUE, and Chi of trifoliate orange seedlings, as well as the oxidative damage. This study firstly reported that low oxidative burst in *Si*-inoculated seedlings exposed to soil drought was associated with increased antioxidant enzyme activities and antioxidant levels, as well as upregulated expression of genes encoding antioxidant enzymes and fatty acid desaturases. *Si* has a high potential as a biostimulator for enhanced plant drought tolerance.

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

Conceptualization, Q-SW and J-LC; data curation, J-LC and YW; methodology, J-LC. resources, Q-SW; supervision, Q-SW; writing—original draft, YW; writing—review and editing, AH, EA, and Q-SW. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Plan in Scientific and Technological Innovation Team of Outstanding Young Scientists, Hubei Provincial Department of Education (T201604). The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP2023R356), King Saud University, Riyadh, Saudi Arabia.

Acknowledgments

The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP2023R356), King Saud University, Riyadh, Saudi Arabia.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1247342/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 24 October 2023
DOI 10.3389/fpls.2023.1278990

OPEN ACCESS

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RECEIVED 17 August 2023
ACCEPTED 09 October 2023
PUBLISHED 24 October 2023

CITATION
Guigard L, Jobert L, Busset N, Moulin L and Czernic P (2023) Symbiotic compatibility between rice cultivars and arbuscular mycorrhizal fungi genotypes affects rice growth and mycorrhiza-induced resistance. *Front. Plant Sci.* 14:1278990. doi: 10.3389/fpls.2023.1278990

Symbiotic compatibility between rice cultivars and arbuscular mycorrhizal fungi genotypes affects rice growth and mycorrhiza-induced resistance

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Introduction: Arbuscular mycorrhizal fungi (AMF) belong to the Glomeromycota clade and can form root symbioses with 80% of Angiosperms, including crops species such as wheat, maize and rice. By increasing nutrient availability, uptake and soil anchoring of plants, AMF can improve plant's growth and tolerance to abiotic stresses. AMF can also reduce symptoms and pathogen load on infected plants, both locally and systemically, through a phenomenon called mycorrhiza induced resistance (MIR). There is scarce information on rice mycorrhization, despite the high potential of this symbiosis in a context of sustainable water management in rice production systems.

Methods: We studied the symbiotic compatibility (global mycorrhization & arbuscules intensity) and MIR phenotypes between six rice cultivars from two subspecies (*indica*: IR64 & Phka Rumduol; *japonica*: Nipponbare, Kitaake, Azucena & Zhonghua 11) and three AMF genotypes (*Funneliformis mosseae* FR140 (FM), *Rhizophagus irregularis* DAOM197198 (RIR) & *R. intraradices* FR121 (RIN)). The impact of mycorrhization on rice growth and defence response to *Xanthomonas oryzae* pv *oryzae* (Xoo) infection was recorded via both phenotypic indexes and rice marker gene expression studies.

Results: All three AMF genotypes colonise the roots of all rice varieties, with clear differences in efficiency depending on the combination under study (from 27% to 84% for Phka Rumduol-RIN and Nipponbare-RIR combinations, respectively). Mycorrhization significantly (α=0.05) induced negative to beneficial effects on rice growth (impact on dry weight ranging from -21% to 227% on Azucena-FM and Kitaake-RIN combinations, respectively), and neutral to beneficial effects on the extent of Xoo symptoms on leaves (except for Azucena-RIN combination which showed a 68% increase of chlorosis). *R. irregularis* DAOM197198 was the most compatible AMF partner of rice, with high root colonisation intensity (84% of Nipponbare's roots hyphal colonisation), beneficial effects on rice growth (dry weight +28% (IR64) to +178% (Kitaake)) and decrease of Xoo-induced symptoms

(-6% (Nipponbare) to -27% (IR64)). Transcriptomic analyses by RT-qPCR on leaves of two rice cultivars contrasting in their association with AMF show two different patterns of response on several physiological marker genes.

Discussion: Overall, the symbiotic compatibility between rice cultivars and AMF demonstrates adequate colonization, effectively restricting the nutrient starvation response and mitigating symptoms of phytopathogenic infection.

KEYWORDS

Oryza sativa, plant-fungi interactions, biological control, *Xanthomonas oryzae*, symbiotic association, biotic stress

Introduction

In recent years, there has been a growing interest in the naturally occurring interactions between plants and the inhabitants of their root microbiome. It has been widely reported that this cohort of microorganisms plays a role in the growth of their host and in its tolerance to biotic and abiotic stresses (Berendsen et al., 2012; Schlaeppi and Bulgarelli, 2015; Vannier et al., 2019; de la Fuente Cantó et al., 2020). These include arbuscular mycorrhizal fungi (AMF), which form a mutualistic association with the roots of various crops such as wheat, maize or rice. (Paszkowski and Boller, 2002; Suzuki et al., 2015; Fiorilli et al., 2018).

The establishment of this symbiosis is mediated by a molecular dialogue between the partners, via the exudation of strigolactones by the plant and the recognition of fungal Myc factors (lipochitooligosaccharides or short-chain chitin oligomers) (Mbodj et al., 2018; Ho-Plágaro and García-Garrido, 2022). When in contact with the root, the hyphae changes into an adhesive structure named hyphopodia, enabling the access to the internal root cortex. It spreads *via* intercellular spaces and colonise cortical cells with highly branched intracellular structures named arbuscules, preferential sites of exchange with their host (Gutjahr et al., 2008; Gutjahr et al., 2009; Jung et al., 2012; Liu et al., 2022). Their number and functioning in a plant root system is recognised as a marker of symbiotic compatibility (Montero et al., 2019).

During these exchanges, plants retribute up to 30% of their produced photosynthates to the fungi in the form of sugars and lipids (Jung et al., 2012; Sugiura et al., 2020). In return, mycorrhizal fungi provide a multitude of beneficial effects. The mere presence of the fungi within cortical root cells can enhance shoot and root biomass, especially stimulating the lateral roots formation and development in wheat, rice or maize (Oláh et al., 2005; Gutjahr et al., 2009; Chiu et al., 2018; Fiorilli et al., 2018). Its hyphal network enables it to cover a large area of soil, mineralising and recovering essential and/or poorly bioavailable nutrients or water for the development of its host (Begum et al., 2019; Kadam et al., 2020). Particularly, phosphorus is a key nutrient in AMF symbiosis with plants (Smith et al., 2011). Its bioavailability (or lack thereof due to complexation with soil particulates) affects the recruitment and functioning of the fungal association with its host (Breuillin et al., 2010; Jiang et al., 2021). Mycorrhization impacts inorganic phosphate (Pi) responsive genes expression in multiple plant species, such as rice or wheat, suppressing for instance the Pi starvation response typically occurring in low Pi soils (Yang et al., 2012; Fiorilli et al., 2018; Campo and San Segundo, 2020). In addition to improving the mineral nutrition of the plant, the AMF symbiosis can help its host tolerate a wide range of stresses. It can increase its host's tolerance to a variety of abiotic stresses, from drought to excessive temperature, or reduce root uptake of heavy metals (de Andrade et al., 2015; Begum et al., 2019; Chen et al., 2019). AMF symbioses also enhance their host's tolerance to pathogen pressure at two complementary levels. Fungal root colonisation protects the host both by competing with soil pathogens for its photosynthates and colonisation sites and by triggering a local defence response (accumulation of callose, ROS, phenols and R proteins) (Schouteden et al., 2015; Gupta et al., 2017; Dowarah et al., 2021). By modulating phytohormonal pathways such as jasmonate, salicylic acid and ethylene, AMF primes its host's defence responses in the shoot via a mechanism called mycorrhiza-induced resistance (MIR) (Jung et al., 2012; Gupta et al., 2017; Fiorilli et al., 2018; Nishad et al., 2020). This MIR results in reduced foliar symptoms and control of pathogen development on a variety of plants and shoot pathogens (Liu et al., 2007; Fiorilli et al., 2018; Kadam et al., 2020). Due to their ability to improve soil fertility and plant health, AMF have great potential as plant bioinoculants in the field.

This potential is as promising as AMF are generally known to have low host specificity, capable to induce growth of a multitude of different crops (Van Geel et al., 2016). However, recent meta-

Abbreviations: A, arbuscules; AMF, arbuscular mycorrhizal fungi; CT, non-mycorrhizal control plants, mock-inoculated with the granular inoculum without any fungal spores; Eh, external hyphae; ET, ethylene; F., *Funneliformis*; FM, *Funneliformis mosseae* FR 140; Hy, hyphopodia; Ih, intercellular hyphae; JA, jasmonic acid; MIR, mycorrhiza-induced resistance; P., *Pyricularia;* Pi, inorganic Phosphate; R., *Rhizophagus*; RT-qPCR, real-time quantitative polymerase chain reaction; RIN, *Rhizophagus intraradices* FR 121; RIR, *Rhizophagus irregularis* DAOM 197198; SA, salicylic acid; Sp, spores; V, vesicules; Xoo, *Xanthomonas oryzae* pv *oryzae* PXO99.

analyses and studies highlighted that there exists different symbiotic compatibilities between AMF species and crop cultivars since different associations results in different phenotypic observations (Pérez-de-Luque et al., 2017; Campos et al., 2018; Silva et al., 2018). A meta-analysis on 115 studies showed that some specific associations between AMF genera and plant host families are more efficient for crop growth promotion, such as the *Poacees* family with the AMF genera *Funneliformis* and *Rhizophagus* (Van Geel et al., 2016). Crop responsiveness to mycorrhization is indeed plant genotype-dependent, as well as AMF species-dependent and is positively linked with AMF colonisation (Lehmann et al., 2012). This meta-analysis explained that the relationship between crop mycorrhizal response and AMF colonisation wasn't significant for wheat or barley, while recent studies have underlined differences in symbiotic compatibility between rice genotypes, both in terms of AMF colonisation and its effect on rice growth (Suzuki et al., 2015; Diedhiou et al., 2016; Davidson et al., 2019).

Rice typical watering mode is flooding, but it has major drawbacks: monopolising a third of the world's freshwater, high levels of methane production, soil polluting streaming of chemical inputs and negative impact on AMF development (Redeker et al., 2000; Chandel et al., 2002; Saito et al., 2018; Chialva et al., 2020). As a substitute to constant flooding, Alternate Wetting and Drying (AWD) rice management practices have been developed, reducing water use by up to 30% and methane emissions by 48% without reducing yield (Richards and Sander, 2014; LaHue et al., 2016). Rice varieties that are AMF-responsive should therefore be selected in fields that are being converted from flooded to AWD rice systems.

Within these paddy fields, initial studies showed little or no colonisation of different rice varieties under flooded conditions (Lumini et al., 2011; Vallino et al., 2014), but recent ones have reported AMF colonisation in experimental and farmers' fields around the world, especially in rainfed lowland systems (Chialva et al., 2020; Sarkodee-Addo et al., 2020; Barro et al., 2022). There are few studies on the natural occurrence of AMF communities and their diversity in rice paddy fields (Wang et al., 2015; Bernaola et al., 2018a; Zhang et al., 2020; Wang et al., 2021). AMF communities rely on the site and the irrigation mode and *Glomerales, Archaeosporales* and *Diversisporales* are generally the predominant orders (Lumini et al., 2011; Chialva et al., 2020; Barro et al., 2022). Members from the *Glomeraceae, Claroideoglomeraceae* and *Paraglomeraceae* families have been found in paddy fields in China and Ghana (Wang et al., 2015; Sarkodee-Addo et al., 2020), including the well-studied *Rhizophagus irregularis* and *Funneliformis mosseae* species. Another analysis on fragrant black rice in Indian fields identified *R. intraradices* and *F. mosseae* in both field and rice root samples (Surendirakumar et al., 2021). Their global distribution in various fields, long-term storage ability, and ability to form symbiosis with a wide range of plant hosts make them excellent models for studying AMF symbiosis (Berruti et al., 2016).

Under greenhouse conditions, there is evidence that rice mycorrhization can improve plant biomass, yield and tolerance to multiple abiotic and biotic stresses (Gutjahr et al., 2009; Campos-Soriano et al., 2012; Li et al., 2016; Campo et al., 2020). These beneficial effects depend on rice developmental stage, variety and AMF genotype (Suzuki et al., 2015; Sisaphaithong et al., 2017; Wang et al., 2021). Surprisingly, studies on the global effect of AMF symbiosis on both rice's growth and defence responses are scarce or limited to a few combinations of rice cultivars and AMF species. Studies by Campos-Soriano & San-Segundo showed that the mycorrhization of a single *japonica* variety, Senia, by the AMF model *R. intraradices* enhances its biomass and resistance to *Pyricularia (P.) oryzae*, both locally and systemically (Campos-Soriano et al., 2010; Campos-Soriano et al., 2012). Two AMF species, *R. irregularis* and *F. mosseae*, inoculated on 12 *japonica* rice varieties showed contrasted effects on rice growth, Pi content in leaves, and resistance to *P. oryzae* infection (Campo et al., 2020). A multi-AMF species inoculant on another two tropical *japonica* varieties also increases their growth but at the same time their susceptibility to insect attacks and *Rhizoctonia solani* infection (Bernaola et al., 2018b). Finally, a large study by Suzuki et al., 2015 showed a range of impact (from improvement to deterioration) of *F. mosseae*'s inoculation on the biomass of 64 rice genotypes.

In order to develop AMF bioinoculants for rice production under AWD conditions, it is necessary to deepen our understanding of the symbiotic compatibility between rice and AMFs. This will assess which combinations are beneficial, negligible, or detrimental to plant growth and responses to environmental stresses.

In this study, the symbiotic compatibility between six varieties of *japonica* and *indica* rice and three AMF genotypes, known to interact with rice, was characterised. Model rice varieties as well as varieties with potential for AWD programs were targeted. We analysed the colonisation rate and intensity as well as the functioning of the interaction between rice and mycorrhizal fungi. We then assessed how AMF inoculation impacts rice's growth and defence responses to a pathogenic infection by *Xanthomonas oryzae* pv. *oryzae (*Xoo). We used a combination of phenotypic and rice gene expression studies to uncover promising compatible associations. How phenotypic responses of rice to AMF symbiosis can be linked to systemic changes in marker gene expression (ranging from growth, phytohormonal balances to defence response) was investigated in the leaves of two rice model cultivars contrasting in AMF establishment and responses.

Materials and methods

Plant and fungal material

Six *Oryza sativa* cultivars and three AMF genotypes belonging to three different species were selected and their characteristics are listed in Table 1. Two *indica* (IR64 and Phka Rumduol) and four *japonica* (Azucena, Kitaake, Nipponbare and Zhonghua 11) subspecies were selected. Seeds were obtained from IRRI and propagated at IRD except for Phka Rumduol which was provided by CIRAD.

TABLE 1 Rice cultivars and AMF genotypes used in this study.

Plant/Fungal Material	Characteristics	Reference
Oryza sativa subsp. *japonica*		
Nipponbare	*japonica* reference, high-quality sequenced genome, AMF-responsive, drought sensitive	Gutjahr et al., 2008; Matsumoto et al., 2016; Degenkolbe et al., 2009
Kitaake	model for rice transformation, short cycle, not light sensitive, AMF-responsive, drought tolerant to a certain extent	Jain et al., 2019; Mubarok et al., 2019; Shi et al., 2021
Azucena	short cycle, not light sensitive, sensitive to phytoparasitic nematodes, not yet tested on AMF symbiosis drought tolerant	Masson et al., 2022; Ghorbanzadeh et al., 2023
Zhonghua 11	short cycle, not light sensitive, resistant to phytoparasitic nematodes, widely used in China for T-DNA mutant sources, AMF-responsive drought sensitive	Phan et al., 2018; Huang et al., 2020; Masson et al., 2022; Nguyen et al., 2022; Xiao et al., 2009
Oryza sativa subsp. *indica*		
IR64	*indica* reference, high yield quality, sensitive to nematodes, AMF-responsive, drought sensitive	Suzuki et al., 2015; Mackill and Khush, 2018; Phan et al., 2018; Ghorbanzadeh et al., 2023
Phka Rumduol	jasmine premium rice, highly cultivated in Cambodia, not yet tested on AMF symbiosis drought sensitive	Masson et al., 2022; Zhao et al., 2016
AMF genotypes		
Funneliformis mosseae FR140	Colonise a large variety of hosts including rice, worldwide presence in fields, induce MIR in rice against *Pyricularia* (P.) *oryzae* and in wheat against *Xanthomonas oryzae*.	Vos et al., 2012; Suzuki et al., 2015; Berruti et al., 2016; Fiorilli et al., 2018; Campo et al., 2020.
Rhizophagus intraradices FR121	Colonise a large variety of hosts including rice, worldwide presence in fields, induce MIR in rice against *P. oryzae*	Gutjahr et al., 2008; Campos-Soriano et al., 2012; Berruti et al., 2016.
Rhizophagus irregularis DAOM197198	Colonise a large variety of hosts including rice, long-term storage, worldwide presence in fields, sequenced genome, induce MIR in rice against *P. oryzae*.	Stockinger et al., 2009; Tisserant et al., 2013; Berruti et al., 2016; Campo et al., 2020.

Funneliformis mosseae FR140 (FM), *Rhizophagus intraradices* FR121 (RIN) and *Rhizophagus irregularis* DAOM 197198 (RIR) were purchased from MycAgro Lab (Technopôle Agro-Environnement, Bretenière, France) in the form of individual granular inoculums (100 spores/g).

Plant growth conditions

Rice seeds were dehusked and surface-sterilised by immersion in 70% ethanol for 3 min, then in 3.8% sodium hypochlorite supplemented with 1% Tween 20 under agitation (180 rpm) for 30 min. Seeds were rinsed three times with sterile water, three times with 2% filtered sodium thiosulfate and three more times with sterile water. They were incubated overnight at 28°C in sterile water in the dark and then germinated on sterile-soaked sand for three days at 28°C. To ensure the absence of contaminants, 100 µL of the last rinse water and imbibition water were plated on tryptic soy agar (Sigma-Aldrich) Petri dishes. Four homogeneous rice seedlings were then transferred to anti-coiling pots (Comptoir Vert, France) filled with 150 mL of clay beads (6-18 mm, Terres & Traditions, France) and 450 mL of sterile inert substrate composed of 70% of sand, 20% sieved perlite and 10% of vermiculite (Campo & San Segundo, 2020). This substrate was inoculated with either the AMF granular inoculum, or the granular inoculum without fungal spores (control) at a volume of 5% per pot.

Rice plants were grown for 2.5 months in a growth chamber (12 h day/night, 28°C day, 26°C night, 75% humidity). The substrate was moistened for one week and then watered three times a week with a Hoagland solution (Hoagland and Arnon, 1938) reduced in phosphate (2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1 mM $MgSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 µM KH_2PO_4 and trace elements, complete recipe in Supplementary Table 1), with the watering volume gradually increased according to the cultivar growth.

Rice growth phenotyping

Maximum height, shoot and root fresh weights were measured for each plant (n = 20 per condition). Shoot dry weight was also measured after drying at 40°C for one week at 48 h (n = 20 par condition). Roots were stored in 70% ethanol at 4°C until mycorrhizal quantification (n= 5 pools of 4 root systems).

Mycorrhizal quantification

The root systems of four plants from the same pot were washed in tap water, placed in 70% ethanol and stored at 4°C until analysis. Fungal structures were stained using a blue ink-based protocol modified from Cao et al., 2013. Roots were heated to 80°C for 45 min in 10% KOH. They were rinsed three times with ultrapure water (MilliQ) and stained with a staining solution consisting of 5% blue ink (Waterman "Bleu Sérénité") in 5% acetic acid at room temperature for 10 min. They were then rinsed three times with ultrapure water and fixed in 5% acetic acid overnight.

Five replicates of 20 to 25 fragments from coronary roots were mounted between slide and glass and the mycorrhizal index (global mycorrhization and arbuscular intensity) were assessed as in Trouvelot et al., 1986, on a Axiozoom Zeiss microscope.

Biocontrol assays against *Xanthomonas oryzae* pv *oryzae*

Infection of *Xanthomonas oryzae* pv. *oryzae* PXO99 (Xoo) was carried out on 50-days old rice plants by leaf clipping as described in Niño-Liu et al., 2005. The extent of chlorosis and necrosis was assessed 14 days post inoculation (dpi) on each leaf (n = 18). Mock inoculations were made with sterile water to assess that the sole clipping of the leaf does not induce any disease symptoms.

RNA extraction and quantitative PCR of rice gene expression

RNA was extracted from leaf samples collected during leaf-clipping (before infection, at 50 days post germination). They were ground to a fine powder using a TissueLyser II (Retsch) at 30 Hz, for 15 s twice. Each biological replicate consisted of two (Nipponbare) to three (IR64) leaf samples from the same pot, with four biological replicates for each condition. Total RNA was extracted using TriReagent (Sigma) and a DNAse (QIAgen) treatment was added in the protocol before purification with the RNA Clean & Concentrator kit (Zymo), according to the manufacturer's instructions. The quantity and quality of total RNA was assessed using a NanoDrop 1000 spectrophotometer (ThermoFisher). Approximately 360 ng of total RNA from each biological replicate was used for retrotranscription into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). cDNAs were diluted 5-fold and RT-qPCRs were performed using the Takyon™ Low ROX SYBR 2X MasterMix blue dTTP (Eurogentec) on a LightCycler 480 qPCR system (Roche). Plate preparation was automated using the epMotion 5070 pipetting robot (Eppendorf). Four independent biological replicates were analysed for each condition, each one analysed in triplicate. Relative gene expression is calculated by comparing each sample to the standard's (number of cycles of EF1a, ΔCt), and then to the control group (FM, RIN & RIR vs. CT, $\Delta\Delta Ct$). The fold change is calculated with $2^{-mean\Delta\Delta Ct}$ and the logFC represents the relative expression of each marker gene as indicated in (Pfaffl, 2001). The list of marker genes, their function and the primers used in this study are listed in Table 2.

Statistical analyses

Statistical analyses were performed with Rstudio (version 2022.2.0.443) and R (version 4.1.3) softwares using the packages "readxl", "tidyverse", "ggplot2", "rstatix", "ggpubr", "multicompView", "car", "pcr", "pheatmap". As phenotypic data of mycorrhization and arbuscular intensity indexes fulfilled normality (Shapiro test, $p>0.05$) and homoscedasticity (Levene test, $p>0.05$) hypotheses, a two-way ANOVA was used to test the significance of rice cultivar and AMF genotype effects on these parameters, and one-way ANOVA with Tukey test were used for pairwise group comparisons. For rice growth and biocontrol traits, these data did not fulfil the normality and homoscedasticity hypotheses, thus a non-parametric ANOVA with Kruskal-Wallis test ($\alpha=0.05$) followed by pairwise comparisons with Wilcoxon tests ($\alpha=0.05$) were used for mean group comparisons of measured phenotypic traits.

For the analysis of the expression of rice marker genes, the "pcr" package was used. Linear regression was used to assess statistical differences between AMF inoculation and marker gene expression. To visualise the relative expression of each marker gene as a function of rice cultivars and AMF inoculation, a heatmap of LogFC was made with the "pheatmap" package.

Results

Mycorrhizal colonisation and arbuscular content in AMF-rice *japonica* and *indica* rice cultivars

To assess the symbiotic compatibility between the three AMF genotypes (RIN, RIR, FM) and the six rice cultivars (listed in Table 1), we analysed the mycorrhization rates in the 18 combinations. We focused on the global fungal colonisation rate and the percentage of visible arbuscules in the mycorrhizal roots (see Material & Methods). The use of mycorrhizal index of global mycorrhization and arbuscular intensity allowed us to quantify the interaction between AMF and rice. The mean of each mycorrhizal index for each combination are presented in Figure 1 and in Supplementary Table 2.

We tested whether rice cultivar and AMF genotype have an impact on rice's global mycorrhization and arbuscular intensity index, with a two-way ANOVA test. Both indexes are statistically significantly affected by either rice cultivar or AMF genotype (Two-Way ANOVA, $F = 66.91593$, $p< 2.10^{-6}$; $F = 4.9315$, $p< 0.01$).

