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Biostimulants for climate-smart and sustainable agriculture

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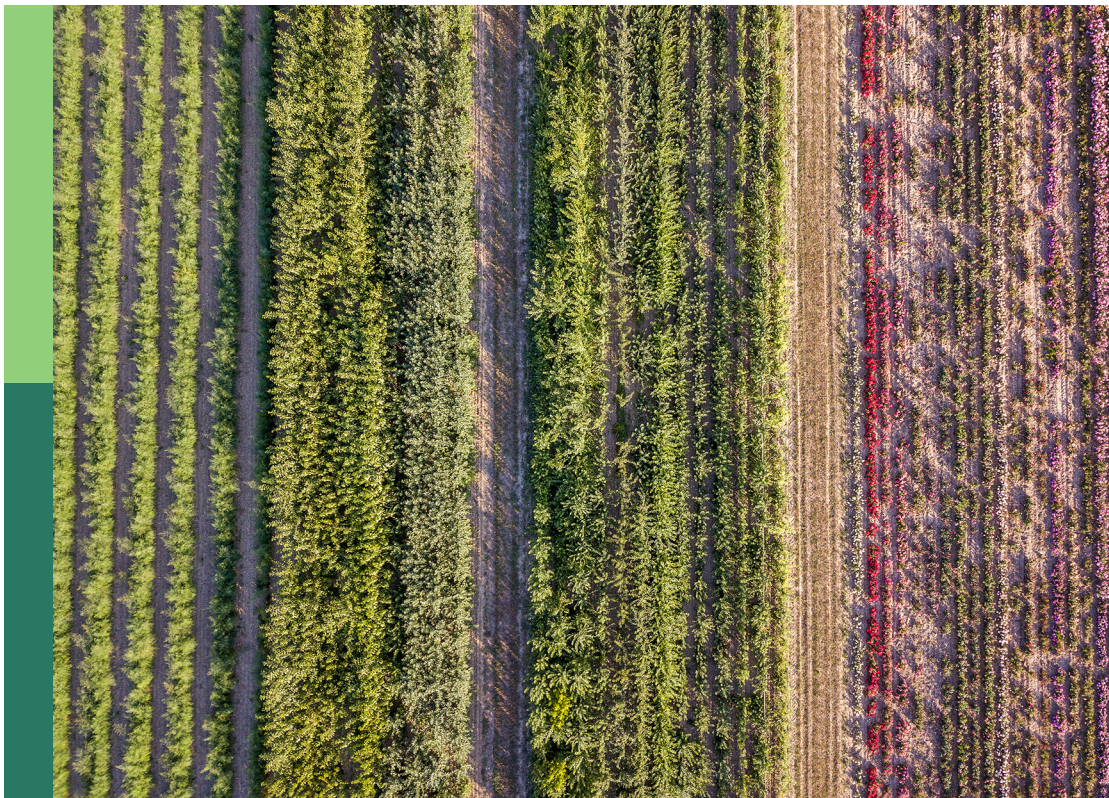

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Biostimulants for climate-smart and sustainable agriculture

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Editorial: Biostimulants for climate-smart and sustainable agriculture

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Editorial on the Research Topic
Biostimulants for climate-smart and sustainable agriculture

In the quest for sustainable and climate-resilient agriculture, a diverse array of innovative strategies has emerged to bolster resilience and productivity, from harnessing biostimulants to unraveling the intricate interplay between plants and their microbiome under environmental stresses. The Research Topic "*Biostimulants for climate-smart and sustainable agriculture*" presents a collection of pioneering