All the rice cultivars tested were root colonised by each AMF genotype (Figure 1; Supplementary Table 2). Each fungal organ (hyphal structures, spores, vesicles and arbuscules) was clearly visible on each combination as shown in Figure 2 and Supplementary Figure 1. The global intensity of mycorrhization ranged from 27.20% (Phka Rumduol with RIN) to 83.90% (Nipponbare with RIR). Independently of the fungal inoculation, *indica* rice varieties have the less intense symbiotic percentage, ranging from 27.20% (Phka Rumduol - RIN) to 46.8% (IR64 - RIN) (Supplementary Table 2). The percentage of mycorrhization of the *japonica* cultivars ranges from 43.20% (Kitaake with FM) to 83.90% (Nipponbare with RIR). Nipponbare is the most intensely mycorrhized cultivar with 79%, 77.90% and 83.90% for FM, RIN and RIR inoculation, respectively (Supplementary Table 2).

The arbuscular percentage of the mycorrhizal roots ranged from 3.22% (Azucena with FM) to 49.40% (Nipponbare with RIR)

10.3389/fpls.2023.1278990

TABLE 2 Rice marker genes used in RT-qPCR expression studies.

Gene name	Annotation RAPDB	Forward primer (5′-3′)	Reverse primer (5′-3′)	References
Reference gene				
OsEF-1A	Os03g0177400 - Rice elongation factor 1A	GAAGTCTCATCCTACCTGAAGAAG	GTCAAGAGCCTCAAGCAAGG	Petitot et al., 2017
Defence response				
OsWRKY30	Os08g0499300 - WRKY transcription factor, Disease resistance against X. Oryzae, Drought tolerance	ATGGCTGTCTGTCAGAGAGGATG	CAGTGGTAGGAGAAGGTTGTGC	Ryu et al., 2006
OsMPK10	Os01g0629900 - Similar to Blast and wounding induced mitogen-activated protein kinase	TCAACTCCAATTCCTGCCAAG	AACAACTCTTCCTGGTCTTGC	Nguyễn et al., 2014
OsPAL4	Os02g0627100 - Phenylalanine ammonia-lyase, Broad spectrum disease resistance	CCTCGCCATCGCTGCCATC	GCCGTTGTTGTAGAAGTCGTTCAC	Petitot et al., 2017
OsPR5	Os12g0628600 - Similar to Thaumatin-like pathogenesis-related protein 3 precursor	CGCTGCCCCGACGCTTAC	ACGACTTGGTAGTTGCTGTTGC	Delteil et al., 2012
OsTGAP1	Os04g0637000 - TGA-type bZIP Transcription Factor, Regulation of diterpenoid phytoalexin production, Defence response	ATGGCCAGTGAAGGATGAAG	CTCTTGTGCCCACATCAGAA	Okada et al., 2009
OsDXS3	Os07g0190000 - Similar to 1-deoxy-D-xylulose 5-phosphate synthase 2 precursor	TGTTCTTGCCAGACAGGTAC	GTCGGCTGATGTGTATATGC	Valette et al., 2020
Hormone (SA)				
OsNPR1	Os01g0194300 - Ankyrin-repeat protein, Herbivore-induced defence response, Blast disease resistance	AGAAGTCATTGCCTCCAG	ACATCGTCAGAGTCAAGG	Kumari et al., 2016
OsWRKY45	Os05g0322900 - WRKY transcription factor, Benzothiadiazole (BTH)-inducible blast resistance	CGGGCAGAAGGAGATCCAAAACT	GCCGATGTAGGTGACCCTGTAGC	Shimono et al., 2012
Hormone (JA)				
OsJAMyb	Os11g0684000 - JA-dependent myb transcription factor	TAGGGGTTCAAAGAGGACCA	TCCTCAGTGCAATTCTGGAG	Yokotani et al., 2013
OsJAZ6	Os03g0402800 - TIFY family protein, JASMONATE-ZIM domain (JAZ) protein, JA signalling, Regulation of spikelet development	TTGATGACTTCCAGCTGAGAA	GCGCTGTGGAGGAACTCTTG	Lu et al., 2016
OsLOX4	Os03g0700400 - Lipoxygenase-3, Generation of stale flavour	TGGTGGAGCAGATCTACGTG	ATCGCCTTGATCGAGTAGCC	Nguyen et al., 2022
Hormone (ET)				
OsACS1	Os03g0727600 - ACC synthase, Ethylene biosynthesis	GATGGTCTCGGATGATCACA	GTCGGGGGAAAACTGAAAAT	Petitot et al., 2017
Nutrient homeostasis				
OsNIA1	Os02g0770800 - NADH/NADPH-dependent nitrate reductase	AAGGTGTCTTGTGCTGGATGGC	AGCTTGTCGAGTTCGTCCTTGC	Tang et al., 2012
OsIRO2	Os01g0952800 - Iron-related bHLH transcription factor 2, Tolerance to Fe deficiency, Regulation of Fe uptake from soil, Fe translocation to grain during seed maturation	ACGAGCTCTACTCCTCCCTC	CTTCTGCAGCTCGGGTATGT	Ogo et al., 2006
OsMGD2	Os08g0299400 - Monogalactosyl diacylglycerol (MGDG) synthase, Adaptation to Pi deficiency, Phosphate utilisation and acquisition	AGACAGGTTGCCAGATGGTT	CTGGAGCTTGTGGATGTCCT	Hasegawa et al., 2010
OsPAP23	Os08g0280100 - Purple acid phosphatase 23	GACTCTGGTTGGTTGTGTGC	GCATCAGCGTGTTCATGGAA	Secco et al., 2013

(Continued)

TABLE 2 Continued

Gene name	Annotation RAPDB	Forward primer (5′-3′)	Reverse primer (5′-3′)	References
OsSPX3	Os10g0392600 - SPX domain-containing protein, Negative regulation of phosphate signalling, Pi homeostasis	CAGTCCATCCGATCCGATCC	TCTCTCAATGACTCGTTTCGT	Secco et al., 2013
Development				
OsXTH17	Os08g0237000 - Xyloglucan endotransglucosylases/hydrolase, Cell wall modification processes during rice growth and development	GCCGACTTCCACACCTACAA	GCCAGGTCGTCGTACTTCTT	Lin et al., 2019
OsYABBY6	Os12g0621100 - Similar to Filamentous flower-like yabby protein	TTCGTCGTCTTCCAAGCTCA	ACCCTTTGCCTCTTCTCTGG	Jiang et al., 2015

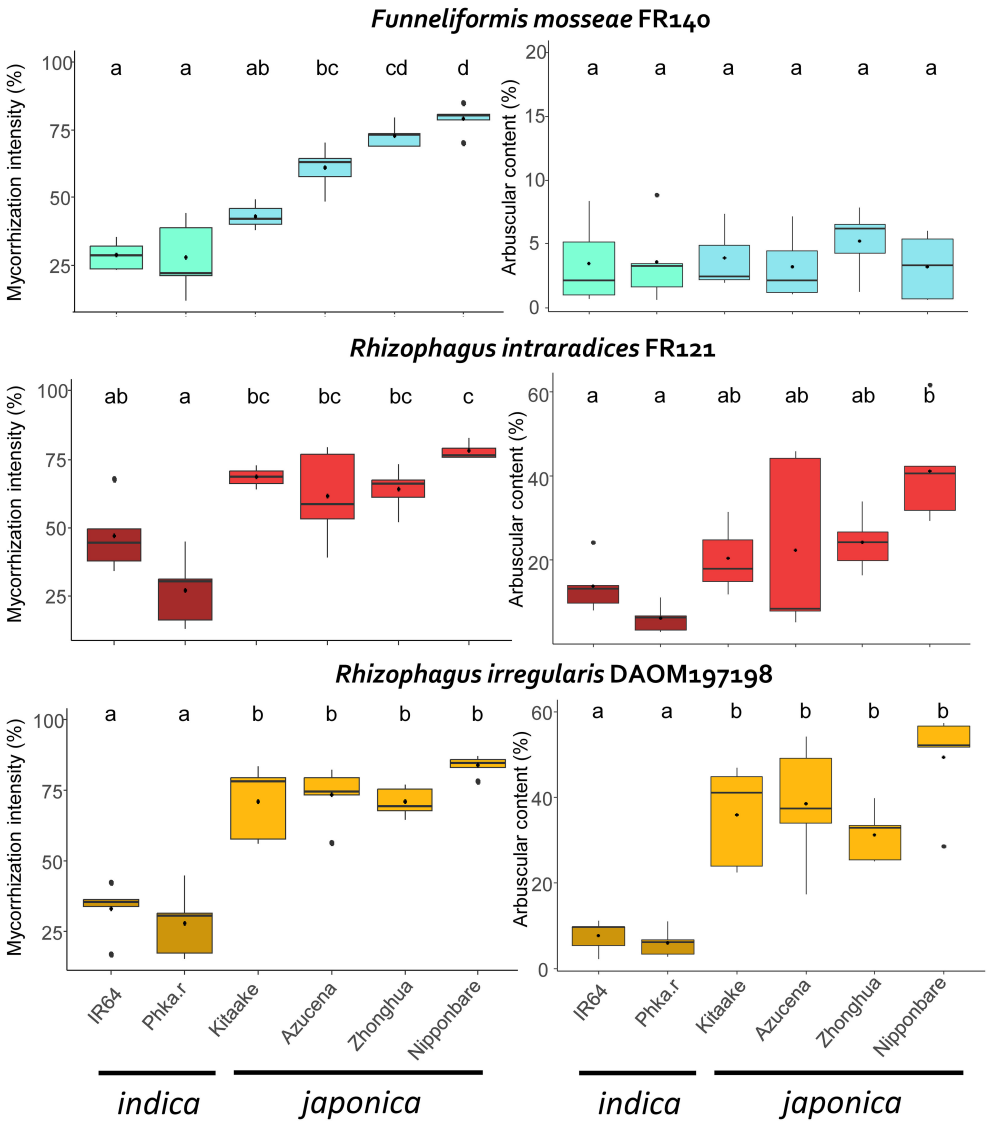

FIGURE 1
Mycorrhization index of each AMF genotype according to rice cultivar. Left: global mycorrhization intensity (%), right: arbuscular content (%). Blue: *F. mosseae*, red: *R. intraradices*, yellow: *R. irregularis*. Turquoise, dark red and dark yellow: *indica* rice. Light blue, red and yellow: *japonica* rice. Letters above boxplots indicate statistical groups according to the results of the ANOVA and Tukey tests (p value< 0.05). Phka.r: Phka Rumduol; Zhonghua: Zhonghua 11.

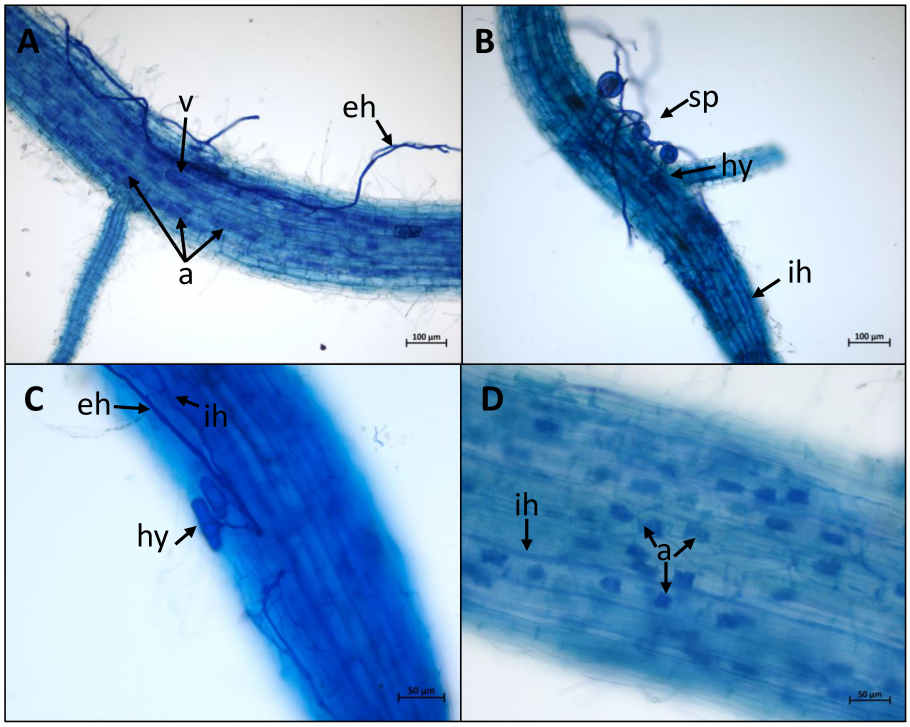

FIGURE 2
AMF colonisation in rice roots. Plants were stained using the ink-acetic acid method. External fungal organs: external hyphae (eh), spores (sp). Symbiotic fungal organs: intercellular hyphae (ih); hyphopodia (hy); vesicule (v); arbuscules (a). **(A)** = Colonisation of Nipponbare roots by *R. intraradices* FR121. **(B)** = Colonisation of Nipponbare roots by *F. mosseae* FR140. **(C)** = Colonisation of Azucena roots by *R. irregularis* DAOM197198. **(D)** = Colonisation of Zhonghua 11 roots by *R. intraradices* FR121.

(Supplementary Table 2). *Japonica* rice cultivars showed the highest percentage of visible arbuscules in the mycorrhized system compared to *indica* rice, with RIN and (Figure 1). On the other hand, each rice genotype in interaction with FM formed almost no arbuscules, with a maximum of 5.24% in Zhonghua 11 (Supplementary Table 2).

Growth response of rice cultivars to AMF colonisation

The phenotypic response of rice to AMF inoculation and symbiosis establishment was assessed by growth measurements. Maximum height, fresh and dry shoot weight and fresh root weight were measured for each combination (n = 20) and are shown as boxplots in Figure 3. All the corresponding measured values are listed in Supplementary Table 2. Developmental stage of each cultivar (at 10 weeks) is shown in Supplementary Figure 2. Plants were still at developmental vegetative stage, except for Kitaake plants which flowered one week before harvest.

The dataset shows that AMF inoculation can result in an increase, decrease or no significant effect on plant growth parameters. We observed a significant decrease in the height of IR64 during the interaction with FM or RIN (-18.81% and -14.12% respectively, Figure 3A and Supplementary Table 2). The RIR genotype resulted in a significant increase in both root and shoot weights for Phka Rumduol, Kitaake, Zhonghua 11 and Nipponbare. However, this effect was not statistically significant for Azucena. The effect on rice's dry weight wasn't as significant as on height or fresh weight, but was still a good proxy for the beneficial effect of AMF on rice growth (Figure 3).

Under our growth conditions, we observed different growth rates depending on the rice cultivar. Uninoculated Kitaake was the smallest rice cultivar both in size (24.65 cm) and weight (0.19 g, 0.11 g, 0.09 g on average for fresh shoot, root and dry shoot weight, respectively; Supplementary Table 2). Still, mycorrhization of Kitaake induced a clear improvement in growth: the highest dry weight among all combinations being obtained with Kitaake in association with RIN (0.30 g, Figure 3D; Supplementary Table 2). This combination showed the greatest positive effect on rice's growth on all variables: 36% taller, 259%, 270% and 221% heavier on biomass of fresh shoots and roots, and dry shoots, respectively (Supplementary Table 2).

Globally, FM inoculation affected rice growth non-significantly (Nipponbare, Phka Rumduol, Zhonghua 11) or negatively (Azucena, IR64), in both height and weight (Figure 3). The only significant positive interaction was with Kitaake: 20% taller, 163%, 177%, 125% heavier on its fresh shoot & root and dry shoot weights, respectively (Figure 3; Supplementary Table 2). The effect of RIN inoculation was contrasting: beneficial on Kitaake and Zhonghua 11, negative on IR64 and Phka Rumduol or non-significant on Azucena and Nipponbare (Figure 3). RIR was the AMF genotype that induce the most positive

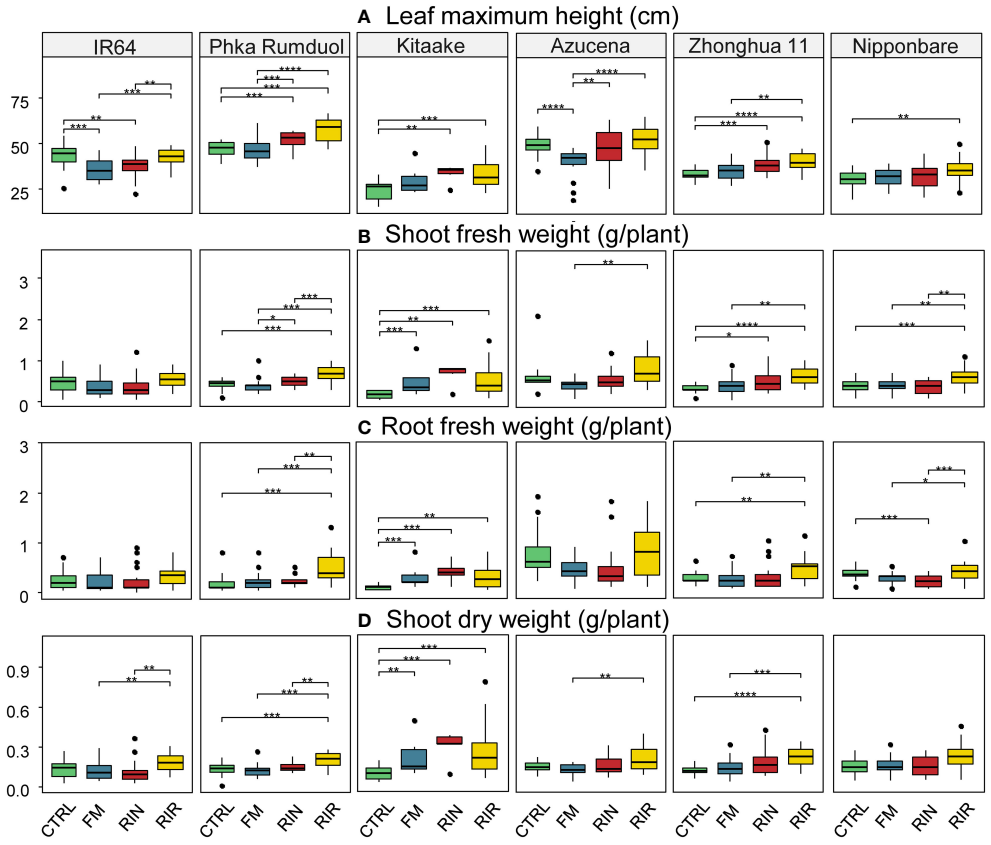

FIGURE 3
Effect of mycorrhization on phenotypic traits for each combination. **(A)** Maximum leaf height (cm). **(B)** Shoot fresh weight (g/plant). **(C)** Fresh root weight (g/plant). **(D)** Shoot dry weight (g/plant), depending on AMF inoculation. Green box plot: CTRL (no AMF), blue: FM (*F. mosseae* FR140), red: RIN (*R. intraradices* FR121), yellow: RIR (*R. irregularis* DAOM197198). n=20 for each combination except Kitaake-RIN and Kitaake-FM with n=6 and n=8, respectively. *: p value< 0.05. **: p value< 0.01. ***: p value< 0.005. ****: p value<< 0.005 (Wilcoxon test; adjusted pvalue with Bonferroni method).

effects among all rice cultivars: +35% and +20% leaf height with Kitaake and Phka Rumduol, respectively (Figure 3A); +182% and 103% fresh shoot weight with Kitaake and Zhonghua 11, respectively (Figure 3B) and +196% and +149% fresh root weight with Kitaake and Phka Rumduol, respectively (Figure 3C).

Our results show that the effect of AMF inoculation on rice growth depends on both rice cultivars and AMF genotypes: ranging from negative, neutral to beneficial outcomes across the 18 combinations under study.

Mycorrhiza-induced resistance

The potential of each fungal inoculum to induce systemic resistance in the leaves of each rice cultivar during a shoot phytopathogen infection was investigated. Rice plants were infected by leaf-clipping with *Xanthomonas oryzae* pv *oryzae* PXO99 (Xoo) and the extent of chlorosis and necrosis was recorded 14 days later. The results are shown as boxplots in Figure 4 and all the corresponding measured values are listed in Supplementary Table 2.

The effect of AMF inoculation on the chlorosis and necrosis symptoms of rice induced by Xoo differed greatly between combinations. Only two combinations, both with RIN, showed a significant increase in leaf symptoms: Azucena on chlorosis (+69%) and Phka Rumduol on necrosis (+106%). Regarding the bio-protective effects of AMF, chlorosis symptoms were significantly reduced on IR64 in combination with FM (-24%) and RIR (-26%), on Zhonghua 11 in combination with FM (-44%), RIN (-28%) and RIR (-29%), and on Nipponbare with FM (-40%) and RIN (-34%) (Figure 4 and Supplementary Table 2). For necrosis, only Zhonghua 11 and Nipponbare showed significant reductions of symptoms. These reductions in the size of necrosis were observed with the three AMF genotypes: -65%, -66% and -64% for Zhonghua 11 with FM, RIN and RIR respectively; and -78%, -87% and -64% for Nipponbare with FM, RIN and RIR, respectively (Figure 4 and Supplementary Table 2).

RT-qPCR analysis of growth and immunity molecular marker genes in contrasting rice-AMF combinations

We observed contrasted patterns of symbiotic compatibility among our AMF-cultivar combinations. In order to link these observed differences with the expression level of leaf marker

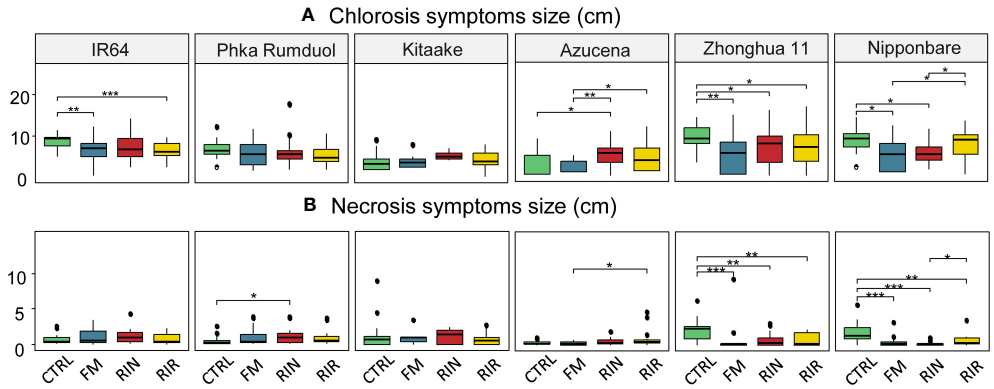

FIGURE 4
Size of chlorosis **(A)** and necrosis **(B)** symptoms following leaf clipping with Xoo, for each rice cultivar-AMF genotype combination. Green: no AMF, blue: *F. mosseae* FR140, red: *R. intraradices* FR121, yellow: *R. irregularis* DOAM197198. n=18 for each combination except Kitaake-RIN and Kitaake-FM with n=5 and n=7, respectively. *: p value< 0.05. **: p value< 0.01. ***: p value< 0.005 (Wilcoxon test).

genes, we selected two rice cultivars with contrasting AMF responses: Nipponbare and IR64. The first is a *japonica* model cultivar and was the most intensely mycorrhized, regardless of the AMF genotype, with the interaction having non-significant to beneficial effects on its growth and tolerance to Xoo infection (Figures 1, 3, 4). The latter is an *indica* model cultivar, that was significantly less mycorrhized, with non-significant to negative effects of the AMF interaction on its growth, but with beneficial effects on its tolerance to Xoo infection (Figures 1, 3, 4). We selected 19 markers genes of development, nutrient homeostasis, hormonal balances and defence and their expression was normalised to that of *EF1a* reference gene. The list of marker genes, their function and the primers used in this study are listed in Table 2. A summary of the statistical comparison of gene expression for each combination, for both Nipponbare and IR64, is provided in Supplementary Table 3 and Supplementary Figures 3, 4. Their expression was visualised as a heatmap in Figure 5.

The expression of two cellular growth marker genes, *OsYABBY6*, responsible for abaxial-adaxial polarity and whose expression is needed for leaf development (Jiang et al., 2015), and *OsXTH17*, a xyloglucan endotransglucosylase/hydrolases involved in primary cell wall formation (Lin et al., 2019) was recorded. A non-significant induction of *OsXTH17* expression was observed for Nipponbare in interaction with RIN (p= 0.055), while that of *OsYABBY6* is repressed with FM (p=0.009) (Figure 5; Supplementary Table 3; Supplementary Figure 3). In IR64, their expression was not significantly affected independently of the AMF genotype (Supplementary Figure 4).

To assess the effect of mycorrhization on mineral homeostasis in leaves, one iron transporter (*OsIRO2*), one nitrate-reductase (*OsNIA1*) and three Pi transporters, marker genes of Pi-starvation response (*OsMGD2*, *OsPAP23* and *OsSPX3*) were selected. The expression of almost all mineral marker genes was significantly reduced in Nipponbare leaves (*OsNIA1*, *OsMGD2*, *OsPAP23* with RIN, *OsSPX3* with either AMF genotype), except for a non-significant strong induction of *OsIRO2* expression when associated with RIN (p= 0,09) (Figure 5; Supplementary Table 3). In leaves of IR64, the expression of *OsMGD2* and *OsPAP23* was not significantly affected. The expression of the other mineral marker genes was reduced only significantly for *OsSPX3* in RIN-mycorrhized leaves, and for *OsNIA1* and *OsIRO2* in FM-mycorrhized leaves (Figure 5; Supplementary Table 3; Supplementary Figures 3, 4).

Mycorrhization is known to affect the hormonal balance in mycorrhized plants. Its effect on the expression of jasmonate (JA: *OsLOX4*, a lipoxygenase responsible for the biosynthesis of JA (Nguyen et al., 2022) and *OsJAMyb* & *OsJAZ6*, both responsible for JA signalling (Yokotani et al., 2013; Lu et al., 2016), ethylene (ET: *OsACS1*, 1-aminocyclopropane-1-carboxylate synthase responsible for ethylene biosynthesis) and salicylic acid (SA: *OsNPR1*, mediating SA biosynthesis and responsives genes (Kumari et al., 2016) & *OsWRKY45*, a transcription factor mediating SA signalling) pathways was investigated. Overall, jasmonate- and ethylene-related genes expression was not significantly repressed in Nipponbare and IR64 leaves (Figure 5; Supplementary Table 3). SA-related genes were not significantly repressed, except for *OsNPR1* in RIN mycorrhized-IR64 leaves (Figure 5; Supplementary Table 3; Supplementary Figures 3, 4).

The expression of defence-related genes was recorded to assess how mycorrhization affects the defence response in healthy leaves. *OsPR5* is a pathogenesis-related protein, *OsPAL4* is a broad-spectrum disease resistance-related gene and *OsTGAP1* & *OsDXS3* are responsible for phytoalexines production in rice (Okada et al., 2009; Delteil et al., 2012; Petitot et al., 2017; Valette et al., 2020). *OsMPK10* and *OsWRKY30* are responsible for early disease-mediated signalling, the latter also responsive to SA and JA treatments (Ryu et al., 2006; Nguyễn et al., 2014). Globally, defence genes appeared to be more induced in mycorrhized IR64 leaves than in Nipponbare leaves (Figure 5; Supplementary Figures 3, 4). In Nipponbare leaves, we observed i) a significant repression of *OsPR5* expression, irrespective of the AMF species, ii) a significant down-regulation of *OsPAL4* expression with FM, iii) a non-significant down-regulation with RIN (Supplementary Table 3). In leaves of IR64, phytoalexin biosynthesis-related genes (*OsDXS3* and *OsTGAP1*) were not significantly induced in plant associated with AMF. When mycorrhized with RIR, the expression of *OsWRKY30*

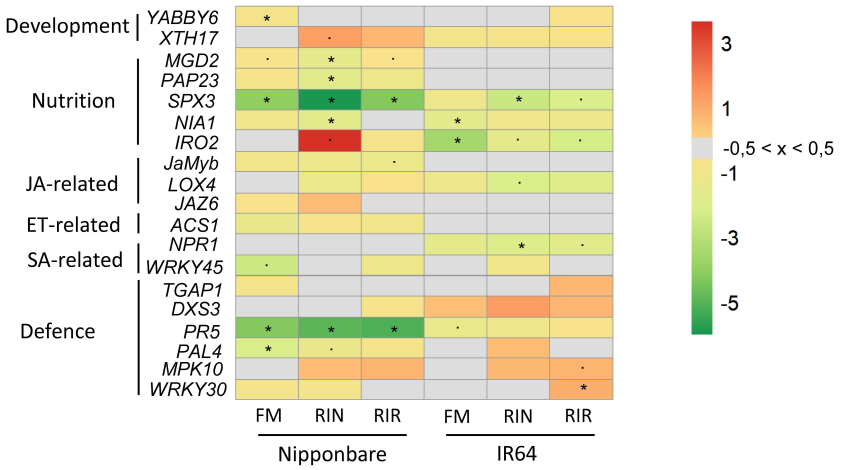

FIGURE 5
Relative expression of rice marker genes in response to mycorrhization, in Nipponbare and IR64 leaves. Marker genes related to development, nutrition, hormone balance and defence were selected from the literature (listed in Table 2). Transcript levels were normalised to that of the reference gene *EF1a*. The log2 fold change values are shown in red (positive), green (negative) and grey (between -0.5 and 0.5) (n = 4). JA= jasmonic acid. ET= ethylene. SA= salicylic acid. "*"= statistically significant (p-value< 0.05). "."= tendencies (p-value< 0.10).

is significantly induced but not the one of *OsMPK10* (p-value = 0.03 and 0.07, respectively) (Figure 5; Supplementary Table 3).

Discussion

Symbiotic compatibility between six rice cultivars and three AMF genotypes depends on the studied combination

In this study we have characterised the symbiotic compatibility between multiple rice cultivars from *japonica* and *indica* subspecies in association with three AMF genotypes (*F. mosseae* FR140, *R. intraradices* FR121 and *R. irregularis* DAOM197198). First, we observed that all rice cultivars selected for this study were colonised by each fungal inoculum, their colonisation intensity differing between rice cultivars (Phka Rumduol being the lowest and Nipponbare the highest in terms of global mycorrhization). A similar pattern was also observed at the arbuscular level, with FM developing fewer arbuscules when interacting with rice, compared to the two *Rhizophagus* genotypes. We also observed an effect of AMF colonisation on rice growth that could be either beneficial, neutral, or negative. However, there was no direct relationship between the level of colonisation and the growth phenotype: both *indica* rice cultivars had similar AMF colonisation and arbuscular levels, but the tendency of IR64 to be negatively affected on growth wasn't observed for Phka Rumduol (Figures 1, 3). The *japonica* cultivars used in this study were generally beneficially affected in their growth (either in height or weight; Figure 3). A special case was observed with the cultivar Kitaake. As stated earlier, early mortality and a general lack of growth were observed for this cultivar in our inert substrate. We hypothesised that Kitaake's growth wasn't optimal under these drastic conditions. However, plants that have managed to grow were well colonised by the three AMF (43%, 69% and 71% of roots colonised by FM, RIN, and RIR respectively). The highest levels of growth improvement were recorded for this cultivar: 37% taller, 259%, 270% and 221% heavier on biomass of fresh shoots and roots and dry shoots (Supplementary Table 2). AMF symbiosis is known to enhance root anchoring and nutrient uptake in poor soils, thereby improving plant health (Paszkowski and Boller, 2002; Gutjahr et al., 2009; Chiu et al., 2018). However, early growth depression following mycorrhization has also been documented in wheat, barley, and soybean (Jacott et al., 2017). Such depression was eventually overcome (or not) in the later life cycle of the crop (Jacott et al., 2017). This growth depression can be explained by the genetic variability of AMF genotypes and their symbiotic effectiveness, but the recurring hypothesis is related to the trade-off between plant photosynthates and soil nutrients recovered by AMF (Jin et al., 2017). Our differences in the phenotypic growth effects of mycorrhization among rice cultivars could be explained by an imbalance between early life stage development and the photosynthetic carbon cost for AMF establishment and function (Jacott et al., 2017; Jin et al., 2017). Our growth conditions were adapted from recent studies on wheat and rice mycorrhization, composed of a mixture of sand, sieved perlite, and vermiculite. It mimics sandy soil and is easy to sterilise. Though, this inert substrate is still poor in essential nutrients, and a modified Hoagland's solution depleted in phosphate was used to water the plants. Several studies have shown that similar conditions allow both crop species to grow and the establishment of an efficient AMF symbiosis (Gutjahr et al., 2008; Fiorilli et al., 2018; Campo and San Segundo, 2020; Campo et al., 2020; Guo et al., 2022).

Japonica cultivars respond better to mycorrhization than *indica* cultivars

Under our growing conditions, *japonica* rice appeared to be more intensely colonised than *indica* rice. (Figure 1; Supplementary Table 2). The result of upland *japonica* rice cultivars being more colonised and responding more to AMF colonisation than flooded *indica* rice cultivars is shared by several studies, considering their different root architecture and the co-evolution between AMF and upland rice in aerobic selective condition, optimizing their interaction (Diedhiou et al., 2016; Davidson et al., 2019). It is important to note that we selected two *indica* rice cultivars compared to four *japonica*, depending on the valuable factors listed in Table 1. This study should be extended to more rice genotypes from both subspecies to understand if this pattern can be generalised. The global understanding of how rice mycorrhization is affected by host genotype is currently limited. In a published report, the mycorrhizal growth response (MGR, corresponding to the ratio between the dry weight of mycorrhized and control plants) was measured for 64 rice genotypes from different subspecies 4 weeks after inoculation with *F. mosseae*. Differences between genotypes were observed but not related to *indica* or *japonica* origin (Suzuki et al., 2015). A possible explanation is that variations in AMF recognition receptors between host genotypes affect their symbiotic compatibility. OsCERK1, a LysM receptor-like kinase that is essential for AMF recognition and activation of the symbiosis pathway, is also responsible for root branching in rice (Chiu et al., 2018; Choi et al., 2018). It has been proposed that natural variation between allelic variants of the *OsCERK1* gene in different rice cultivars may affect their symbiotic compatibility with *R. irregularis* DAOM 197198 (Huang et al., 2020). A higher level of AMF colonisation, 14 days post inoculation, was reported for eleven *indica* rice cultivars, compared to eight *japonica*. The difference was proposed to be related to an underrepresented *OsCERK1* haplotype, absent in *japonica* rice and present in their selected *indica* cultivars (Lefebvre, 2020). In our study, we obtained opposite results, but we chose to harvest the rice after two months of growth, which, in addition to the different substrate used, may explain the difference. Several reports have shown that root colonisation of Nipponbare becomes clearly visible after 14 days post inoculation, and arbuscules after 3 to 4 weeks (Gutjahr et al., 2015; Guo et al., 2022; Liu et al., 2022). It would be interesting to assess whether rice cultivar also affects the kinetics of AMF establishment and functioning in the long term.

Beneficial effects on resistance to Xoo are highly dependent on rice cultivar and AMF genotypes

As plant colonisation by AMF has often been shown to be associated with a better protection against pathogens, we conducted biocontrol trials against Xoo. We observed a general tendency for symptoms to decrease, which was statistically significant for some AMF-rice cultivar combinations (Figure 4; Supplementary Table 2). Only two among 18 showed an increase in the leaf symptoms when associated with an AMF (Phka Rumduol and Azucena associated with RIN). Almost all well-colonised *japonica* rice species showed a significant reduction of at least one Xoo-induced symptoms thanks to MIR, particularly with Zhonghua 11 and Nipponbare cultivars. The most-colonised rice cultivar, Nipponbare, see chlorosis and necrosis symptoms reduced in both FM and RIN conditions. With the most compatible one, this reduction of symptoms can be noticed but is not statistically significant. This reduction of symptoms becomes statistically significant in a repeated study on 20 non-mycorrhizal Nipponbare and 20 associated with RIR (Additionnal file 6: Supplementary Figure 3). An induction of 39%, 107% and 187% of Nipponbare's maximum height, shoot and root dry weight, respectively was shown, linked with a reduction of chlorosis symptoms by 32% at 14 days post-clipping. It is noteworthy that IR64, a rice variety three times less intensively mycorrhized, also shows reduced chlorosis symptoms when mycorrhized with FM or RIR. A possible explanation could reside in the differences between rice genotypes themselves, being more or less sensitive to AMF colonisation and their established interaction, having then important or little to no effects on rice growth phenotype, but notable impacts on rice nutrient physiology and defence responses. Our results highlight here that symbiotic compatibility between AMF and rice species states for a sufficient amount of colonisation allowing significant nutrient starvation response inhibition and phytopathogen-induced symptoms reduction.

The potential of biological control by AMF symbiosis is well documented in the literature, although pathogen-dependent. Mycorrhization of *japonica* rice cultivars by AMF genotypes such as *R. intraradices, R. irregularis* or *F. mosseae* showed both a local and a systemic defence response against *Pyricularia oryzae*, sometimes associated with an increase in shoot biomass (Campos-Soriano et al., 2010 ; Campos-Soriano et al., 2012; Campo et al., 2020). In *Triticum aestivum* cv. Chinese Spring associated with *F. mosseae*, the AMF symbiosis confers both a positive effect on growth and resistance to *X. translucens* (Fiorilli et al., 2018). Two American rice cultivars inoculated with a mixture of AMF genotypes showed increased susceptibility to two insects and *Rhizoctonia solani* infections, but without growth defects or nutrient losses, suggesting an effect of symbiosis on defence vs growth trade-off (Bernaola et al., 2018b).

Oryza sativa cv *japonica* Nipponbare is the best AMF-responsive rice cultivar

Many research studies focus on the association between *R. irregularis* and Nipponbare (Gutjahr et al., 2009; Gutjahr et al., 2015; Campo et al., 2020; Guo et al., 2022; Liu et al., 2022). Since they are both models (sequenced genomes, easy to grow, transform and store, controlled lifecycle), deepening our knowledge of their interaction at different life stages of both organisms may shed light on how rice interacts with and benefits from AMF and *vice-versa*. Under our conditions, Nipponbare was the rice cultivar with the

best level of mycorrhization, regardless of the AMF inoculated (Figure 1; Supplementary Table 2). There is no significant negative effect of mycorrhization on its growth, with a tendency of AMF symbiosis to increase both height and weight (Figure 3). After Xoo infection, a significant reduction of symptoms was observed with FM and RIN (Figure 4). To further confirm this, the experiment was repeated (n = 20) for Nipponbare in combination with *R. irregularis* DAOM197198 and showed then a clear positive effect on growth promotion (height, root and leaf weight) and on biocontrol against Xoo (Supplementary Figure 5). At the molecular level, RT-qPCR analysis showed that the expression of cellular development marker genes was induced by mycorrhization, even after two months of growth (Figure 5; Supplementary Table 3). Under these conditions, the overall expression of nutrient transporter and defence genes was down-regulated. As these genes are known to be induced upon starvation, Nipponbare in symbiosis with AMF could then be considered to be in a healthy state.

Our results are in agreement with previous work. On Nipponbare, *Glomus intraradices*, which could be classified as *R. irregularis* according to the current AMF phylogeny, colonises more than 60% of the large lateral roots and increases both dry weight and coronary root length (Gutjahr et al., 2009). Colonisation kinetics of *R. irregularis* on this cultivar showed high colonisation rates (up to 87% of root length, of which 59% contained arbuscules) and the presence of viable arbuscules and vesicles up to six weeks after inoculation (Gutjahr et al., 2015; Liu et al., 2022). Here we show that even after ten weeks, arbuscules and vesicles were still clearly visible and in larger numbers. A study on the effect of pre-inoculation of *F. mosseae* on Nipponbare before transplanting in the field showed a root colonisation rate of 65%, an arbuscule content of 1.3% and a vesicle content of 6% (Sisaphaithong et al., 2017), consistent with our results. Growing conditions and practices may have an impact on this low arbuscule content. After 10 weeks of association with *F. mosseae*, eight temperate *japonica* cultivars used in Spanish or Italian fields had between 20 and 60% of arbuscules in their mycorrhized roots, with non-significant to beneficial effects on both rice growth and *P. oryzae* tolerance (Campo et al., 2020). The geographical origin and genotype of the host may determine its symbiotic compatibility with different AMF genotypes.

Defence potential imprint revealed by systemic molecular analyses

The description of the phenotypic responses (rice growth and tolerance to Xoo) of different rice cultivars to inoculation with each AMF genotype allowed us to assess the symbiotic compatibility between them. To understand how AMF symbiosis affects the molecular functioning in two rice cultivars with contrasting phenotypic responses to AMF, the leaf molecular responses of Nipponbare and IR64 after six weeks of interaction were studied. Local molecular responses to the establishment of the mycorrhization have been well studied in rice roots (Güimil et al., 2005; Gutjahr et al., 2009; Campos-Soriano et al., 2010, reviewed in Choi et al., 2018), but the systemic effects on the responses of rice leaves are still poorly described. A panel of rice marker genes involved in development, nutrient status, phytohormone signalling and defence responses (listed in Table 2, detailed in the results) was selected, and their expression analysed in healthy 50-day old leaves of Nipponbare and IR64.

First of all, the response of Nipponbare leaves to root mycorrhization was consistent with our phenotypic results. We observed a modulation of developmental gene expression, coupled with a reduced starvation response (repression of Pi starvation's marker genes and a nitrate reductase marker gene, Figure 5; Supplementary Table 3). Defence related genes expression was not significantly to negatively affected by AMF symbiosis in Nipponbare, suggesting that mycorrhization does not induce a systemic defence response under healthy conditions. Overall, mycorrhization of the *japonica* rice Nipponbare highlights an improvement of rice's development and nutritional status, to be linked with the reported increase in growth in this most intensely mycorrhized rice. This imprint on rice's expression pattern hints for a better overall state and tolerance against abiotic as well as biotic stressors. The study of the effect of either one of them on mycorrhized Nipponbare is interesting to assess which specific systemic mechanisms will act on the trade-off between growth and tolerance.

Mycorrhized IR64 plants showed a different response to AMF symbiosis. A non-significant repression of developmental and nutrient starvation response marker genes was observed, coupled with a non-significant induction of the defence response (Figure 5; Supplementary Table 3). Under our growth conditions, mycorrhization of IR64 does not seem to be as beneficial as of Nipponbare and this is still to be linked with phenotypic results (i.e. a non-significant to negative impact of mycorrhization on IR64's growth with a less mycorrhized cultivar).

The nutritional status of both our rice cultivar were investigated thanks to iron, phosphate and nitrate-related genes expression. There is a strong trend of induction of *OsIRO2* expression in leaves of Nipponbare interacting with RIN (Figure 5; Supplementary Table 3; Supplementary Figure 3). This transcription factor is induced under Fe deficiency, modulating key genes involved in iron uptake in rice (*OsNAS1*, *OsTOM1* or *OsYSL15*), with *IRO2*-overexpressing rice showing improved Fe-deficiency tolerance compared to the non-transgenic lines (Ogo et al., 2006; Ogo et al., 2011). Mycorrhization may affect iron homeostasis, *via* its effect on *IRO2* expression, affecting Fe uptake and translocation to shoots and grains (Ogo et al., 2011) but little research has been done on this subject. Leaves of the *japonica* rice Senia show an increase of *IRO2* expression during mycorrhization with RIR, while wheat symbiosis with FM triggers an accumulation of Fe-uptake related proteins in roots, linked with a translocation of iron from roots to shoots, suggesting both a beneficial effect of mycorrhization on iron

homeostasis in mycorrhized hosts (Campos-Soriano et al., 2012; Fiorilli et al., 2018).

AMF have been demonstrated to increase the bioavailability of essential nutrients such as nitrogen and phosphate to their host roots. This capacity can be linked with the repression of *OsNIA1* and Pi-starvation-related genes expression in the leaves of our two studied rice cultivars. (Figure 5; Supplementary Table 3; Supplementary Figures 3, 4). These results are in line with previous reports, and specifically results showing the repression of *OsSPX3, OsPAP23* and *OsMGD2* expression in Loto rice leaves in association with FM (Liu et al., 2007; Gutjahr et al., 2008; Fiorilli et al., 2018; Campo and San Segundo, 2020; Vannini et al., 2021).

The expression of marker genes for phytohormones biosynthesis or signalling in leaves was not significantly affected by AMF symbiosis, except for a repression of the SA perception gene (*OsNPR1*) in leaves of IR64 (Figure 5; Supplementary Table 3; Supplementary Figure 4). Recent literature reports contradictory results on the effect of mycorrhization on SA pathways in wheat or rice: the first is not affected on any SA-related pathways during FM mycorrhization, while *OsNPR1* is induced in some *japonica* rice cultivars (Campos-Soriano et al., 2012; Fiorilli et al., 2018; Tian et al., 2019). Mycorrhization effect on SA-related pathways may be plant species-dependent (Campo and San Segundo, 2020).

Under our conditions, ethylene and jasmonate related genes were not significantly repressed in both cultivars (Figure 5; Supplementary Table 3). Ethylene biosynthesis is known to be induced in tomato and wheat and repressed in rice leaves under mycorrhization, not significantly in our case (Fiorilli et al., 2018; Campo and San Segundo, 2020). Jasmonate-related genes are previously reported to be modulated by mycorrhization in different rice cultivars, both on its biosynthesis or signalling (Campos-Soriano et al., 2012; Campo and San Segundo, 2020). MIR is reported to occur via JA-related pathways, leading to reduced symptoms and pathogen load in multiple host-pathogen interactions (Dowarah et al., 2021). The molecular responses to Xoo infection may allow us to understand if the reduction of symptoms occurring in both our rice cultivars are linked with MIR, a primed induction of defence responses, responding faster and more efficiently than non-mycorrhized controls, or by an overall better rice health state (related to both growth and nutritional status improvement). If the MIR hypothesis appears to be true, deeper transcriptional analyses are to be conducted to understand by which mechanisms MIR occurs.

Overall, the systemic response of rice to AMF symbiosis is dependent on rice cultivar and AMF genotype but can be linked to an overall improvement in rice health.

Conclusion

In this study we showed that the establishment of the AMF symbiosis and its effects on rice depends on both the rice cultivar and the AMF genotype for each variable studied. We found that mycorrhizal growth enhancement and induced resistance to Xoo strongly depends on both rice variety and AMF genotype. In our study and under our conditions, *japonica* rice chosen subspecies tend to be more colonised and have more benefits on growth and defence responses than *indica* ones. In the model rice cultivars Nipponbare and IR64, root colonisation rate, growth enhancement or shortage in both shoot and roots can be associated with a systemic modification of molecular pathways in leaves. These differences in rice response raise the question of how beneficial the AMF symbioses really are. In some cases, AMF interactions are detrimental to the growth of the plant host or its response to the environment (Jin et al., 2017; Bernaola et al., 2018b). The assumption that AMF symbiosis can be viewed as an equilibrium between mutualism and parasitism, with symbiont considered as more or less efficient and cooperative partners (Kiers et al., 2011; Kaur et al., 2022), may be closer to biological reality. In our study, we have identified rice-AMF combinations that are able to develop into a functional symbiosis with positive effects on both rice growth and tolerance to phytopathogens. These combinations should now be tested in unflooded rice field conditions with low Pi to unravel their true potential.

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

LG: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft. LJ: Formal Analysis, Investigation, Methodology, Writing – original draft. NB: Investigation, Methodology, Writing – original draft. LM: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. PC: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The authors gratefully acknowledge financial support from the Consultative Group for International Agricultural Research (CGIAR) research program (CRP) RICE. LG and LJ were supported by fellowships from the French Ministry of Higher Education, Research and Innovation.

Acknowledgments

The authors thank Isabelle Rimbault for technical help in the experiments, as well as Florent Tivet from CIRAD for providing Phka rumduol rice seed.

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.2023.1278990/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 15 November 2023
DOI 10.3389/fpls.2023.1250020

OPEN ACCESS

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RECEIVED 29 June 2023
ACCEPTED 30 October 2023
PUBLISHED 15 November 2023

CITATION
Pandey P, Tripathi A, Dwivedi S, Lal K and Jhang T (2023) Deciphering the mechanisms, hormonal signaling, and potential applications of endophytic microbes to mediate stress tolerance in medicinal plants.
Front. Plant Sci. 14:1250020.
doi: 10.3389/fpls.2023.1250020

Deciphering the mechanisms, hormonal signaling, and potential applications of endophytic microbes to mediate stress tolerance in medicinal plants

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The global healthcare market in the post-pandemic era emphasizes a constant pursuit of therapeutic, adaptogenic, and immune booster drugs. Medicinal plants are the only natural resource to meet this by supplying an array of bioactive secondary metabolites in an economic, greener and sustainable manner. Driven by the thrust in demand for natural immunity imparting nutraceutical and life-saving plant-derived drugs, the acreage for commercial cultivation of medicinal plants has dramatically increased in recent years. Limited resources of land and water, low productivity, poor soil fertility coupled with climate change, and biotic (bacteria, fungi, insects, viruses, nematodes) and abiotic (temperature, drought, salinity, waterlogging, and metal toxicity) stress necessitate medicinal plant productivity enhancement through sustainable strategies. Plants evolved intricate physiological (membrane integrity, organelle structural changes, osmotic adjustments, cell and tissue survival, reclamation, increased root-shoot ratio, antibiosis, hypersensitivity, etc.), biochemical (phytohormones synthesis, proline, protein levels, antioxidant enzymes accumulation, ion exclusion, generation of heat-shock proteins, synthesis of allelochemicals. etc.), and cellular (sensing of stress signals, signaling pathways, modulating expression of stress-responsive genes and proteins, etc.) mechanisms to combat stresses. Endophytes, colonizing in different plant tissues, synthesize novel bioactive compounds that medicinal plants can harness to mitigate environmental cues, thus making the agroecosystems self-sufficient toward green and sustainable approaches. Medicinal plants with a host set of metabolites and endophytes with another set of secondary metabolites interact in a highly complex manner involving adaptive mechanisms, including appropriate cellular responses triggered by stimuli received from the sensors situated on the cytoplasm and transmitting signals to the transcriptional machinery in the nucleus to withstand a stressful environment effectively. Signaling pathways serve as a crucial nexus for sensing stress and establishing

plants' proper molecular and cellular responses. However, the underlying mechanisms and critical signaling pathways triggered by endophytic microbes are meager. This review comprehends the diversity of endophytes in medicinal plants and endophyte-mediated plant-microbe interactions for biotic and abiotic stress tolerance in medicinal plants by understanding complex adaptive physiological mechanisms and signaling cascades involving defined molecular and cellular responses. Leveraging this knowledge, researchers can design specific microbial formulations that optimize plant health, increase nutrient uptake, boost crop yields, and support a resilient, sustainable agricultural system.

KEYWORDS

plant-microbe interaction, medicinal plants, biotic-abiotic stress, signaling pathways, ethylene, salicylic acid, jasmonic acid

1 Introduction

Medicinal plants are crucial in the pharmaceutical and drug industries for providing many pharmaceutically vital bioactive molecules for herbal medicine. Rising consumer demand for herbal drugs and natural products has significantly increased the cultivation acreage of medicinal plants, competing with fixed land resources for cereals and other horticultural crops. The intent of increasing productivity per unit area from the limited land resources has led to excessive usage of agrochemicals (fertilizers, insecticides, pesticides, weedicides, etc.) consumption over the past few decades. Their redundant usage has critically affected soil microbiome and environmental health. Therefore, developing green, efficient, affordable, and eco-friendly agrotechnologies is essential for improving medicinal plants' health and productivity. Sustainable agricultural production is a significant challenge in the global climate change paradigm. In this context, harnessing endophytic microbes as biostimulants can be an effective, sustainable approach. Endophytes are microorganisms (bacteria or fungi) that spend at least a portion of their life cycle forming an association with an asymptomatic plant (Vanessa and Christopher, 2004). Medicinal plants are strongly influenced by microbial endophyte association. In general, endophytic microbes can modify their structure and diversity depending on genotypes, organs, health conditions, and growth stages of host medicinal plants in order to obtain a constant supply of nutrients. Medicinal plants have a range of physiological characteristics, metabolites, and growth patterns that influence their ability to attract different endophytic microbes. Environmental factors considerably impact the quality and yield of medicinal plants. They not only affect the distribution of a medicinal plant but also determine the species of microbial endophytes that can colonize the host during its life cycle.

Plants grown in biologically diverse soil abundant with beneficial microbes have better survival under harsh conditions. The plant's roots anchor it to the soil, enabling it to absorb minerals and essential nutrients and synthesize chemical substances mediating various plant-microbe interactions. These interactions comprise mutualistic relationships with beneficial microbes; however, parasitism occurs with harmful microbes (Badri et al., 2009). The plant deploys surface-localized receptor proteins to recognize self-modified or microbe-derived molecules to recognize microbial invaders are potentially harmful or beneficial microbes. The recognition of β-glucan chains and plant immunity depends on the degree of polymerization and β-1,3-glucan receptor systems perception by a specific plant species (Wanke et al., 2020). The positive interactions have practical implications useful in pharmaceutical, biotechnological, and agricultural applications, but the negative interactions lead to severe plant diseases that endanger global agricultural productivity. Utilizing plant-microbe interactions eliminates the need for synthetic inorganic pesticides and fertilizers, which lowers input costs and, thus, minimizes the impact of synthetic agrochemicals on vital existing ecological communities (Whipps and Gerhardson, 2007). Furthermore, plant-microbe symbiosis produces crucial compounds of industrial and pharmaceutical interest, which eliminates the need for costly catalysts and synthetic derivatives (Wu et al., 2007).

Integrating plant-associated microbes into farming to support agricultural production mitigates a series of biotic and abiotic perturbations (Tanaka et al., 2005; Vega et al., 2008; Wani et al., 2016; Lata et al., 2018; Mukherjee et al., 2021; Siddique et al., 2022). Biotic and abiotic factors influence many morpho-physiological disturbances in plants, including stunted growth and development, senescence, altered gene expression, cellular metabolism, etc., reducing overall crop yield and quality (Purohit et al., 2019). Abiotic stresses are caused by non-living factors such as drought, salinity, waterlogging, temperature extremes (heat, cold, and freezing), metal toxicity, etc., while biotic stresses (caused by living organisms, especially bacteria, fungi, viruses, insects, nematodes, and weeds, etc.), directly starve the hosts of their nutrients limiting the growth or plant death resulting in the pre- and post-harvest crop losses. Plants can mitigate biotic stressors even if they lack an adaptive immune system by adjusting to specific, sophisticated strategies such as antibiosis, hypersensitivity, allelochemical synthesis, membrane integrity, organelle modifications, etc. Plants' genetic makeup controls the defensive schemes that respond to these stresses. Numerous genes

in the plant genome are either tolerant or resistant to various biotic stressors. Being sessile, plants have no choice to escape these environmental cues; however, they alter their genetic architecture for stress adaptation. Specifically, by inducing immunological responses, generating antioxidants, and inhibiting pathogen growth, endophytic microorganisms help plants cope with biotic and abiotic stress. Notably, the interaction between plants and microbes results in the production of a wide range of bioactive substances, including artemisinin, taxol, phenolic acid, huperzine, azadirachtin, vindoline, guanosine, inosine, serpentine ajmalicine, curcumin, and camptothecin, which are profoundly utilized in agriculture and medicine.

Endophytes modulate levels and activity of phytohormones, viz., gibberellins, cytokinins, ethylene (ET), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA), which play a crucial role in plant growth, fitness, and stress amelioration (Barnawal et al., 2016; Egamberdieva et al., 2017; Xu et al., 2018; Sabagh et al., 2021; Chaudhary et al., 2022; Tripathi A. et al., 2022). In stressful conditions, plant defense systems trigger appropriate cellular responses by responding to stimuli from sensors situated on the cytoplasm or cell surface and transmitting signals to the transcriptional machinery in the nucleus with the help of various signaling pathways. Signaling pathways are crucial for sensing stress and establishing the proper molecular and cellular responses (Mir et al., 2022). Phytohormones are an integral part of the plant defense system, commonly known as the plant's systemic acquired resistance (SAR) and induced systemic resistance (ISR). These plant hormones operate as plant protective agents against different phytopathogens. In addition to regulating plant physiological and morphological responses, phytohormones also shape the plant microbiome. Different phytohormones induce distinct effects on plant microbiomes. Plants constantly face a wide range of biotic and abiotic stresses that lead to specific transcriptional variations at the individual gene level, with high variability and stress specificity. Therefore, more practical and fundamental studies are required to address the processes and functioning of hormonal signaling and crosstalk. Hence, this review focuses on a detailed overview of the diversity of endophytes in medicinal plants and defense mechanisms at the cellular level associated with endophyte-mediated plant-microbe interactions for biotic-abiotic stress alleviation, including different signaling pathways.

2 Diversity of endophytic microbes in medicinal plants

Endophytic microbes live in various plant habitats that communally shape the plant endomicrobiome and are most frequently found in plant roots, stems, leaves, fruits, and seeds. Generally, they establish communities in intercellular spaces; nevertheless, certain species can penetrate cells (Toubal et al., 2018). The primary habitat and colonization of endophytic microbes are roots, and their preferred entry points are root hairs, cracks, or wounds caused by phytopathogen infection; this permits the leakage of metabolites that attract more endophytes. Nevertheless, the other vital regions for root colonization are the cortex and epidermis intercellular gaps (Compant et al., 2005). For instance, the root colonization of *Piriformospora indica*, commences in the cortical area with a biotrophic development stage and proceeds to a cell death-dependent step. Rhizospheric microbes associated with Fenugreek (*Trigonella foenumgraecum*) stimulate host plant growth via soil nutrient uptake and recycling (Kumari et al., 2020). Different endophytes may serve as the primary root mutualistic symbionts in stressful situations where mycorrhizae are often scarce (Mandyam et al., 2010; Rat et al., 2021). Sometimes, endophytes enter within the xylem vessels that migrate from the root zones; several harbor-diversified communities penetrate the aerial regions utilizing the soil surface. The majority of endophytic microorganisms embrace an array of entryways, especially the leaves (phyllosphere), above ground stem (caulosphere), below ground stem (laimosphere), flowers (anthosphere), fruits (carposphere), and seeds (spermasphere) (Lindow and Brandl, 2003; Ritpitakphong et al., 2016; Abdullaeva et al., 2020; Sun et al., 2023). Upon arriving leaves and stems from openings like stomata, they grow and create a thin biofilm (Frank et al., 2017). In addition, several microbes might penetrate the inner regions and establish where other microorganisms may invade the xylem. They continue to colonize and grow in various organs, such as the caulosphere, phylloplane, anthrosphere, and carposphere (Meyer and Leveau, 2012). These microbes are inherently advantageous in that they serve as a marker for the beginning of the community structure in the seedling and the end of the community assemblage in the seed (Shahzad et al., 2018). They are pretty intriguing since they transmit their personalities to subsequent generations vertically and can generate endospores, uphold plant growth, control cell motility, and regulate endogenous phytohormones, which improve the structure of the soil, disrupt seed dormancy, and degrade xenobiotics. However, seed endophytes developed multiple paths; few penetrate through the xylem, stigma, and the extrinsic route, wherein an external factor contaminates seeds. The floral components of plants have not been comprehensively investigated to study endophytic diversity; nevertheless, Qian et al. (2014) isolated an endophytic fungus, *Lasiodiplodia* sp., from floral parts of *Viscum coloratum*, which is involved in the synthesis of vital metabolites. Therefore, the diversity of endophytic communities is primarily determined by a series of transforming factors, including the host genetic makeup and immune system, the environment, microbe-microbe interactions, types of soil, and nutrition. Figure 1 depicts the schematic representation of the diversity of endophytic microbes in various plant parts.

3 The complexity of the plants-microbes relationship

Plant-microbe interactions bear a complex relationship depending on the biological and physicochemical ecology of soil, seed surface, phyllosphere, and rhizosphere. While

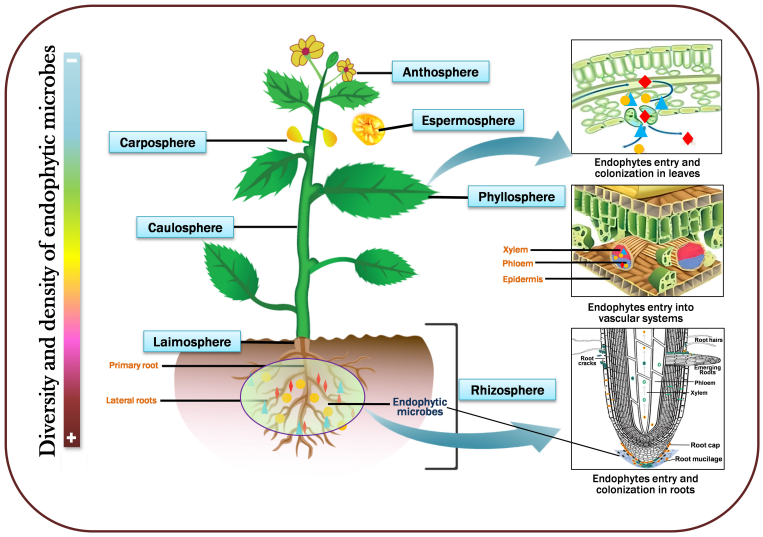

FIGURE 1
A simplified diagramm showing microbial diversity in various plant parts viz., leaves (phyllosphere), above ground stem (caulosphere), below ground stem (laimosphere), flowers (anthosphere), fruits (carposphere), and seeds (spermasphere). Sidebar color intensities represent microbial density and diversity; dark red represents high, and light blue indicates low diversity and density.

"obligate" microbes interact with living cells in order to develop and complete their life cycle, "epiphytes" grow upon another plant merely for physical support, and "opportunistic" microbes occasionally penetrate the endosphere of plants (Hardoim et al., 2008). The plant and the endophyte coexist in this interaction and greatly benefit one another (Ting et al., 2009). These endophytes are frequently rhizospheric; basal root zones with tiny crevices and the apical root zone may be the ideal sites for their linkage and subsequent entrance into the host (Gagne et al., 1987). They multiply throughout the host plant (Hallmann et al., 1997) and dwell in the cells, vascular system, or intercellular regions (Bell et al., 1995). While roots have the most excellent chance of colonization through the epidermis created by the lateral root system, endophytic microbes could penetrate through the stomata and transmit vertically to offspring via maternal seeds (Agarwal and Shende, 1987). It is indisputable from the "balanced antagonism" during asymptomatic colonization among the host and endophytic microorganisms that endophytes can survive inside the host without invoking any innate immunity and enhance their ability to sustain themselves by producing substances that are similar to those of plants (Schulz and Boyle, 2005). According to extensive research on the symbiotic association between endophytic microbes and their host plant, the plant safeguards and sustains the endophytes, which 'in return' deliver natural compounds with therapeutic potential (antiviral, antifungal, antibacterial, insecticidal, etc.) to uplift the former's productivity and sustainability in their natural habitat. Additionally, they defend host plants from phytopathogens by triggering the synthesis of plant secondary metabolites under adverse conditions (Azevedo et al., 2000; Strobel, 2003). Hence, they are now considered an essential component of biodiversity; the distribution of endophytic microflora varies depending on the host. They have been found inside nearly all vascular plants, notably those with medicinal properties that have been assumed to be linked to drug synthesis; several studies have shown that these endophytes represent a significant source of medicinal compounds (Zhang et al., 2006).

Endophytic microbes have a wide and diverse niche in plants, which leads to a complex relationship that implies mutualism, antagonism, and rarely parasitism (Nair and Padmavathy, 2014). They reside within the plant tissue, wherein numerous bacteria and fungi species constitute the "plant endomicrobiome," capable of triggering a number of cellular and physiological changes in the plant. Some relationships between plants and microbes are commensalism, whereby the plant incurs no harm, but the microbe benefits. The microbes and the plant interact through chemical signaling molecules released by the plants and discharge of corresponding microbial substances (phenols, steroids, taxol, xanthones, terpenoids, benzopyranones, isocoumarins, chinones, tetralones, cytochalasins, and enniatines, etc.), resulting in a two-way "crosstalk" that employs signal transduction. Once a link between plant and microbe is established, both organisms continue to monitor each others' physiology and adjust their behavior accordingly. Endophytic bacteria have a considerable advantage over plants' rhizospheric bacteria and provide more benefits than microorganisms outside of the plants and in the rhizosphere because they are in direct contact with the plant tissues (Araujo et al., 2002; Hardoim et al., 2015). Fungal endophytes spread into progeny via hyphal fragments or spores in above-ground tissues by pathogens (biotic dispersal agents) or air or water (abiotic dispersal agents) through parent plants, whereby the progeny become infected (Hodgson et al., 2014; Gagic et al., 2018), growing in the rhizosphere's nutrient-rich environment, harboring airborne pathogenic organisms (Sasse et al., 2018), enabling transmission of fungal endophytes across different host species (Wiewiora et al., 2015).

4 Interaction of secondary metabolites of the host and metabolites from endophytic microbes

The interaction between secondary metabolites of the host and metabolites from endophytic microbes is a complex and dynamic process that can result in diversified effects from beneficial to detrimental. One of the most fascinating aspects of endophytic microbes is their potential to synthesize bioactive compounds that might interact with secondary metabolites of their host. Plant secondary metabolites perform diverse functions in plants, including growth and development, inherent immunity (Piasecka et al., 2015), defense responses (Isah, 2019), stress adaptation (Yang et al., 2018), phytopathogen control, operating as signals for plant-microbe symbiosis, and transforming microbial communities linked to hosts (Guerrieri et al., 2019). Similarly, plant microbiomes are involved in many of the abovementioned processes, directly or indirectly modulating plant metabolism (Trivedi et al., 2020; Adeleke and Babalola, 2021; Ayilara et al., 2022). Plants can shape their microbiome by secreting an array of metabolites; consequently, the microbiome could affect the host plants' metabolome. Perhaps in medicinal plants, the stimulation of secondary metabolites through endophytes is a common phenomenon that can transform the rhizobiome (Sasse et al., 2018; Cotton et al., 2019). Recent research suggested that interactions between plants and their microbiomes could increase the biomass of *Salvia miltiorrhiza*, having a unique microbiome (*Sphingomonas, Pantoea, Dothideomycetes*, and *Pseudomonas*), as well as affect the synthesis of a novel bioactive compound "tanshinone" (Chen et al., 2018; Huang A. C. et al., 2019). Similarly, *Marmoricola* sp. and *Acinetobacter* sp. enhanced morphine content in *Papaver somniferum* via modulating expression of morphine biosynthesis genes (Ray et al., 2019), and *Phialemoniopsis cornearis, Fusarium redolens*, and *Macrophomina pseudophaseolina* influenced forskolin biosynthesis in a medicinal plant *Coleus forskohlii* (Mastan et al., 2019). Using a chemical recognition framework, plants can also recognize specific molecules released by microbiomes that trigger plants to build signaling networks, modify associated gene functions, and accumulate specific secondary metabolites (Tidke et al., 2019). Nevertheless, it is likely that a portion of these so-called "secondary metabolites" are actually the metabolic by-products of their endophytic microbes. Endophytic microbes can synthesize numerous secondary metabolites, such as paclitaxel (taxol), podophyllotoxin, camptothecin, and deoxypodophyllotoxin, which are also generated by plants (Etalo et al., 2018; Furtado et al., 2019; Pang et al., 2021). Consequently, it is crucial to distinguish which metabolites originated from the plant microbiome and which ones from the host.

The effects of microbial secondary metabolites on plants have been well-documented. Even though some pathogenic microbes secrete toxins that harm plants, such as fumonisins and AAL-toxins made by the *Fusarium* sp. and *Alternaria alternata f.* sp. (Chen et al., 2020), many microbes synthesize valuable secondary metabolites that promote plant growth; for example, *Bacillus tequilensis* SSB07 produces several phytohormones viz., gibberellins, IAA, and ABA which boosted growth and thermotolerance in soybean (Kang et al., 2019). Plant microbiomes can also produce numerous volatile organic compounds (aldehydes, alcohols, ammonia, ketones, terpenes, esters, etc.) that can influence plant development, communication, pathogen defense, and prevent herbivorous insects and parasitic nematodes (Kai et al., 2009; Ortíz-Castro et al., 2009; Zhang et al., 2020). Maggini et al. (2017) reported that the influence of the interaction between the medicinal plant *Echinacea purpurea* (L.) Moench and its endophytic microbes revealed that microbes could affect the synthesis of volatile organic compounds, phenylpropanoid, and alkamides in the host. Besides, plant-derived non-volatile secondary metabolites like flavonoids and coumarins shape the root microbiota. Furthermore, secondary metabolite "benzoxazinoids" could act as allelochemicals and natural pesticides on the root microbiome (Hu et al., 2018; Schütz et al., 2019; Jacoby et al., 2020). The symbiotic relationships of plants and endophytic microbes enable them to sustain safely, regardless of extremely harsh environments. The long-term coevolution within ecosystems due to this mutual association, each endophyte evolved a distinct range of hosts, allowing them to colonize a specific host group. The production of secondary metabolites, crucial for endophyte-host communication for mutual survival and their sensitivity to various habitats, is hypothesized to be influenced by the coevolution of endophytes and their host (Lind et al., 2017). Endophytes and their host plants share precursors in their corresponding secondary metabolite in biosynthesis pathways. However, endophytes may mimic the host pathways to establish their own metabolic route for secondary metabolites (Alam et al., 2021). Overall, it has been confirmed that despite their diversity, secondary metabolites are synthesized via a few shared biosynthetic, and the metabolomic pathways of endophytic microbes and their host are similar. Determining whether these secondary metabolites are produced by plants or due to symbiosis with endophytic microorganisms remains disputed. Therefore, understanding the processes influencing plant-microbiome assembly, signaling crosstalk in plant-microbiome communications, genetic controls on secondary metabolites, and how microbiomes and environment alter them are exciting research areas for the future.

5 Endophytes-mediated plant-microbe interactions to mitigate environmental cues

Plant phenotypic performance is determined by its genotype, environment, and interactions between genotype x environment. The phenotypic potential of a crop is fully expressed in a stress-free environment with no interference from any environmental factors. However, plants endure a range of perturbations categorized into two major groups: (i) weather extremes or abiotic stresses (drought, soil salinity, waterlogging, low and high temperatures, etc.) and (ii)

pathogenesis or biotic stresses (bacteria, viruses, fungi, insects, nematodes, etc.). Endophytes improve plants' stress tolerance by stimulating the synthesis of secondary metabolites (comprising or clinically useful molecules) through various sophisticated strategies (Tripathi A. et al., 2022; Liu et al., 2023). Moreover, they decrease the pressure caused by toxic heavy metals, reduce hazardous greenhouse gases, and limit pests' growth on plants through a plethora of other specific methods (through extracellular sequestration, modulating antioxidative enzyme activities, mineral nutrient uptake, degradation of pathways for reducing phytotoxicity, etc.) (Azevedo et al., 2000; Stępniewska and Kuźniar, 2013). Remediation by conventional strategies is quite expensive, laborious, and unsustainable, whereas plant-microbe-based approaches for remediation are remarkably potent, less intrusive, and sustainable (Anderson et al., 1993; Radwan, 2009). Additionally, endophytic plants with pertinent metabolic frameworks and degradation pathways toward diminishing phytotoxicity and optimizing decay can rejuvenate groundwater and wastelands (Weyens et al., 2009). Polyaromatic hydrocarbon (PAH) removal by endophytes is also successful; decreasing atmospheric carbon by storing carbon in plants' rhizospheres is likely a viable strategy (Wu et al., 2009). The schematic representation of the impact of biotic and abiotic perturbations on plants and how the integration of endophytic microbes helps to alleviate these perturbations is illustrated in Figure 2.

5.1 Endophytic microbes for abiotic stress tolerance in host medicinal plants

Abiotic factors like drought, salt, heat, freezing, heavy metal toxicity, hypoxia and anoxia, waterlogging, and nutritional imbalance are the most severe constraints leading to a drastic decline in crop production (about 51–82%), which hampers global food and nutritional security (Khare and Arora, 2015; Cooke and Leishman, 2016; Yadav et al., 2020; Del Buono, 2021; Raza et al., 2022; Kaur et al., 2023). These stressors have become more common over the past several decades, mainly as a result of the aberrant weather fluctuations triggered by climate change. Plants tolerate these stresses by modifying their physiological, molecular, and biochemical architecture to maintain homeostasis, including osmotic adjustment, nutrient absorption and assimilation, enzyme activity, membrane integrity, metabolic alterations, and most notably, photosynthesis (Moradtalab et al., 2018; Ahanger et al., 2019; Raza, 2021). Most of these imbalances in response to stress conditions are linked to phytohormone synthesis and distribution in plants' underground and aerial regions (Verma et al., 2016; Arif et al., 2021). Plants generate reactive oxygen species (ROS) as a consequence of these abiotic stresses, which cause severe cell injuries (Oktem et al., 2008; Hasanuzzaman et al., 2020). To counteract the damaging effects of these cues, plants respond physiologically and molecularly, which includes the synthesis of

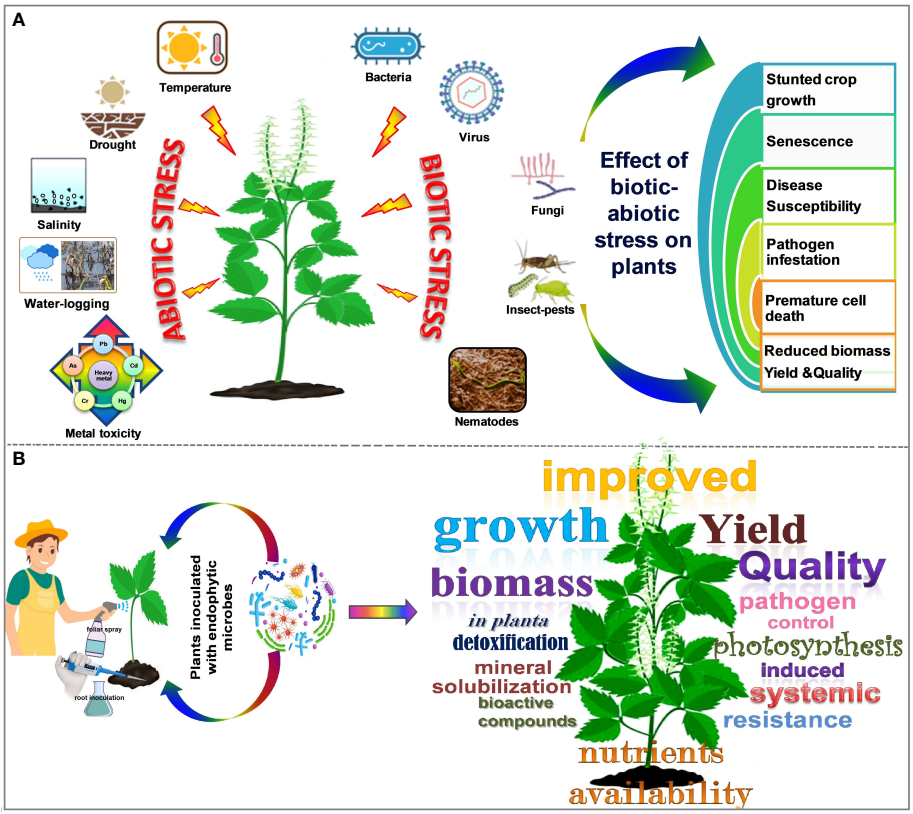

FIGURE 2
Impact of biotic and abiotic stresses on plants **(A)**, integration of endophytic microbes in plants for improving yield quality and tolerance against different stresses **(B)**.

essential proteins associated with metabolism, stimulation of cell signaling, and transcription factors governed through the expression of the majority of stress-tolerant genes that, in turn, are driven by multifaceted biomolecules (Hasanuzzaman et al., 2020; Raza, 2022).

Drought stress has a detrimental effect on plant growth and development, physiological, biochemical, and cellular metabolism, viz., cell membrane elasticity, fluidity, integrity, stomatal conductance, water potential, the structure of enzymes, proteins, amino acids, nucleic acids, etc. and, as well as the homeostasis of the agroecosystems (Kutasy et al., 2022; Noor et al., 2022). Plants modulate diverse cellular signaling pathways, including phytohormones, stress response proteins, osmolytes, and antioxidant enzymes for drought adaptation (Kosar et al., 2021). Numerous endophytes generate ACC deaminase (1-Amino Cyclopropane-1-Carboxylate), which assists its host plant in combating drought by interrupting the ET biosynthesis pathway and diminishing the ET levels, which in turn restricts stress signals. *Bacillus licheniformis* K11, having auxin and ACC deaminase-producing activities, mitigated drought's detrimental effects without using synthetic agrochemicals (Lim and Kim, 2013). Nevertheless, drought drastically reduces photosynthesis compared to plants' respiration (Vanlerberghe et al., 2016). Crop plants activate regulons like dehydration-responsive element-binding protein (DREB2) in response to temperature and drought stress (Nakashima et al., 2012). Furthermore, plants produce defensive chemicals in response to drought by mobilizing the metabolites critical for their osmotic adjustment. ABA-mediated stomatal closure may be crucial in controlling plant development by lowering other abiotic stressors, including osmotic stress (Waqas et al., 2012). An endophytic microbe, *Sinorhizobium meliloti* increased FeSOD and CU/ZnSOD, improving drought tolerance in alfalfa (Naya et al., 2007). Likewise, Meng and He (2011) reported an arbuscular mycorrhizal fungus (AMF) maximizes nutrient uptake and modulates metabolic activities (soluble sugar, chlorophyll, leaf subsurface, total phosphorous, total underground nitrogen and tanshinone content, and decreases the content of total aerial nitrogen) to boost drought tolerance in *Salvia*. Moreover, *Trichoderma hamatum* promoted drought tolerance in the Theobroma cacao plant by delaying drought-related stomatal conductance and net photosynthesis adjustments (Bae et al., 2009). Sziderics et al. (2007) claim that a fungus called *Piriformospora indica* increases resistance to osmotic stress by expressing the enzymes ACC-oxidase and lipid transfer protein. The synthesis of ROS under drought conditions often leads to premature cell death (Cruz de Carvalho, 2008), and antioxidant enzymes like catalase (CAT), polyphenol oxidase (PPO), and peroxidase (POD) scavenge ROS to prevent stress-induced damage (Zandalinas et al., 2018). These antioxidants also facilitate rejuvenation from water deficit and dehydration (Laxa et al., 2019). Similarly, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* improved drought tolerance in *Mentha piperita* (L.) by enhancing antioxidant enzymes, total phenolic content, and decreasing malondialdehyde (MDA) and proline content (Chiappero et al., 2019). Therefore, antioxidant-producing endophyte microbes are being explored further for favorable eco-friendly gains. Recent research demonstrates the beneficial effects of antioxidant enzymes in peppermint under severe drought (Chiappero et al., 2019; Asghari et al., 2020). Proline accumulation is a key strategy for promoting drought tolerance as it helps in the maintenance of protein structure and function to preserve membrane integrity (Kishor et al., 2005). Besides enhancing antioxidant activity, *Pseudomonas strains* and *Bacillus subtilis* also considerably increased proline levels and total soluble sugars in sweet corn (Zarei et al., 2020). Endophytic microbes have an inherent property to produce phytohormones such as gibberellins (GA), auxin, JA, SA, and ABA. These hormones could also be directly responsible for stimulating various defensive systems in host plants. It has been demonstrated that SA performs an important role in drought stress by altering nitrogen metabolism, inducing the generation of antioxidants, and glycine betaine accumulation, thereby conferring protection from stress (Khan et al., 2022). Shah et al. (2019) reported that *Piriformospora indica* promotes drought tolerance by synthesizing auxins and bioactive compounds in *Cymbidium aloifolium* (L.) Sw. Similarly, *Azospirillum brasilense* and *A. Chroococcum* enhanced drought stress tolerance *via* improving ABA, proteins, phenolic, soluble sugars, flavonoid, and oxygenated monoterpenes while reducing the activity of CAT and GPX in Peppermint (Asghari et al., 2020). An endophyte, *Paenibacillus polymyxa* strain CR1, increased Arabidopsis's dehydration-responsive genes (RD29), enabling the plants to face drought environments effectively (Liu et al., 2020). Likewise, the GOT9 strain of *Bacillus subtilis* in *Arabidopsis* stimulated the upregulation of several genes related to drought stress, specifically response-to-desiccation (*RD20* and *29B*), encodes dehydrin protein (RAB18), as well as 9-cis epoxy carotenoid dioxygenase (*NCED3*), consequently mitigating the physiological damage caused by drought (Woo et al., 2020). An erratic rainfall pattern due to climate change often functions as an acute stressor, leading to a rapid increase in available soil water, ultimately resulting in premature plant death. Wang et al. (2009) showed that *Penicillium griseofulvin* reduces water stress injury by improving the function of protective enzymes and osmotic levels, thereby increasing the ability to withstand salt, drought, and water stress in *Glycyrrhiza uralensis*. Furthermore, Orchard et al. (2016) claimed that the AMF *Glomus tenue* enhanced the tolerance of ryegrass (*Lolium rigidum*) plants during waterlogging stress. *Pseudomonas putida* inoculation in *Arabidopsis* regulated linked to key polyamine synthetic genes [*ADC* (arginine decarboxylase), *CPA* (N-carbamoyl putrescine amidohydrolase, *AIH* (agmatine iminohydrolase), SPMS (spermine synthase), *SPDS* (spermidine synthase) and *SAMDC* (S-adenosyl methionine decarboxylase)] affecting the amounts of polyamine in cells. The higher level of putrescine and free cellular spermidine is positively linked with water stress (Sen et al., 2018). Recently, *Endostemon obtusifolius* plant inoculated with *Paenibacillus polymyxa* and *Fusarium oxysporum* showed enhanced drought tolerance (Ogbe et al., 2023). In other studies *Streptomyces dioscori* SF1 strain enhanced drought, salinity and phytopathogen resistance in *Glycyrrhiza uralensis* via the production of ammonia, IAA, enzyme activities, potassium solubilization, nitrogen fixation and *Sphingomonas paucimobilis* ZJSH1 strain ameliorate drought, salt, and heavy metal toxicity in

Dendrobium officinale plants (Li X. et al., 2023; Li J. et al., 2023). Fungal endophytes, *Acrocalymma aquatic* and *Alternaria alstroemeriae* provide tolerance against drought-induced damage in *Isatis indigotica* simply because of synergistic effects on soil enzymatic activity, soil organic material, the biomass of roots, as well as epigoitrin levels (Li W. et al., 2023).

Salinity stress is the most critical abiotic stress that limits crop growth, development, and metabolism, resulting in reduced yield and productivity (Khan et al., 2020**;** Han et al., 2021). Worldwide, over 6% of the land is classified as saline; this percentage by 2050 is predicted to rise drastically owing to climate change, further aggravating the situation for farming systems. Salinity triggers osmotic pressure, inadequate nutrient supply, and increased ion accumulation beyond critical levels (Hasegawa et al., 2000; Hafeez et al., 2021; Saddiq et al., 2021). Human-generated causes such as irrigation with saline water, industrial pollution, and excessive use of harmful agrochemicals often increase salt stress (Zhu et al., 2019). Different strategies for enhancing plant development under salt stress are triggered by microbial inoculation, including the synthesis of ACC-deaminase, antioxidant enzymes, phytohormones, volatile organic compounds, osmoprotectant metabolites (glycine, proline, alanine, glutamic acid, threonine, serine, choline, betaine, aspartate, and organic acids), modifying ion transporters, which in turn preserves ionic, osmotic, and water homeostasis (Choudhary et al., 2022; Gamalero and Glick, 2022; Kumawat et al., 2022). When sodium ions accumulation reaches toxic levels, ROS is produced that severely damages cellular organelles, viz., mitochondria, chloroplasts, cell membranes, and peroxisomes, impairing plants' metabolic systems (Munns and Gilliham, 2015). Furthermore, high salinity declined the plant's water absorption capacity, resulting in poor stomatal activity and reduced cell growth as a consequence of lower cellular water levels. According to Liu et al. (2011), during salt stress, soluble protein content and peroxidase activity (POD) are modulated by endophytic fungi Botrytis *sp.* and *Chaetomium globosum* in Chrysanthemum morifolium. Recently, Jan et al. (2019) claimed salt stress tolerance in Euphorbia milii is promoted by the fungus *Yarrowia lipolytica*. An endophyte, *Brachybacterium paraconglomeratu* strain SMR20, ameliorates salt stress in *Chlorophytum borivilianum* via delaying chlorosis and senescence, enhanced foliar nutrient uptake, deamination of ACC, modifying ET, IAA, ABA, proline, and MDA (Barnawal et al., 2016). Similarly, *Glutamicibacter halophytocola* enhanced tolerance to high NaCl levels in *Limonium sinense* (Qin et al., 2018). de Zélicourt et al. (2018) have demonstrated that an endophyte *Enterobacter* sp. conquers the root and shoot tissues of *Arabidopsis* and promotes salt stress tolerance via producing 2-keto-4-methylthiobutyric. For instance, a bacterial endophyte, *Burkholderia phytofirmans* modified the gene expression for encoding signaling of cell surface component that signals bacteria of environmental stimuli and subsequently enhances their metabolism (Pinedo et al., 2015; Sheibani-Tezerji et al., 2015). Additionally, numerous bacteria in the plant endosphere modify ABA-mediated cell signaling systems as well as their production during salt stress, which may promote plant development. Similarly, *Pseudomonas* PS01 induced salinity tolerance by modulating the expression of stress-responsive genes *LOX2* (lipoxygenase) while reducing *GLY17* (glycogen synthase 17) and *APX2* (ascorbate peroxidase 2*)* in *Arabidopsis* (Chu et al., 2019). A critical factor in managing the nutrient profile and promoting plant growth during salt stress is enhanced microbe-mediated soil enzymatic activity (Shabaan et al., 2022). Recent research revealed that applying *Kosakonia sacchari* to soil can lower antioxidants like CAT, APX, GR (glutathione reductase), and SOD (superoxide dismutase) levels and oxidative stress markers like proline, MDA, and H_2O_2 (Shahid et al., 2021). Similarly, *Pseudomonas putida, Klebsiella* sp., *Alcaligenes* sp., and *P. cedrina* enhanced salt stress tolerance by decreasing the accumulation of MDA, proline, and H_2O_2 in *Medicago sativa* (Tirry et al., 2021). Karthikeyan et al. (2012) demonstrated that the inoculation of *Achromobacter xylosoxidans* in *Catharanthus roseus* reduced ET levels and increased the content of antioxidants such as APX, CAT, and SOD under salinity stress. Moreover, halophilic microorganisms control critical stress signaling pathways, such as proline, ABA, and MDA synthesis, ultimately minimizing stress impacts (Ayaz et al., 2022). Likewise, Semwal et al. (2023) reported that *Bacillus* strains NBRI HYL5, NBRIHYL8, and NBRIHYL9 with ACC deaminase activity, biofilm, phosphate solubilization, exo-polysaccharide and alginate generation properties enhanced abiotic stress tolerance in *Gloriosa superb*. Endophytic microbes, *Streptomyces umbrinus* EG1 *and S. carpaticus* EG2 promote root-shoot growth and chlorophyll content, thereby enhancing salt tolerance in *Iris persica and Echium amoenum* plants (Oloumi et al., 2023).

Like drought, salinity, and water stress, global agricultural production is greatly constrained by temperature extremes (heat, cold, and freezing). Heat stress alters the rate of osmotic adjustment, resulting in a disparity in water potential and a negative impact on metabolism and tissue damage. Plants have developed several tolerance mechanisms to cope with such temperature extremes, including the synthesis of heat-shock proteins (HSPs), pathways for eliminating ROS, and the stimulation of certain phytohormones (Khan et al., 2020; Haider et al., 2021; Raza et al., 2021a). The consequences of cold stress, including chilling temperatures of 15°C and freezing temperatures below 0°C, also severely impact the growth and development of plants (Habibi, 2015). Cold-induced abiotic stress profoundly affects all cellular processes in plants, including several signal transduction pathways by which these stressors are transduced, such as ABA, protein kinase, Ca^{2+}, protein phosphate, ROS components, etc. The plants' gene expression is altered in response to surviving cold stress, which modifies osmolytes levels, membrane lipids, phytohormones, proteins, ROS scavenging enzymes, and phenolic content (Ritonga et al., 2021; Saleem et al., 2021; Hwarari et al., 2022; Wei et al., 2022). For example, Fernandez et al. (2012) demonstrated that by balancing carbohydrate metabolism, stress-induced gene expression, and increased metabolite levels, *Burkholderia phytofirmans* PsJN bacterized grapevine showed enhanced tolerance against low temperature. Similarly, Su et al. (2015)) discovered that treating *Arabidopsis thaliana* with *Burkholderia phytofirmans* PsJN during cold stress curtailed the plasmalemmas' disruption and strengthened the mesophyll cell wall. In other studies, PsJN ameliorated cold tolerance in *Vitis vinifera* with an improved accumulation of proline, aldehydes (ALD), and MDA

along with *PAL* (phenylalanine ammonia-lyase) and *STS* (stilbene synthase) genes (Theocharis et al., 2012) as well as improved CO2 fixation, starch and phenolics (Barka et al., 2006). However, the *Dichanthelium lanuginosum* plant relies on endophytic fungi Curvularia protuberate *in three-way mutualistic interactions with a virus (virus-fungal endophyte-plant)* for survival at high soil temperatures (Márquez et al., 2007).

Metal toxicity is increasing globally due to anthropogenic activities that have not only polluted the soil but also pose a severe threat to human health when they reach the food chain and are biomagnified. Heavy metals like arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), aluminum (Al), copper (Cu), and zinc (Zn) supplied through irrigation significantly influenced soil dynamics (Nazli et al., 2020; Mehmood et al., 2019; Bashir et al., 2021; Haseeb et al., 2022). The deleterious effects of heavy metal ions on tissues, such as the stimulation of necrosis and chlorosis, inhibition of chlorophyll biosynthesis, and membrane lipid degradation, may significantly impact crop productivity (Takasaki et al., 2010; Raza et al., 2021b; Raza et al., 2022). Plants have evolved sophisticated mechanisms, including hyperaccumulation, tolerance, exclusion, and chelation with organic compounds as the fundamental strategies. Research findings have suggested that endophytic microorganisms play a significant role in boosting resilience to metal toxicity via complex mechanisms, including intracellular accumulation, sequestration, extracellular precipitation, and conversion of toxic metals to a negligible or non-toxic form (Rajkumar et al., 2009; Ma et al., 2016; Mishra et al., 2017). Interestingly, Domka et al. (2019) discovered a fungal endophyte called *Mucor* sp. significantly strengthens the ability of *Arabidopsis arinosa* to tolerate metal toxicity. Furthermore, an endophyte, *Bacillus* sp. *SLS18* diminishes the toxicity of heavy metals by accumulating biomass in the root tillers and leaves of *Solanum nigrum* and *Phytolacca acinosa* (Luo et al., 2012). Similarly, microbial endophytes *Paenibacillus hunanensis* strain CIMAP-A4 and BAC-7 improved arsenic tolerance in *Bacopa monnieri* (L.) via IAA production and biofilm formation (Tripathi P. et al., 2022). Xu et al. (2016) claimed that *Agrobacterium* spp. *and Bacillus* spp. reduced arsenate to arsenite in *Pteris vittata* (L.). An endophyte, *Paenibacillus* relieved heavy metal toxicity in *Tridax procumbens* (Govarthanan et al., 2016) as well as helped in the removal of PAHs phytotoxicity via biodegradation of phenanthrene through co-metabolism in *Plantago asiatica* (Zhu et al., 2016). Endophytic microorganisms can also diminish heavy metal-induced oxidative-stress damage (Wan et al., 2012). The toxic effects of Cd accumulation were synergistically controlled by various plant metabolic defensive systems, including hyperaccumulators, detoxification routes, and antioxidative processes by bacterial endophytes, *Klenkia*, *Modestobacter, Sphingomonas* in *Lonicera japonica* (Xie et al., 2023) *Pseudomonas* strain E3 in *Solanum nigrum* (Chi et al., 2023).

5.2 Endophytic microbes for biotic stress tolerance in host medicinal plants

Biotic stresses are known to be affected by abiotic stress conditions in terms of their incidence and dissemination (Scherm and Coakley, 2003; McDonald et al., 2009; Ziska et al., 2010; Peters et al., 2014). Through modifications to plant physiology and defense mechanisms, these stress conditions also directly impact plant-pest interactions (Scherm and Coakley, 2003; Duveiller et al., 2007; Gimenez et al., 2018). Several biological agents, including bacteria, fungi, viruses, weeds, insects, and nematodes, are the major stress factors that tend to increase ROS, affecting how well plants operate physiologically and molecularly and decreasing agricultural productivity. Plant-parasitic nematodes can attack all parts of the plant, although they predominantly harm the root system and spread disease through the soil. They cause stunting and wilting, which are symptoms of inadequate nutrition. Although they seldom kill, their hosts' viruses can harm plants systemically, producing stunting, chlorosis, and malformations in different regions of the plant. Piercing-sucking insects can spread viruses to plants via their styles. In combination with bacteria, fungi cause a more severe impact, resulting in vascular wilts, leaf spots, and cankers (Schlenker and Roberts, 2009). Insects may physically harm plants severely, including the leaves, stems, bark, and flowers, while infected plants can transmit viruses and bacteria to healthy plants via insects.

In many cases, weeds can take over habitats faster than certain attractive plants because they proliferate and generate many viable seeds. Inhibiting the growth of desirable plants, such as crops or flowers, is not done directly by weeds, which are viewed as undesired and unproductive plants, but rather through competing with the desirable plants for nutrients and space. Through antagonistic action, endophytic microbes can strengthen plants' defense systems against pathogen invasion (Miller et al., 2002; Gunatilaka, 2006). Additionally, they are said to improve the health of the soil and crops by assisting plants in coping with biotic stress. Therefore, using endophytic microbes as biofertilizers and biocontrol agents has established a natural alternative to harmful chemicals for crop production and alleviating biotic stress. In general, two mechanisms, systemic-acquired resistance (SAR) and induced systemic resistance (ISR), confer plant resistance to pathogens. ISR is defined as the plants' innate resistance primarily mediated by beneficial microbes via modulating root immunity, root colonization, and the production of specific elicitors like volatile organic compounds, siderophores, polysaccharides, enzymes, and phytohormones, whereas SAR is considered as the plants' acquired resistance (Olowe et al., 2020; Hamid et al., 2021).

A wide range of pests and pathogens can be successfully combated using the SAR and ISR mechanisms (Vlot et al., 2021; Meena et al., 2022; Yu et al., 2022). Even though multiple studies have shown that endophytic microbes regulate diversified physiological, cellular, and molecular functions in plants and aid in their survival when attacked by pathogens (Teixeira et al., 2019; Olowe et al., 2020; Castiglione et al., 2021; Yu et al., 2022), unfortunately, the fundamental mode of action of pathogenesis has yet to be discovered. The results of comprehensive investigations show that developing resistance to several pathogens, such as bacteria, viruses, and fungi, relies on complex mechanisms that may operate simultaneously (Yu et al., 2022), including stimulation of several defense response genes and

enzymes (CAT, GPX (guaiacol peroxidase), APX, GR, SOD, and POD), accumulation of hormones (auxin, GA, ET, JA, and SA), glucanases, sugars, chitinases, PR proteins, secondary metabolites and osmolytes which in turn play a direct role in limiting the growth and spread of pathogens (Baxter et al., 2014; Pieterse et al., 2014; Conrath et al., 2015; Camejo et al., 2016; Guo et al., 2019; Olowe et al., 2020; Luo et al., 2022). Previous research confirmed that endophytes significantly control the host's gene expression, physiological responses, and defense-related processes in plants (Van Bael et al., 2012; Estrada et al., 2013; Salam et al., 2017). For instance, JA and SA prove to be very helpful in plant stress responses against phytopathogens (Mejía et al., 2008; Ren and Dai, 2012; Khare et al., 2016). Furthermore, the gibberellins synthesized by endophytes boost insect and pathogens' resistance via SA and JA pathways (Waqas et al., 2015a). *Fusarium solani*, an endophyte, induces systemic resistance to the pathogenic fungi *Septoria lycopersici* by promoting the expression of genes associated with the pathogenesis (Kavroulakis et al., 2007). Additionally, some endophytic microbes produce an array of bioactive compounds that might improve the plants' resistance to different phytopathogens such as *Macrophomina phaseolina*, which causes charcoal rot disease via siderophores-synthesizing (Arora et al., 2001), *Vertcillium* wilt (Mercado-Blanco et al., 2004), *Cadosporium sphaerospermum and C. cladosporioides* through the synthesis of pathogen-toxic cadinane sesquiterpenoids (Silva et al., 2006), antagonistic to pathogenic fungi by toxic chemical "trichothecin" (Zhang et al., 2010), *Fusarium oxysporum* and *F. Solani* (Yang et al., 2012), *Rhizoctonia solani, Pythium myriotylum, Phytophthora capsici, Colletotrichum gloeosporioides*, and *Radopholus similis* by producing volatile substances(Sheoran et al., 2015) as well as inhibiting pathogenic fungi by releasing some toxins (Wang et al. (2012). According to Strobel et al. (1999), an endophytic microbe *Cryptosporiopsis cf. quercina* in *Triptergyium wilfordii* (thunder god vine) produces "cryptocin" and "cryptocandin," which are poisonous to the host plant's pathogenic fungus *Pyricularia oryzae*. Moreover, Cao et al. (2009) reported endophytes, *Stachybotrys elegans, Choiromyces aboriginum, and Cylindrocarpon* linked with cell wall-disruptive enzymes combat pathogenic fungi in *Phragmites australis* plant. Microbial endophytes viz., *Cohnella* sp., *Paenibacillus* sp., and *Pantoea* sp. induced plant defense mechanism against anthracnose disease in *Centella asiatica* (Rakotoniriana et al., 2013). In other studies, *Bacillus amyloliquefaciens* improved tolerance to root-rot in *Panax notoginseng* (Ma et al., 2013), phytophthora blight resistance in *Ginkgo biloba* (Yang et al., 2014), and inhibited multiple phytopathogens in *Curcuma longa* via synthesizing 'iturin' and 'surfactin' (Jayakumar et al., 2019). Hong et al. (2018) reported that microbial endophytes, *Stenotrophomonas maltophilia* and *Bacillus* sp. suppressed phytopathogens growth in *Panax ginseng*. Fungal endophytes, *Penicillium chrysogenum* and *Alternaria alternate* enhanced tolerance against pathogenic microorganisms in *Asclepias sinaica* by producing extracellular enzymes viz., amylase, pectinase, xylanase, cellulase, gelatinase, and tyrosinase (Fouda et al., 2015). In another study, *Withania somnifera* plants inoculated with *Talaromyces trachyspermus* effectively combat phytopathogens which resulted from the antagonistic activity of endophytes and enhanced IAA, phosphate solubilization, and siderophore synthesis (Sahu et al., 2019). Similarly, Jiang et al. (2018) showed that *Bacillus velezensis* increased plants' resistance to gray mold disease caused by *Botrytis cinerea* by activating antioxidant-mediated defense signaling genes SOD, POD, CAT, and SA-signaling genes viz., NPR1 (non-expressor of pathogenesis-related genes) and PR1 (pathogenesis-related protein1). These findings suggest that endophyte priming triggers molecular and biochemical changes that prevent pathogen invasions of plants. Interestingly, Kumar et al. (2016) identified that the inoculation of the endophyte *Peanibacillus lentimorbus* in *Nicotiana tabacum* reduced the prevalence of CMV (cucumber mosaic virus) by augmenting the expression of genes related to stress *PR1*, *AsSyn* (asparagine synthetase), *Gluc* (b-1,3-glucanase), *BR-SK1*(brassinosteroid signaling kinase 1), *TCAS* (tetra-hydrocannabinolic acid synthase), *ZF-HD* (zinc finger-homeodomain), *RdRP2* (RNA dependent RNA polymerase), and antioxidants (CAT, SOD, APX, and GPX). Recently, Azabou et al. (2020) reported that an endophyte *Bacillus velezensis* OEE1 prevents *Verticillium* wilt disease in olive plants by producing antifungal volatile organic molecules (benzene acetic acid, 1-decene, phenyl ethyl alcohol, tetradecane, and benzaldehyde). Likewise, *Microbacterium* sp. SMR1 enhanced downy mildew tolerance in *Papaver somniferum* (L.) via protein modification, differential expression of transcripts related to signal transduction, transcription factors, and SA-dependent defense pathway (Ray et al., 2021). Many researchers showed the ability of both bacterial and fungal endophytes to control diseases and phytopathogens by synthesized volatile and non-volatile compounds, soluble antifungal metabolites and by specific mechanisms including activation of defense enzymes and PR proteins associated with ISR, JA/ET mediated disease resistance, antagonism, antimicrobial, antioxidant, and anti-proliferative properties, production of IAA, siderophores.and β-1,3-glucanase, proteolytic activity, chitinase and cellulose synthesis in diverse medicinal plants including *Chloranthus elatior*, *Taxillus chinensis, Salvia miltiorrhiza, Curcuma longa, Dioscorea bulbifera, Viola odorata, Cremastra appendiculata*, *Angelica sinensis*, *Cornus florida, Nicotiana tabacum, Zingiber zerumbet and Piper betle* (Harsha et al., 2023; Jiao et al., 2023; Manasa et al., 2023; Mei et al., 2023; Rotich and Mmbaga, 2023; Salwan et al., 2023; Santra and Banerjee, 2023; Sharma et al., 2023; Song et al., 2023; Thankam and Manuel, 2023; Wang et al., 2023; Yehia, 2023; Zou et al., 2023).

It is well documented that endophytic microbes improve host plant resistance to insect herbivores primarily by synthesizing a variety of alkaloid-based protective chemicals in the plant tissue or by changing the nutritional quality of the plant. Eventually, endophytes such as *Chaetomium cochliodes, Trichoderma viride*, and *Cladosporium cladosporioide* are known to facilitate insect resistance in creeping thistle (Gange et al., 2012) and red spruce (Sumarah et al., 2010). An endophyte, *Epichloë coenophiala* AR584, showed enhanced herbivore resistance in *Lolium arundinaceum* (Schreb.) *via* the production of alkaloids which provide anti-herbivore defenses, stoichiometry, photosynthesis, and transpiration rates, and stomatal conductance (Johnson et al., 2023). Endophytes function as an acquired plant immune system,

taking up space, fighting diseases that may otherwise attack the host, and delaying or deterring herbivores' infection. For instance, Bittleston et al. (2011) showed that an endophyte *Leucocoprinus gongylophorus* produces compounds that are antagonistic fungal-ants' symbionts to boost insect resistance. Furthermore, an endophyte *Chaetomium Ch1001* increases resistance to the root-knot nematode by synthesizing ABA that affects the insect juveniles' second-stage motility (Yan et al., 2011). Additionally, endophytes *Beauveria bassiana* and *Lecanicillium dimorphum* improve insect resistance by altering cell division-related protein expressions in the host plant (Gómez-Vidal et al., 2009). Daungfu et al. (2019) found that bacterial endophytes *Bacillus subtilis* LE24, B. amyloliquefaciens LE109, and B. tequilensis PO80 from the citrus plant with antagonistic properties against phytopathogens might be helpful in the biocontrol of diseases. Diab et al. (2023) recently claimed that endophytic microbes, *Streptomyces* sp. *ES2, Streptomyces, Nocardioides*, and *Pseudonocardia* produce metabolites that act as natural biocontrol agents against insects in *Artemisia herba-alba* and *A. judaica* plants. A list of endophytic microbes enhancing abiotic and biotic stress tolerance and associated mechanisms in the host plants are shown in Table 1 (bacterial endophytes) and Table 2 (fungal endophytes).

These studies confirm that endophytes may increase the hosts' tolerance to pathogens through diverse methods. In summary, while endophytes invade plant tissues, they impact the interactions between both the endophytes and the pathogens, perhaps causing facilitation (positive stimulation of pathogens), negatively reinforcing host resistance, or exhibiting merely no effect (Suryanarayanan et al., 2009; Adame-Alvarez et al., 2014; Schmidt et al., 2014). Nevertheless, it is unclear how endophytic entomopathogenic fungi invade and are colonized; this requires additional research for confirmation. Plants sense the information signal of stresses and respond accordingly to activate specific molecules to combat such stressors. Furthermore, the behavior of a given plant species or cultivar may vary, plant responses are frequently organ-dependent, and findings acquired with whole plants are sometimes misleading.

6 Mechanisms mediating plant-microbe interactions to alleviate biotic-abiotic stresses

Plants have developed a multitude of physiological (membrane integrity, organelle structural changes, osmotic adjustments, photosynthesis, and respiration, cell and tissue survival, reclamation, increased root-shoot ratio, increased root hair length and density, photosynthates translocations, antibiosis, hypersensitivity, etc.), biochemical (phytohormones synthesis, proline, protein levels, increased chlorophyll accumulation, ACC-deaminase production, antioxidant enzymes accumulation, ion exclusion, generation of heat-shock proteins, protein denaturation, membrane lipid saturation/unsaturation, synthesis of allelochemicals. etc.), and cellular (sensing of stress signals, signaling pathways, ROS generation, SAR, ISR, modulating expression of stress-responsive genes and proteins, regulation of transcriptional factors, etc.) adaptive mechanisms to withstand stressful environments (Figure 3). Endophytes live close interactions with plants and penetrate host plants through their roots, seeds, leaves, and stems to colonize their internal tissues. During the initial phases of colonization, endophytes produce exopolysaccharides (EPS), which aid in adhesion to the root surface and shield them from oxidative damage (Wan et al., 2012). During the fungal transmission of phosphate and nitrogen, the AMF mycelial system mainly spreads around plant roots and facilitates nutrient intake that promotes plant growth in adverse circumstances. Moreover, by maintaining plants' homeostasis, endophytes diminish water stress damage and trigger regulons like DREB2, stress-induced gene expression, better CO_2 fixation, starch and phenolics, HSPs generation, balancing carbohydrate metabolism, disrupting plasmalemmas, and reinforced cell walls to face of drought and temperature (heat and cold) and strengthen the functioning of protective enzymes and osmosis delivering plants more resilience plants to various abiotic stressors including drought, waterlogging and salinity (Barka et al., 2006; Nakashima et al., 2012; Raza et al., 2021a). Different strategies for enhancing salt stress tolerance triggered by microbial inoculation are synthesis of antioxidant enzymes, phytohormones, ACC-deaminase, volatile organic compounds, osmoprotectant compounds (glycine, proline, alanine, glutamic acid, threonine, serine, choline, betaine, aspartate, and organic acids), altering ion transporters, resulting in water, ionic, and osmotic homeostasis. They further strengthen plant resistance to heavy metal toxicity through transport, cell wall development, redox communication, and intra/extra-cellular trapping. Most of these abnormalities in reaction to stressful situations are attributed to the creation and dissemination phytohormones in plants' subterranean and aerial parts (Verma et al., 2016; Arif et al., 2021). Phytohormones also operate as signal molecules between endophytic microbes and plants, regulating structural and morphological changes necessary for plant growth and to accelerating total root biomass through expanding root length and surface (Spaepen et al., 2007). For instance, *Sphingomonas* sp. isolated from *Tephrosia apollinea* augment host plant growth through IAA production (Khan et al., 2014), *Pseudomonas spadiceum* lowers osmotic stress by producing GA (Waqas et al., 2012) and *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas*, and *Arthrobacter* sp. generate cytokinins that perform an indispensable function in plants including apical dominance, chloroplast development, cell growth and transformation, senescence prevention, and plant-pathogen interactions (De Hita et al., 2020). Endophytes, including *Rhizobium* sp., *Azospirillum brasilense*, *Burkholderia cepacia*, *Acetobacter diazotrophicus*, and *Klebsiella oxytoca* have the potential of biological nitrogen fixation that supply alternate nitrogen for farming (Kong and Hong, 2020). Additionally, some endophytes, such as *Pseudomonas fluorescens* have the potential to dissolve insoluble phosphates or to liberate organic phosphates through the manufacturing of citric, malic, and gluconic acids (Otieno et al., 2015). Endophytes are also successful in bioremediation (Ayilara et al., 2023) through various methods, such as reducing heavy metal stress (Zhang et al., 2012) and removing dangerous greenhouse gases (Stępniewska and Kuźniar,

10.3389/fpls.2023.1250020

TABLE 1 Biotic-abiotic stress tolerance and plant defense mechanism conferred by endophytic bacteria in host medicinal plants.

Endophytic microbes	Host medicinal plants	Stress type	Plant defense mechanism	References
Sinorhizobium meliloti	*Medicago sativa* (L.)	Drought	FeSOD and CU/ZnSOD are up-regulated	Naya et al., 2007
Bacillus amyloliquefaciens, Pseudomonas fluorescens	*Mentha piperita* (L.)	Drought	Enhance antioxidant enzymes (POX and SOD), total phenolic content, decrease MDA and proline	Chiappero et al., 2019
Azospirillum brasilense, Azotobacter chroococcum	*Mentha piperita* (L.)	Drought	Improve ABA, proteins, SOD, phenolic, soluble sugars, flavonoid, and oxygenated monoterpenes, while reducing the activity of CAT and GPX	Asghari et al., 2020
Fusarium oxysporum (EOLF-5)	*Endostemon obtusifolius* (E. Mey. ex Benth.) NE Br.	Drought	Production of ammonia and siderophore, free radical scavenging ability	Ogbe et al., 2023
Acrocalymma aquatica Alternaria alstroemeriae	*Isatis indigotica* Fortune	Drought	Via synergistic effects on soil enzymatic activities, organic matter, root biomass, epigoitrin content	Li W. et al., 2023
Pseudomonas putida, Klebsiella sp., *Alcaligenes* sp., *P. cedrina*	*Medicago sativa* (L.)	Salinity	Decrease accumulation of MDA, proline and H_2O_2	Tirry et al., 2021
Enterobacter sp. *SA187*	*Citrus* (L.)	Salinity	Ethylene stimulation	de Zélicourt et al., 2018
Burkholderia phytofirmans	*Arabidopsis Thaliana* (L.) Heynh.	Salinity	Improve proline and modulate genes responsible for ABA signaling (RD29, RD29B), antioxidant linked(APX2), glyoxylate pathway (GYLI7), reduce expression of JA signaling gene (LOX2)	Pinedo et al., 2015
Bacillus megaterium	*Arabidopsis Thaliana* (L.) Heynh.	Salinity	Enhanced CYP94B3 (linked with JA-Ile catabolism)	Erice et al., 2017
Bacillus amyloliquefaciens	*Arabidopsis Thaliana* (L.) Heynh.	Salinity	Up-regulation of genes responsible for antioxidant (POX and GST), ET-signaling (ACS7, ACS11, ACS2, and ACS8), JA-signaling (LOX), down-regulating ABA-signaling (NCED3, ABI1, NCED4, and MARD1)	Liu et al., 2017
Brachybacterium paraconglomeratu strain SMR20	*Chlorophytum borivilianum Santapau* & R.R.Fern.	Salinity	Deamination of ACC, delayed chlorosis and senescence, reducing stress ethylene, modifying IAA and ABA levels, alteration of leaf pigments, proline, malondialdehyde, and enhanced foliar nutrient uptake	Barnawal et al., 2016
Achromobacter xylosoxidans	*Catharanthus roseus* (L.) G. Don	Salinity	Increased germination percentage and root weight under saline conditions	Karthikeyan et al., 2012
Glutamicibacter halophytocola	*Limonium sinense* (Girard) Kuntze	Salinity	Improved tolerance to high NaCl concentration	Qin et al., 2018
Streptomyces umbrinus EG1 and *Streptomyces carpaticus* EG2	*Iris persica* L. and *Echium amoenum* Fisch. & C.A.Mey.	Salinity	Promotes root and shoot growth and chlorophyll content	Oloumi et al., 2023
Bacillus, *Brevibacillus*, *Agrobacterium*, and *Paenibacillus*	*Vicia faba* L.	Salinity	By decreasing growth parameters and metabolic activities, and increasingcproline content and of antioxidant enzymes activity	Mahgoub et al., 2021
Bacillus subtilis,B. tequilensis, B. licheniformis, B. sonorensis Burkholderia sp., *Acinetobacter pittii*	*Artemisia annua* (L.)	Water, drought, and salinity	Improving artemisinin yield and content by siderophore production, phosphate solubilization, IAA production, ACC deaminase activity and nitrogen fixation	Tripathi et al., 2020
Bacillus sp. *strain* NBRI HYL5, NBRIHYL8, NBRIHYL9	*Gloriosa superba* L.	Abiotic stress	ACC deaminase activity, biofilm, phosphate solubilization, IAA, exo-polysaccharide and alginate generation	Semwal et al., 2023
Burkholderia phytofirmans strain PsJN	*Vitis vinifera* (L.)	Chilling	Enhancement of chilling tolerance	Barka et al., 2006

(Continued)

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TABLE 1 Continued

Endophytic microbes	Host medicinal plants	Stress type	Plant defense mechanism	References
Burkholderia phytofirmans (PsJN)	*Vitis vinifera* (L.)	Cold	Balancing carbohydrate metabolism	Fernandez et al., 2012
Bacillus sp. SLS18	*Solanum nigrum* (L.), *Phytolacca acinosa* Roxb.	Heavy metal toxicity (Mn and Cd)	Improving biomass and root tillers accumulation	Luo et al., 2012
Pseudomonas koreensis AGB-1	*Miscanthus sinensis* Andersson	Heavy metal toxicity (Zn Cd As and Pb)	Through extracellular sequestration, increased Catalase and SOD activities	Babu et al., 2015
Serratia nematodiphila LRE07	*Solanum nigrum* (L.)	Heavy metal promoted oxidative injury	Improving essential mineral nutrient uptake and antioxidative enzymes activities	Wan et al., 2012
Paenibacillus hunanensis strain CIMAP-A4, BAC-7	*Bacopa monnieri* (L.)	Heavy metal toxicity (Arsenic)	IAA production and biofilm formation	Tripathi P. et al., 2022
Bacillus gaemokensis strain CIMAP-A7	*Andrographis paniculata* (Burm.f.) Nees	Phytotoxicity (Atrazine)	By reducing stress enzymes, proline, and malondialdehyde accumulation	Tripathi et al., 2021
Paenibacillus sp.	*Tridax procumbens* (L.)	Heavy metal toxicity	Relieved heavy metal stress in plants	Govarthanan et al., 2016
Agrobacterium spp. *and Bacillus* spp.	*Pteris vittata* (L.)	Heavy metal toxicity (Arsenic)	Reduced arsenate to arsenite	Xu et al., 2016
Paenibacillus sp.	*Plantago asiatica* (L.)	Phytotoxicity Polycyclic aromatic hydrocarbons (PAHs)	Biodegradation of phenanthrene through co-metabolism	Zhu et al., 2016
Klenkia, Modestobacter, Sphingomonas	*Lonicera japonica* thunb	Heavy metal-toxicity	The toxic effects of Cd accumulation were synergistically controlled by various plant metabolic defensive systems viz., detoxification routes and antioxidative processes	Xie et al., 2023
Pseudomonas strain E3	*Solanum nigrum* L.	Heavy metal-toxicity	By increasing cadmium (Cd) extraction via hyperaccumulator	Chi et al., 2023
Pseudomonas fluorescence	*Olea europaea* (L.)	Disease	Antagonism	Mercado-Blanco et al., 2004
Penicillium citrinum LWL4, *Aspergillus terreus* LWL5	*Helianthus annuus* (L.)	Disease	Modulation of antioxidants, defense hormones, and functional amino acids	Waqas et al., 2015b
Bacillus amyloliquefaciens	*Nicotiana tobaccum* (L.)	Disease	Regulate expression of PPO, JA/ET signaling	Jiao et al., 2020
Microbacterium sp. SMR1	*Papaver somniferum* (L.)	Disease (Downy mildew)	By protein modification, differential expression of transcripts related to signal transduction, transcription factors, SA-dependent defense pathway	Ray et al., 2021
Bacillus amyloliquefaciens	*Panax notoginseng* (Burkill) F.H. Chen.	Disease (Root-rot)	Antagonistism	Ma et al., 2013
Cohnella sp., *Paenibacillus* sp. *and Pantoea* sp.	*Centella asiatica* (L.) Urban	Disease (Anthracnose)	Induction of plant defense mechanism, antagonism	Rakotoniriana et al., 2013
Bacillus amyloliquefaciens	*Ginkgo biloba* (L.)	Disease (*Phytophthora* blight)	Produced antibiotics and induced systemic resistance	Yang et al., 2014
Bacillus sp.	*Curcuma longa* (L.)	Disease	Induced host disease resistance	Jayakumar et al., 2019

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TABLE 1 Continued

Endophytic microbes	Host medicinal plants	Stress type	Plant defense mechanism	References
Stenotrophomonas sp., *Serratia marcescens, Bacillus thuringiensis*	*Cornus florida* L.	Disease	Activation of defense enzymes and PR proteins associated with induced systemic resistance	Rotich and Mmbaga, 2023
Bacillus amyloliquefaciens	*Nicotiana tabacum* L.	Disease	By activation of JA/ET mediated disease resistance	Jiao et al., 2023
Bacillus spp., *Klebsiella aerogenes, Pseudomonas fuscovaginae, Enterobacter tabaci, Pantoea* spp., *Kosakonia* spp.	Zingiber zerumbet (L) Smith	Disease	Antagonism, biocontrol agents for soil-borne soft-rot disease (*Pythium* spp.)	Harsha et al., 2023
Bacillus velezensis	*Piper betle* L.	Disease	Through induction of defense enzymes	Manasa et al., 2023
Peanibacillus lentimorbus B-30488	*Nicotiana tobaccum* (L.).	Virus	Targets antioxidant enzymes and PR genes	Kumar et al., 2016
Streptomyces sp. *ES2, Streptomyces, Nocardioides, and Pseudonocardia*	*Artemisia herba-alba* Asso, *A. judaica* L.	Insect	By producing metabolites that acts as natural biocontrol agents	Diab et al., 2023
Bacillus subtilis, Myxormia sp.	*Angelica sinensis* (Oliv.) Diels	Pathogenic fungi	Secretes some toxic chemicals harmful to pathogens *viz., Fusarium oxysporum, F. Solani*	Yang et al., 2012
Bacillus subtilis LE24, B. amyloliquefaciens LE109, B. tequilensis PO80	*Citrus* (L.)	Phytopathogen	Pathogen biocontrol	Daungfu et al., 2019
Pseudomonas putida BP25	*Piper nigrum* (L.)	Phytopathogen	Suppression of pathogens	Sheoran et al., 2015
Bacillus velezensis OEE1	*Olea europaea* (L.)	Pathogenic fungi: *Verticillium dahliae*	Producing antifungal lipopeptides and secondary metabolites	Azabou et al., 2020
Phyllobacterium myrsinacearum	*Epimedium brevicornu* Maxim	Phytopathogenes	Antagonistism	He et al., 2009
Stenotrophomonas maltophilia and Bacillus sp.	*Panax ginseng* C.A. Meyer	Phytopathogenic fungi	Suppressed pathogen growth	Hong et al., 2018
Pantoea, Agrobacterium, Pseudomonas, Bacillus sp., *Colletotrichum* sp., *Trichothecium roseum, Phomopsis liquidambari*	*Artemisia annua* L.	Phytopathogens	Antagonistic activity	Zheng et al., 2021
Pseudomonas sp. SWUSTb-19	*Aconitum carmichaelii* Debx	Pathogenic fungi	Antagonism, bio-control agent against southern blight	Zou et al., 2023
Bacillus amyloliquefaciens SNMB1	*Salvia miltiorrhiza* Bunge	Phytopathogens and salinity	Antifungal activity	Mei et al., 2023
Kocuria rocea, Bacillus subtilis, Brevibacterium casei, Actinobacterium JS14 strain, B. Amyloliquefaciens, B. velezensis	*Curcuma longa* L.	Phytopathogens and salinity	Antimicrobial properties, producing hormones viz., IAA, GA, CT and secondary metabolites	Thankam and Manuel, 2023
Clonostachys pseudochroleucha, Parathyridaria percutanea, Curvularia lunata	*Dioscorea bulbifera* L.	Phytopathogens	Phosphate solubilisation, siderophore, IAA, and HCN production, amylase, lipolytic, protease, cellulolytic and chitinase activity	Sharma et al., 2023

2013). In heavy metal-contaminated soil, bacterial root endophytes associated with the medicinal plant *Festuca rubra* produce siderophores (hydroxamate and catechol) that accelerate host plant development (Grobelak and Hiller, 2017).

Biocontrol strategies by endophytic microbes exist directly through pathogen control or indirectly utilizing systemic plant resistance (Santoyo et al., 2016). They produce different kinds of siderophores (phenolate, hydroxamate, carboxylate, etc.) to

TABLE 2 Biotic-abiotic stress tolerance and plant defense mechanism conferred by endophytic fungi in host medicinal plants.

Endophytic microbes	Host medicinal plants	Stress type	Plant defense mechanism	References
Piriformospora indica	*Cymbidium aloifolium* (L.) Sw.	Drought and pathogen	By synthesizing auxins and bioactive compounds	Shah et al., 2019
Trichoderma hamatum DIS 219b	*Theobroma cacao* (L.)	Drought	Drought-induced adaptation in stomatal closure and net photosynthesis	Bae et al., 2009
Paenibacillus polymyxa (EORB-2)	*Endostemon obtusifolius* (E. Mey. ex Benth.) N.E. Br.	Drought	Production of ammonia and siderophore, free radical scavenging ability	Ogbe et al., 2023
Streptomyces dioscori SF1	*Glycyrrhiza uralensis* Fisch. ex DC.	Drought, salinity, phytopathogens	Via production of ammonia, IAA, enzymes activities, potassium solubilization, nitrogen fixation	Li X. et al., 2023
Sphingomonas paucimobilis ZJSH1	*Dendrobium officinale* Kimura et. Migo	Drought, salt, and heavy metal toxicity	By hormones (IAA, SA, ABA and zeaxanthin), phosphate cycle, antioxidant enzymes, and polysaccharides	Li J. et al., 2023
Funneliformis mosseae, Rhizophagus intraradices, Claroideoglomus etunicatum	*Sesbania sesban* (L.) Merr.	Salinity	Secrets phytohormones	Abd Allah et al., 2015
Yarrowia lipolytica	*Euphorbia milii* Des Moul.	Salinity	By producing IAA, IAM (indole-3-acetamide), phenol, and flavonoid	Jan et al., 2019
Chaetomium globosum, Botrytis sp.	*Chrysanthemum morifolium* (Ramat.) Hemsl.	Salinity	Increase POD activity and soluble protein content	Liu et al., 2011
Glomus mosseae, G. microcarpum, G. fasciculatum, G.intraradices, Gigaspora margarita, and *Gigaspora heterogama*	*Jatropha curcas* (L.)	Salinity	By improving physiological parameters (leaf relative water content, chlorophyll, proline, and soluble sugar), antioxidant enzymes (SOD, POD, APX, GR), and by reducing oxidative damage to lipids	Kumar et al., 2015
Arbuseular mycorrhiza, Penicillium griseofulvum	*Glycyrrhiza uralensis* Fisch. ex DC.	Water, drought, and salinity	Improving the activity of protective enzymes and osmotic levels	Wang et al., 2009
Glomus tenue	*Lolium rigidum* Gaud.	Waterlogging	By improving root length and other morpho-physiological mechanisms	Orchard et al., 2016
Piriformospora indica	*Capsicum annum* (L.)	Osmotic stress	Encoding lipid transfer protein and ACC-oxidase enzyme	Sziderics et al., 2007
Curvularia protuberate	*Dichanthelium lanuginosum* (Ell.) Gould	Heat	Mutualism	Márquez et al., 2007
Mucor sp.	*Arabidopsis arenosa* (L.) Lawalrée	Heavy metal-induced oxidative stress	Down-regulating catalase activity	Domka et al., 2019
Preussia africana, Bjerkandera adusta, Schizophyllum commune, Alternaria embellisia, Trichaptum biforme, Septoria malagutii, A. consortiale, Verticillium dahliae, Fusarium avenacearum, Trametes versicolor	*Anthemis altissima* L., *Matricaria parthenium* L., *Cichorium intybus* L., *Achillea millefolium* L., *A. filipendulina* Lam.	Abiotic stress	Produced the highest level of IAA-like compounds which enhances seed germination	Hatamzadeh et al., 2023
Epulorhiza sp.	*Anoectochillus formosanus* Hayata	Abiotic stress	Strengthen enzyme activities which enhances survival rate of seedlings	Tang et al., 2008
Sclerotium sp.	*Atracty lancea* (Thunb.) DC.	Abiotic stress	Improving the protection of cells from desiccation and metabolism of the host, enhancing survival rate of seedlings	Chen et al., 2008
Colletotrichum tropicale	*Theobroma cacao* (L.)	Disease (frosty pod rot, witches broom, black pod rot)	Antagonism	Mejía et al., 2008
Epulorhiza sp. AR-18	*Anoectochilus roxburghii* (wall.) Lindl	Disease	Production of siderophore	Arora et al., 2001

(Continued)

TABLE 2 Continued

Endophytic microbes	Host medicinal plants	Stress type	Plant defense mechanism	References
Colleto trichum gloeosporioides, Trichoderma tomentosum, Colletotrichum godetiae, Talaromyces amestolkiae	*Cremastra appendiculata* (D.Don) Makino	Disease	Antagonism, production of IAA, siderophores.and β-1,3-glucanase, proteolytic activity, chitinase and cellulose synthesis	Wang et al., 2023
Colletotrichum acutatum	*Angelica sinensis* (Oliv.) Diels	Disease	Antimicrobial, antioxidant, and anti-proliferative properties	Yehia, 2023
Leucocoprinus gongylophorus	*Cordia alliodora* Cham.	Insect	Release some toxins, antagonism	Bittleston et al., 2011
Chaetomium cochliodes, Cladosporium cladosporioides, Trichoderma viride	*Cirsium arvense* (L.) Scop.	Insect	Release some toxic chemicals harmful to pathogens	Gange et al., 2012
Beauveria bassiana, Lecanicillium dimorphum, L. cf. *Psalliotae*	*Phoenix dactylifera* (L.)	Insect	Regulate cell division-related proteins expression in the host	Gómez-Vidal et al., 2009
Penicillium citrinum LWL4, Aspergillus terreus LWL5	*Helianthus annuus (L.)*	Insect	Salicylic and jasmonic acid pathways	Waqas et al., 2015a
Penicillium rubens (150 strains)	*Picea glauca* (Moench) Voss	Insect	Release toxic chemicals	Sumarah et al., 2010
Epichloë coenophiala AR584	*Lolium arundinaceum* (Schreb.)	Biotic (Herbivore attack)	Stoichiometry, secretion of certain alkaloids which provide anti-herbivore defences	Johnson et al., 2023
Paraphaeosphaeria sp.	*Vaccinium myrtillus*	Pathogenic fungi	Flavonoid biosynthesis and degradation	Koskimäki et al., 2009
Choiromyces aboriginum, Stachybotrys elegans, Cylindrocarpon	*Phragmites australis* (Cav.) Steud.	Pathogenic fungi	Produce cell wall-degrading enzymes to kill pathogenic fungi	Cao et al., 2009
Gilmaniella sp. AL12.	*Atractylodes lancea* (Thunb.) DC.	Pathogenic fungi	Production of JA-inducing defense responses	Ren and Dai, 2012
Chaetomium globosum L18	*Curcuma wenyujin* Y.H.Chen & C.Ling	Pathogenic fungi	Produce some toxic chemicals harmful to pathogens	Wang et al., 2012
Trichothecium roseum	*Maytenus hookeri* Loes.	Pathogenic fungi	Release "trichothecin" toxic to phytopathogens	Zhang et al., 2010
Phomopsis cassia	*Cassia spectabilis* DC.	Pathogenic fungi	Produce cadinane sesquiterpenoids toxic to pathogens	Silva et al., 2006
Cryptosporiopsis cf. *quercina*	*Triptergyium wilfordii* Hook. f.	Pathogenic fungi	Produce cryptocin and cryptocandin toxic to pathogens *Pyricularia oryzae*	Strobel et al., 1999
Penicillium chrysogenum Pc_25, *Alternaria alternata* Aa_27	*Asclepias sinaica* (Bioss.)	Pathogenic microorganisms	Synthesizing extracellular enzymes viz., amylase, pectinase, xylanase, cellulase, gelatinase, and tyrosinase.	Fouda et al., 2015
Talaromyces trachyspermus	*Withania somnifera* (L.)	Phytopathogenes	Via antagonistic activity to pathogens and enhancing IAA, phosphate solubilization, and siderophore synthesis	Sahu et al., 2019
Diaporthe sp. CEL3, *Curvularia* sp. CEL7	*Chloranthus elatior* Sw.	Pathogenic fungi	Synthesized volatile and non-volatile compounds, soluble antifungal metabolites	Santra and Banerjee, 2023
Pestalotiopsis sp., *Neopestalotiopsis parvum and Hypoxylon investiens*	*Taxillus chinensis* (DC.) Danser	Pathogenic fungi	Antifungal activity	Song et al., 2023
Enterobacter, Microbacterium, Pseudomonas, Rhizobium, and Streptomyces	*Viola odorata* L.	Phytopathogenes	Synthesis of antimicrobial and antioxidant products, free radical scavenging capacity	Salwan et al., 2023

converse security against pathogens (Rajkumar et al., 2010). Competition for habitats and food resources, the formation of cell wall-degrading enzymes, lytic enzymes, antibiotic compounds, the commencement of ISR, and the quenching of pathogens' quorum sensing, among some of the other mechanisms (Rajesh and Rai, 2014). The majority of endophytes are recognized for synthesizing secondary metabolites, notably phenols, terpenoids, alkaloids, flavonoids, steroids, and peptides, which have potent antifungal and antibacterial effects and restrict the spread of harmful pathogens. There have been numerous reports of endophytes producing a variety of lytic enzymes, including chitinase, amylase, proteases, cellulose, and hemicelluloses (Bodhankar et al., 2017).

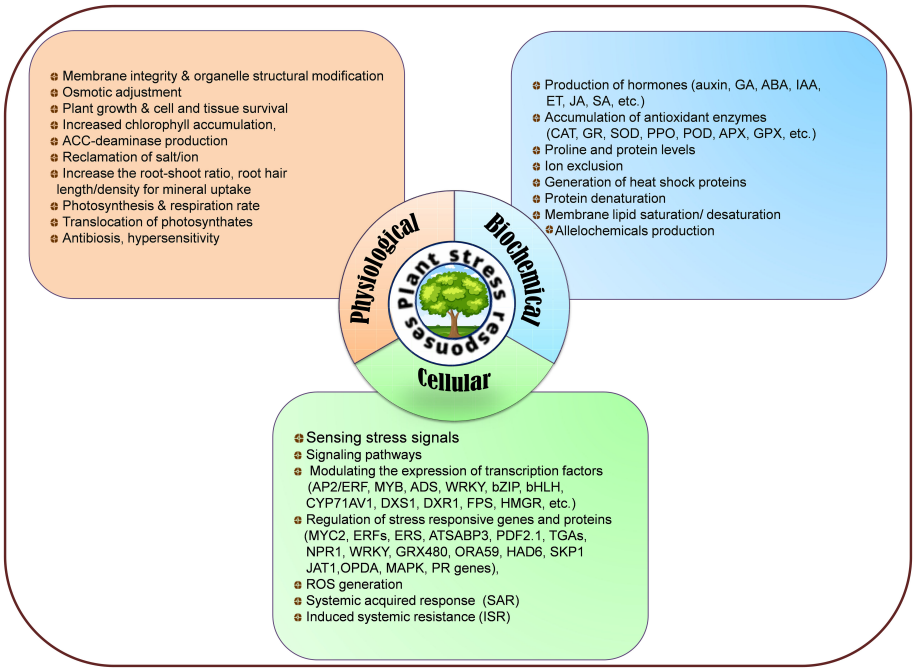

FIGURE 3
Physiological, biochemical, and cellular responses to mitigate biotic and abiotic stresses.

Lytic enzymes are critical for establishing endophytes in host cells by the formation of protein biofilms as well as polysaccharides, which lend phytopathogens' cell walls structural rigidity (Limoli et al., 2015). Nevertheless, it is also beneficial in managing plant diseases through cell wall breakdown while causing cell death (Cao et al., 2009). The virulence-associated factors, viz., biofilm creation, toxin synthesis, antibiotic resistance, and secretions of degradative exoenzymes, are closely governed by quorum sensing. Several pathogenic microbes, *Pseudomonas* and *Ralstonia*, effectively employ acylated homoserine lactones for communication, causing significant crop damage (Mansfield et al., 2012). In order to prevent infection, the antiquorum sensing mechanism could be employed (Chen et al., 2013). Moreover, once a pathogen attacks, the inherent immune system is triggered, which blocks the pathogen's invasion and stops its spread. It is an early defense system against phytopathogens, which involves physical barriers like trichomes, stiff cell walls, and waxy cuticles. Plants release exudates from their roots, comprising proteins, amino acids, and organic acids, which interact among the host plant and endophytes (Kawasaki et al., 2016; Shen et al., 2019; Inbaraj, 2021). Hyperparasitism is a novel biocontrol mechanism where the parasitic host is a plant pathogen; probably the most common hyperparasite is a well-known necrotrophic mycoparasite called *Trichoderma* species that feeds on host mycelium (Qualhato et al., 2013).

In summary, plant-microbe interactions are an efficient, eco-friendly way for plants to cope with severe environmental conditions. Plants evolved multifaceted relationships with diverse groups of microbes to combat biotic-abiotic stresses. Generally, microbes stimulate plant growth by optimizing the physiology and metabolism of the host through different mechanisms. The symbiosis relationships of microbes on host plants might encourage their recruitment through responsive feedback regarding plant health. Endophytes strengthen crop yield by promoting plant growth via regulating nutrient supply and metabolism, enhancing abiotic stresses (heat, drought, waterlogging, salinity, metal-toxicity etc.) tolerance by generating phytohormones, osmotic adjustment, photosynthesis, and respiration rate while controlling biotic stresses (phytopathogens) through antibiosis, SAR, ISR, competition with pathogens, hyperparasitism, and synthesizing toxins and currently extensively utilized in sustainable agriculture. The mechanism strategies whereby endophytic microbes promote plant growth and control phytopathogens, resulting in increased yields, have been schematically illustrated in Figure 4.

7 Hormonal signaling and crosstalk to mitigate biotic-abiotic stresses

Plants' defense mechanism is influenced by many factors, primarily genetic makeup and the physiological condition of the plant. Each cell in a plant's defense system has figured out how and where to respond to stressors, thereby creating an inherent immunity. Among these strategies, phytohormones substantially impact plants' ability to endure stresses. Generally, cytokinins, gibberellins (GAs), and auxins (IAAs) are linked to plant growth and development, whereas ET, JA, and SA are related to plant defense (Koo et al., 2020; Hossain et al., 2021). GAs and IAAs play a significant role in abiotic and biotic stress tolerance, whereas ET, JA, and SA promote abiotic stress tolerance (Kazan, 2013; Santino et al.,

2013; Colebrook et al., 2014). When carried directly to the appropriate cells or transmitted to distant tissues, these hormones influence various physiological networks at low concentrations, increasing resistance to environmental stresses (Colebrook et al., 2014). A comprehensive phytohormone network's tweaking enables plants to respond in a balanced way to developmental and environmental stimuli.

7.1 Ethylene signaling

ET, the gaseous phytohormone, has diversified functions in plants, including cell division and elongation (Love et al., 2009), apical dominance (Yeang and Hillman, 1984; De Martinis, 2000), senescence and abscission (Pierik et al., 2006), flowering (Ogawara et al., 2003; Wang et al., 2013), fruit ripening (Barry and Giovannoni, 2007), breaking seed dormancy and promoting seed germination (Corbineau et al., 2014; Wang et al., 2018; Ahammed et al., 2020), as well as a critical role in programmed cell death (Bouchez et al., 2007). It is a crucial player in both harmful and advantageous plant-microbe interactions (Pierik et al., 2006; Schaller, 2012; Ravanbakhsh et al., 2018; Liu et al., 2019), either through interactions with other phytohormones (Leon-Reyes et al., 2009; Leon-Reyes et al., 2010; Zander et al., 2010) or by controlling the expression of ethylene-responsive genes (Broekaert et al., 2006; Teixeira et al., 2019). Since many biotic and abiotic perturbations influence plants' physiological and developmental processes, ET synthesis plays a pivotal role in the plant's adaptation to these environmental threats (Arraes et al., 2015; Sun et al., 2016; Fröhlich et al., 2023). The sensing of ET signaling occurs at the endoplasmic reticulum membrane, triggering a signaling cascade that controls the transcription of ethylene-responsive genes in the nucleus via ERFs (ethylene-responsive factors) (Ju and Chang, 2015). However, the ET-signaling pathway in *Arabidopsis* is negatively regulated by the ET-receptors viz., ethylene response sensors (ERS1, ERS2), ethylene response (ETR1, ETR2), and ethylene insensitive4 (EIN4) (Liu and Wen, 2012). These ET-receptors stimulate constitutive triple response1 (CTR1) in the absence of ET-signaling, which restricts EIN2, a positive regulator of ET-signaling, through phosphorylating EIN2's C-terminus. Conversely, the presence of ET renders the ET receptors inactive, thereby preventing CTR1 activation. Subsequently, dephosphorylated and cleaved EIN2 C-terminus (CEND) reaches the nucleus, where it stimulates the function of ethylene-insensitive3/ethylene-insensitive3-like1 (EIN3/EIL1), which modulates the expression of ethylene-responsive genes like ERFs. ERFs constitute transcription factors (TFs) with AP2domains that control various genes associated with stress tolerance, growth, development, and hormone-related pathways (Chen et al., 2010; Shakeel et al., 2015; Zhao et al., 2021).

The up-regulation of ET-biosynthesis genes following interactions with advantageous microbes reveals that ET-signaling is activated not only in response to pathogenic microbes but also to helpful endophytic microbes before they are recognized as friends, possibly to optimize the colonization of adequate levels of beneficial microbes (Ravanbakhsh et al., 2018; Eichmann et al., 2021). Owing to inherent physiological reactions to abiotic stressors, plants can instantly produce an enormous amount of ET, which helps the plants to withstand external challenges, but it can also jeopardize growth and development, thereby reducing crop yield and productivity since increased ET levels can cause senescence, abscission, and chlorosis. Research on plant growth-promoting rhizobacteria (PGPR) has shown that they can prevent soil-borne pathogen infections in plants in an ET-dependent way. Furthermore, beneficial microbes can stimulate ISR and SAR in plants to control diseases (Ton et al., 2001).

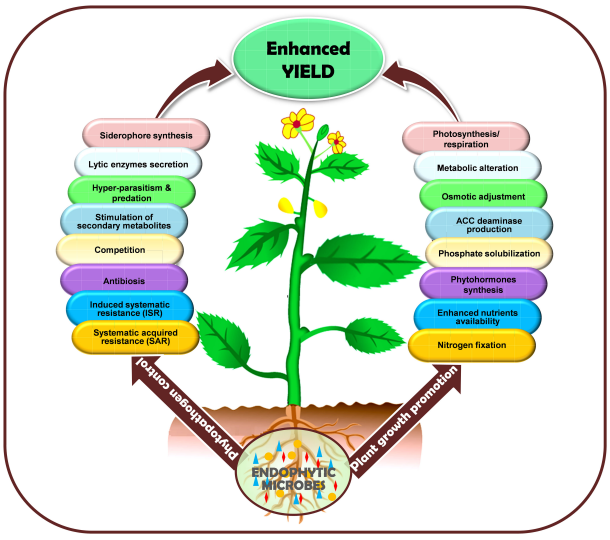

FIGURE 4
Schematic representation of the endophytes mediated mechanisms in biotic-abiotic stress amelioration in plants. The figure depicts endophytes boosting crop yield through enhancing abiotic stress tolerance by promoting plant growth via regulating nutrient supply and metabolism, phytohormones, osmotic adjustment, photosynthesis, and respiration rate while controlling biotic stress (phytopathogens) through antibiosis, SAR, ISR, competition with pathogens, hyperparasitism, and synthesizing toxins.

7.2 Salicylic acid signaling

SA, a key phytohormone, has crucial physiological and cellular impacts on plants, including membrane permeability and photosynthetic metabolism, and absorption and transport of ions during stress (Noreen et al., 2009). Furthermore, SA is recognized to outwit various abiotic stresses like ROS, pathogens attacks, drought, and salinity (Hara et al., 2012). Additionally, it regulates plant responses to infection by diversified pathogens, viz., bacteria, fungi, viruses, etc. (Fujita et al., 2006; Loake and Grant, 2007), and is necessary for developing resistance strategies like host cell death, ISR, and SAR. The expression of various genes, including those encoding PR-proteins (pathogenesis-related proteins), might be a mechanism whereby SA induces stress tolerance (Nakashima et al., 2009). The cytoplasm contains an oligomer of *NPR1*, a crucial regulator of SA-induced plant resistance. Once a disease has occurred, it monomerizes and transports to the nucleus,

activating a series of genes involved in pathogenesis (Kinkema et al., 2000). But in normal plants, Cys156's S-nitrosylation, which prevents its monomerization, controls the oligomer to monomer switch. Following infection, nitrous oxide (NO) accretion causes the *Arabidopsis thaliana* SA-binding protein 3 (ATSABP3) to become S-nitrosylated at Cys280, which reduces the protein's capacity to bind to SA and inhibits its carbonic anhydrase function (Wang F. et al., 2019). In contrast, S-nitrosylation regulates SAR by focusing on the *NPR1/TGA1* system. As mentioned earlier, SA activates thioredoxin (*TRX*), which helps denitrosylate *NPR1* so that it may be monomerized throughout the plant immune response (Kneeshaw et al., 2014). This facilitates *NPR1* to enter the nucleus and interact with the primary leucine zipper transcription factor *TGA*, which in turn makes it easier for *TGA* to bind to the gene-expression promoters. Upon sensing and detecting stimuli of stresses, mitogen-activated protein kinase (MAPK) cascades are triggered that regulate the stress-modulatory systems and are responsible for the signaling of diverse cellular activities under different stressors (Brader et al., 2007). SA facilitates the activation of MAPK pathways driven by pathogen infection and the subsequent production of PR genes for host defense (Xiong and Yang, 2003). Following MPK3 phosphorylation, the *Arabidopsis* protein VIP1 is translocated into the nucleus and functions as a covert inducer of *PR1* genes (Pitzschke et al., 2009). Similarly, MAPKs such as MPK3, MPK4, and MPK6 are confronted with different stresses (Ichimura et al., 2000; Gudesblat et al., 2007). Moreover, pathogen-associated molecular patterns (PAMPs), such as flagellin, activate MAPK cascades to develop pathogen response signaling (Chinchilla et al., 2007). In addition to interacting with ABA-signaling pathways and ROS to improve plant defense, MAPK cascades also play a crucial role in modulating cross-tolerance (Miura and Tada, 2014; Zhou et al., 2014).

7.3 Jasmonic acid signaling

JA is another hormone crucial for eliciting responses against various biotic and abiotic perturbations by triggering plant defense signaling systems (Berendsen et al., 2012; Broekgaarden et al., 2015; Wang J. et al., 2020; Yadav et al., 2021). It is ubiquitously present in plants, having multiple regulatory functions, notably root growth inhibition (Han et al., 2023), axis elongation and root formation (Huang P. et al., 2019), leaf senescence (Wang T. et al., 2020), stomatal opening (Suhita et al., 2003), and flower formation (Niwa et al., 2018). Research findings have shown that JAs boost plant growth and development and various adverse environmental circumstances using JA-signaling pathways. Microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and herbivore-associated molecular patterns (HAMPs), which are predominantly derived from attacking organisms, cell damage, and abiotic stresses, are some plant-environment interaction models linked to JA-signaling pathways (Newman et al., 2013; Basu et al., 2018; Hou et al., 2019). The most functional JAs in plants' cells is jasmonyl isoleucine (JA-Ile); however, under normal conditions, its concentration is relatively low (Fonseca et al., 2009). It is recognized that the formation of JA-Ile in plant leaves during stressful situations serves as a physiological defensive system. Jasmonates are transported to the apoplast and nucleus from the cytoplasm by JA-transfer protein1 (JAT1), located in both cell and nuclear membranes (Wang Y. et al., 2019). Even in distant regions, the presence of JAs in the apoplast triggers the JA-signaling system, and the signals are sent to neighboring cells via the vascular bundles and air transmission (Thorpe et al., 2007). Different JAs synthases are localized in the sieve component of vascular bundles, which enables the re-syncretization of JAs throughout their movement (Heil and Ton, 2008). The biosynthesis of the JA precursor 12-oxo-PDA (*OPDA*) in the phloem sieve component has confirmed the theory of re-synthesis. Owing to the reduced level of JA-Ile under normal situations, specific transcription factors (TFs) are unable to activate the promoters of jasmonates-responsive genes. Owing to the reduced level of JA-Ile under typical conditions, specific transcription factors (TFs) cannot trigger the promoters of jasmonates-responsive genes.

The expression of the jasmonates sensitive genes is inhibited by the efficient transcriptional repression complex, composed of the proteins rendering and the putative JAZ (jasmonate-zim domain) interactor. This complex is further activated by histone deacetylase 6 (*HAD 6*), which closes the open complex (Hause et al., 2003). Thirteen JAZ proteins from *Arabidopsis* have been identified to contain the main ZIM domain and the C-terminal JA-associated domain. Different parts of JAZ proteins promote protein complexes (Gimenez-Ibanez et al., 2015). JAZ links with TFs and NINJA (novel interactor of JAZ) [comprising ethylene-responsive element binding factor associated with amphiphilic repression (EAR) motif and recruits TPL (topless)] to form the JAZ-NINJA-TPL repressor complex (Pauwels and Goossens, 2011). The amino acid sequence, JAZ degron, known as JAZ degron seems to have a bipartite structure with a loop and amphipathic alpha hexyl that bind coronatine or JA-Ile and coronatine insensitive 1 (COI1), respectively (Sheard et al., 2010). SKP1 (Suppressor of kinetochore protein1) and SCF (cullin-F-box) create the ubiquitin-proteasome complex. Establishing an SCF-type E3 ubiquitin ligase is the outcome of the interaction between SKP1 and cullin with the F-box protein. In stressful conditions, this F-box protein COI can identify the JA-Ile and deliver it to the nucleus. JA-Ile facilitates JAZ and COI1 communication inside the SCF complex, with inositol pentakisphosphate functioning as a cofactor in the formation of the CO1-JAZ co-receptor complex (Mosblech et al., 2011). JAs-mediated defenses are modulated by the proteasome-mediated degradation of the JAZ protein and the release of transcription factors (TFs) under environmental perturbations. According to Qi et al. (2011), there is solid proof that the expression of the genes that respond to jasmonates is primarily dependent on the linkage of transcription factors (TFs) with JAZ repressors.

7.4 Crosstalk between ethylene, jasmonic and salicylic acid

Hormonal signaling crosstalk triggers plants to develop certain specific traits that make them tolerant against the plethora of biotic and abiotic stresses via distinct molecular pathways with a complex

network of regulatory interactions (complementary, antagonistic, and or synergistic). Specifically, ET modulates plant defense by controlling the levels of JA and SA (Leon-Reyes et al., 2009; Zander et al., 2010). In such defense responses, ET and JA act synergistically (Penninckx et al., 1998; Zhu, 2014), nevertheless, it has also been reported that they mutually antagonize functions of each other in some specific circumstances (Turner et al., 2002; Bodenhausen and Reymond, 2007). Lorenzo et al. (2003) documented that the ERFs integrate signals from ET and JA. Eventually, other prominent genes that are expressed following the detection of ET and JA include *PDF1.2*, *POTLX3*, *ACS* (ethylene synthesis gene), *THI2.1* (thionin), *PR-3* (chitinase), *PR-4* (hevein-like protein), *PR-6* (proteinase inhibitor), and *PR-9* (peroxidase) (Kolomiets et al., 2000; Norman-Setterbald et al., 2000; Kondo et al., 2007; Chen et al., 2009). However, ET shows antagonistic effects with SA, and they can both suppress each other's biosynthetic pathways. The direct interaction between *NPR1* and *EIN3* prevents the transcription of genes activated by EIN3, a crucial element of SA signaling (Huang P. et al., 2019). As a result, *EIN3* and *EIL1* bind directly to the SID2 promotor, decreasing pathogen-induced SA production and increasing disease susceptibility in host plants (Chen et al., 2009).

Likewise, it is quite interesting that the crosstalk between the antagonistic pathways of hormones JA and SA also results in plant tolerance to various stresses. Several genes, including MYC2, plant defensin 2.1 (*PDF2.1*), TGAs, MAPK, *NPR1*, *ERF1*, *WRKY62*, *WRKY70*, *glutaredoxin 480* (*GRX480*), and octadecanoid-responsive *Arabidopsis* (*ORA59*), play a critical role in JA-SA inter-modulation (Wang et al., 2021). Three NAC (TF family) genes-*ANAC019*, *ANAC055*, and *ANAC072* interact with MYC2 in different ways to prevent SA accumulation. These TFs also regulate the expression of genes that produce SA. *GRX480* preferentially binds to TGAs, modulating *PR1* gene expression, and MPK4 controls *GRX480* positively (SA-signaling pathway), while *MYC2* is negatively regulated (JA-signaling pathway). However, *GRX* genes can prevent the activation of the JA response gene *ORA59* (Wang et al., 2020). The hormonal changes between interactions of JA and SA enhance plants' tolerance against chilling, drought, and oxidative stress. Methyl jasmonate (MeJA) possesses excellent permeability to cell membranes than JA and is very volatile by nature, and it might quickly diffuse nearby plants (Munemasa et al., 2011). External MeJA supplementation controls the formation of ROS and the immune systems by promoting antioxidant enzyme activity in *Panax ginseng* (Wahab et al., 2022). Following stress sensing, plants rapidly generate ROS (Wojtaszek, 1997; Foyer and Noctor, 2005). Furthermore, the plant meticulously regulates ROS synthesis to prevent tissue damage (Vinocur and Altman, 2005; Mittler et al., 2011; Bhattacharjee, 2008). It has been recognized that although higher levels of ROS are toxic and harmful to organisms and can cause permanent cell death, its lower levels are primarily responsible for controlling stresses. Perhaps ROS could be the critical factor facilitating cross-tolerance between biotic and abiotic stress-responsive stimuli (Choudhury et al., 2013; Kissoudis et al., 2014). A diagrammatic representation of ET, JA, and SA signaling cascade and pathway genes for biotic and abiotic stress tolerance is illustrated in Figure 5.

8 Endophytic microbes as biostimulants in sustainable agriculture

8.1 Benefits

Endophytes are an array of ubiquitous microorganisms that inhabit different niches in plant tissues. In addition to the fact that endophytic microbes can help plants to lessen the negative effects of abiotic stresses, research has shown that endophytes have functional traits with linked detrimental impacts of environmental factors on the continued existence and development of susceptible plant species by synthesizing bioactive compounds, triggering resistance that results from gene expression, and altering the metabolism of certain enzymes. They can inhibit the growth of phytopathogens via the production of antifungal compounds, thereby augmenting crop yields by facilitating plants to acquire nutrients while synthesizing phytohormones. Moreover, they reduce heavy metal stress, eliminate hazardous greenhouse gases, and degrade PAHs in the bioremediation process (Stępniewska and Kuźniar, 2013). Additionally, in recent years, endophytes have gained more recognition for their use in the phytoremediation of a range of environmental pollutants and could be helpful in developing effective cleanup systems (McGuinness and Dowling, 2009; Weyens et al., 2009; Segura and Ramos, 2013; Anyasi and Atagana, 2018; Adeleke et al., 2022). The diversity of endophytes, their ability for stress adaptation, and their synthesis of metabolites make them an endless supply of novel metabolites that can reduce harmful chemicals in agriculture. To illustrate, several studies have reported the beneficial effects of microbial endophytes on a wide range of medicinal plants, including *Withania somnifera, Artemisia annua, Papaver somniferum, Cymbidium aloifolium*, *Salvia miltiorrhiza*, *Catharanthus roseus, Bacopa monnieri*, *Nicotiana tobaccum, Andrographis paniculata, Chlorophytum borivilianum, Panax ginseng, Panax notoginseng, Curcuma longa, Curcuma wenyujin*, etc. (Meng and He, 2011; Karthikeyan et al., 2012; Wang et al., 2012; Ma et al., 2013; Barnawal et al., 2016; Kumar et al., 2016; Hong et al., 2018; Jayakumar et al., 2019; Sahu et al., 2019; Shah et al., 2019; Jiao et al., 2020; Ray et al., 2021; Zheng et al., 2021; Mei et al., 2023; Salwan et al., 2023; Sharma et al., 2023; Song et al., 2023; Wang et al., 2023; Zou et al., 2023). Thus, unquestionably, these endophytes have demonstrated tremendous potential as a green and eco-friendly alternative for boosting food production in sustainable agricultural systems.

8.2 Potential applications

Biostimulants are a class of substances or microbes derived from natural resources that are applied to soil or plants to boost crop yield and quality by stimulating plants' biological processes or enriching the soil microbiome for better nutrition and stress tolerance. Biostimulants have emerged as a boon for sustainable agriculture because they significantly accelerate the process of agronomic trait advancement in plants without jeopardizing yield,

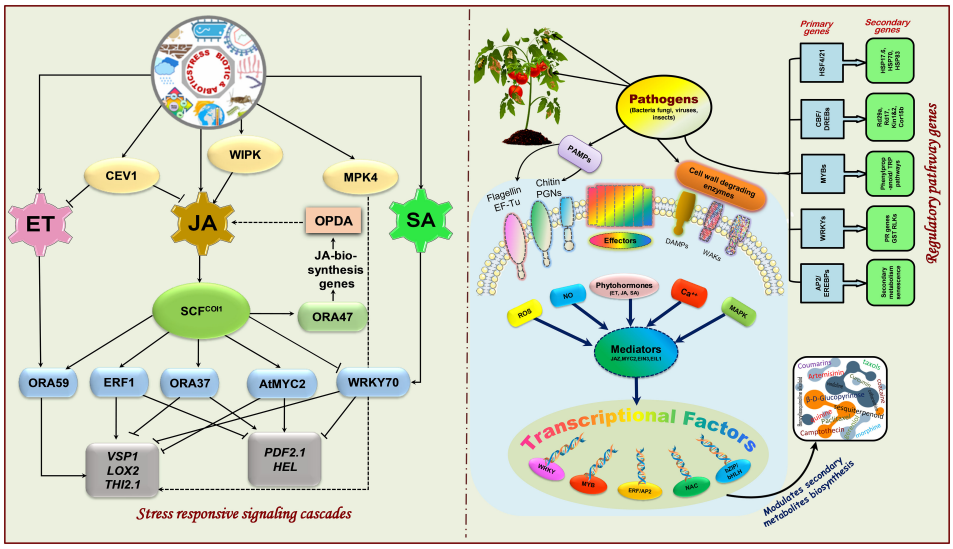

FIGURE 5
Signaling pathways and regulatory genes to mitigate biotic and abiotic stresses. This figure show a simplified depiction of biotic/abiotic stress-induced signaling pathways like jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) signal transduction and their cross-talk with each other. JA has a central hub position acting with ET and SA. ET, in turn, primarily regulates SCF biosynthesis, transport, and signaling, which is crucial for establishing other genes, like ORA59, ORA37, ERF1, AtMMYC2, and WRKY70, and activation of downstream signaling genes for resistance to different type stresses. Furthermore, a cascade of early (primary) and late (secondary) genes is activated in response to pathogen and insect-induced damage. Genes of herbivory resistance, plant disease resistance, JAs, and endogenous signaling molecules are not only involved in the pathogen resistance mechanism of plants but also have an apparent defensive effect on necrotrophic pathogens. Significant changes in defensive enzymes and secondary metabolites occur, which play essential roles in plant resistance against pathogens. CEV1, Cellulose synthase family protein; WIPK, wound-induced protein kinase; OPDA, 12-oxo-PDA; SCF^{COI1}, (Skp, Cullin, F-box containing complex); ORAs, octadecanoid-responsive Arabidopsis; ERFs, ethylene-responsive genes, AtMYC2, Arabidopsis thaliana MYC2; VSP1, vegetative storage protein1; LOX2, lysyl oxidase-like 2; THI2.1, thionin 2.1; PDF2.1, plant defensin2.1; HEL=, AP2: adipocyte protein 2, EREBPs, ethylene-responsive element binding proteins; MYBs, Myeloblastosis; CBF, C-repeat binding factors; DREBs, dehydration responsive element binding protein; HSF4/21, heat shock factor protein, GST, plant glutathione S-transferases; RLKs, receptor-like kinases; TRP, transient receptor potential; Rd, responsive to desiccation, Kln, kallikreins; Cor15b, cold-responsive15b.

quality, or biodiversity. In recent years, endophytic microorganisms have been thoroughly explored for the possibility of being utilized as biostimulants for minimizing the usage of harmful chemicals in agriculture, thereby fulfilling the WHO's envisioned sustainable development goals while ensuring food and nutritional security (Omotayo and Babalola, 2020). To exemplify, investigations using endophytic microorganisms have demonstrated their potential roles as biostimulants (Kumar et al., 2015; Wani et al., 2016; Hashem et al., 2017; Vyas et al., 2018; Saia et al., 2021; Tharek et al., 2022), biofertilizers (Arora and Mishra, 2016; Santoyo et al., 2016), biopesticides (Gange et al., 2012; Waqas et al., 2015a; Lugtenberg et al., 2016), and biocontrol agents (Hashem et al., 2017; Halecker et al., 2020; Jiao et al., 2020). Likewise, da Silva et al. (2017) developed an inexpensive and efficient biostimulant formulation made with endophytic diazotrophic bacteria and humic acids that boosts crop production while ensuring the finest use of fertilizers. Considering the practical implications, microbial formulations promote plant growth and development by restoring soil minerals, improving plant nutrient uptake, or making nutrients easily accessible (Bashan et al., 2014; Mishra et al., 2015). In addition, they also affect the host's other beneficial effects, such as osmotic adjustment, stomatal regulation, shaping root architecture, and adjustment of nitrogen accumulation and metabolism (Compant et al., 2005). Bioinoculants facilitate seed treatment by distributing inoculants evenly over seeds, causing systemic acquired resistance (Ma, 2019), and assisting in bioremediation using a metabolic engineering approach (Dangi et al., 2019). In terms of agrochemical and metal pollutants solubilization, bioabsorption, and mineralization, endophytes have also proven effective in environmental remediation (Gavrilaş et al., 2022). Studies have advanced further the potential implementation of microorganisms as traditional biological control agents (BCAs) by inundating inoculation in plants. Tahir et al. (2017) found that *Bacillus subtilis* volatiles negatively impact *Ralstonia solanacearum's* physiology and ultrastructure and elicit systemic resistance in tobacco against bacterial wilt. The best characterized and most frequently microbial endophytes in biological control programs are *Beauveria bassiana* and *Metarhizium anisopliae* have antagonistic activities on plant pathogens via an array of mechanisms, including the synthesis of metabolites (volatile compounds, antibiotics, and enzymes), competition, parasitic relationships, triggering systemic resistance by the plant, and improvements in plant growth (Vidal and Jaber, 2015; Vega, 2018; Moraga, 2020; Baron and Rigobelo, 2021). In another study, endophytes frequently assist plants in reinforcing their defense mechanisms by facilitating the stimulation of induced systemic resistance, which occasionally overlaps with those of acquired systemic resistance, considering both of them may foster the growth and development of plants (Busby et al., 2016) and protect against phytopathogens (Chadha et al., 2015). Therefore, implementing microbial formulations as biocontrol or biofertilizers

might be an effective alternative to the overuse of agrochemicals. Perhaps the most environmentally and farmer-friendly step toward sustainability might be developing consortia from aspiring endophytic strains from native agricultural fields, resulting in multifaceted bio-solutions.

8.3 Challenges

Despite the widespread interest in endophyte research, there are still certain challenges in designing efficient microbial formulations, such as:

i. Endophytes are tissue-specific; identifying suitable host plants, their healthy tissues or organs is critical.
ii. Isolating novel endophytes and investigating the relevant complementary or antagonistic signaling pathways during symbiosis.
iii. Pecularity of microbial consortia in terms of their modes of action. Some endophytes have aseptic or uncultivable properties, making synthetic cultivation challenging. Therefore, developing new bioengineering systems or modifying traditional isolation methods is crucial.
iv. The biological constraint still exists even though some endophytes' facultative nature offers the possibility of continued colonization, provided they can survive in the rhizosphere.
v. The interactions of microbial biostimulants with the micro-climate (temperature, pH, water, humidity, nutrients, etc.), host plants (defense system and exudates), and native microbes should also be considered.
vi. The inoculants' concentration, functionality, and survivability during storage as well as maintaining sterility, are critical for designing efficient formulations.
vii. Limitation of biological adjuvants as bio-careers.
viii. Artificially inoculated endophytes may begin acting as latent pathogens by disseminating toxins through the food chain.
ix. The potential of exogenously applied endophytic microbes to establish a habitat beneficial to both entities is contingent upon their ability to compete successfully with native microbes. Thus, inoculating crops with consortia rather than a single strain will increase their persistence.
x. Licensing/registration of formulations before arriving on the market is complicated.

Screening of endophytic microbes in a greenhouse, either solely or in combined applications, has proven to be efficient in maximizing crop yields. Designing formulations with high microbial concentrations and survivability is crucial for developing potent biostimulants. However, finding the most critical factors and ensuring sterility during the formulation process is challenging because testing every possible combination is not feasible. Therefore, the commercial success of endophyte-based biostimulants requires a comprehensive knowledge of molecular plant-microbe interaction, methods of transmission, and strategies for establishing a symbiotic relationship between the endophyte and host plant. The research efforts aimed at discovering microbial biostimulants are beginning, which might result in significant advancement in this emerging field. In modern agriculture, methods to increase the use of endophytic microorganisms are desired to use these microbes alone or in combination with bioprospecting as bioinoculants in crop systems. The most effective methods for using endophytic microorganisms in agriculture have not yet been identified. However, applying endophytes as seed dressings or directly into the soil is the most frequent and common method utilized by farmers. Meanwhile, the implementation of these endophytes-based inoculations is unsuccessful on field sites owing to issues with the endophytes' establishment.

Therefore, the manifold characteristics of endophytes make them possible alternatives to harmful agrochemicals, and thus, they are now being utilized more frequently throughout the world. Endophyte-based biostimulants are cost-effective, preserve natural soil microbiota, have few or no hazardous byproducts, enrich soil organic matter, and ensure ecosystem sustainability. Utilizing improved microbial inoculants can be one of the best input components for green farming. Although endophytic microorganisms can be engineered, little is known about their use as bioinoculants in contemporary farming situations. Therefore, more research is required to determine the effectiveness of microbial bio-input for commercialization before these endophytes can be used as bioinoculants to improve soil health and crop yield.

9 Conclusion

The yield and quality of medicinal plants are considerably influenced by various edaphic and climatic factors such as soil characteristics, soil microbiota, light, humidity, temperature, drought, salinity, etc. To adapt to a stressful environment, plants acclimatize themselves by modulating the genes responsive to stress, transcriptional factors, and biosynthesis signaling pathways. Furthermore, in stressful conditions, plant defense systems trigger appropriate cellular responses by stimuli from the sensors situated on the cytoplasm or cell surface and transmitting signals to the transcriptional machinery in the nucleus with the help of various signaling pathways. Sustainable production is still a significant challenge; perhaps specific strategies might be helpful in such scenarios as rescue measures like integrating plant-associated microbes into farming systems, supporting agricultural production through various interventions, and mitigating biotic and abiotic perturbations. Utilizing endophytic microbes as biostimulants not only eliminates the need for synthetic inorganic pesticides and fertilizers but also lowers input costs and, more importantly, minimizes the impact of these agrochemicals on vital existing ecological communities. Nevertheless, its practical application suffers some limitations, viz., endophytes are tissue-specific, and tissue type, the host, and the environment mainly influence their functionality. However, the information gap of their multifaceted

nature in plant tissues has hampered the advancement of endophyte research in various fields. Furthermore, the underlying mechanisms governing these interactions are still not fully explored; several studies have raised the hope of their potential exploitation of plant-microbe interactions in managing various stresses. Therefore, to promote the practicality of endophyte-assisted biological applications as biostimulants, particularly in the field, comprehensive research is necessitated to demonstrate an insight into the microorganisms in its host medicinal plants. Modern high-throughput genomic studies have revolutionized the field of microbiome research by unveiling the enigmatic realms of endophytism, facilitating the pursuit of endophytes, enabling the sequencing of a broader range of microbes, and enticing a comprehensive examination of microbial ecosystems by taxonomic classification, phylogeny, and evolutionary studies, In the future, advanced omics approaches such as genomics, transcriptomics, proteomics, and metabolomics can support an in-depth knowledge of plant-microbe interactions and stress signaling pathways, leading to its potential exploitation in agriculture for improving yield, quality, and resistance of medicinal plants, drug development, and management of the environment.

Author contributions

PP: Conceptualization, Writing—original draft preparation, Writing - review and editing, Visualization. AT: Conceptualization, Writing—original draft preparation. SD: Writing - review and editing. KL: Writing - review and editing. TJ: Writing - review and editing. All authors contributed to the article and approved the submitted version.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We are thankful to the Director, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India. We also thank the peer reviewers for their critical review and valuable suggestions for improving this manuscript. The CIMAP publication communication number is CIMAP/PUB/2023//007.

Conflict of interest

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

Publisher's note

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Glossary

CAT	Catalase
PPO	Polyphenol oxidase
POD	Peroxidase
ACC deaminase	1-Amino Cyclopropane-1-Carboxylate deaminase
SOD	Superoxide dismutase
AMF	Arbuscular mycorrhizal fungus
MDA	malondialdehyde
POD	Peroxidase activity
GR	Glutathione reductase
ALD	Aldehydes
HSPs	Heatshock proteins
PAL	Phenylalanine ammonia-lyase
STS	Stilbene synthase
SAR	Systemic-acquired resistance
ISR	Induced systemic resistance
ROS	Reactive oxygen species
GPX	Guaiacol peroxidise
NPR1	Non-expressor of pathogenesis-related genes
PR1	Pathogenesis-related protein1
CMV	Cucumber mosaic virus
AsSyn	Asparagine synthetase
Gluc	b-1,3-glucanase
BR-SK1	Brassinosteroid signaling kinase 1
TCAS	Tetra-hydrocannabinolic acid synthase
ZF-HD	Zinc finger-homeodomain
RdRP2	RNA dependent RNA polymerase
GAs	Gibberellins
IAAs	Auxins
ERS	Ethylene response sensors
ETR	Ethylene response
EIN4	Ethylene insensitive4
CTR1	Constitutive triple response1
CEND	Cleaved EIN2 C-terminus
EIN3/EIL1	Ethylene-insensitive3/ethylene-insensitive3-like1
TFs	Transcription factors
PGPR	Plant growthpromoting rhizobacteria
PR-proteins	Pathogenesis-related proteins
THI2.1	Thionin2.1
ATSABP3	Arabidopsis thaliana SA-binding protein 3
TRX	Thioredoxin
PAMPs	Pathogen-associated molecular patterns
MAMPs	Microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
DAMPs	Damage-associated molecular patterns
HAMPs	Herbivoreassociated molecular patterns
JA-Ile	Jasmonyl isoleucine
JAT1	JA-transfer protein1
OPDA	12-oxo-PDA
JAZ	Jasmonate-zim domain
HAD 6	Histone deacetylase 6
NINJA	Novel interactor of JAZ
TPL	Topless
COI1	Coronatine insensitive 1
SKP1	Suppressor of kinetochore protein 1
SCF	Cullin-F-box
PDF2.1	Plant defensin 2.1
GRX480	Glutaredoxin 480
ORA59	Octadecanoidresponsive Arabidopsis
MeJA	Methyl jasmonate

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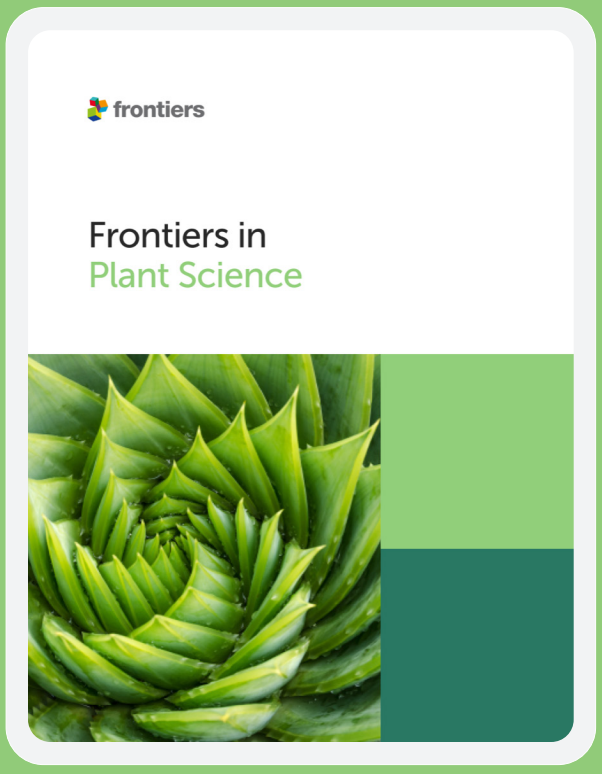

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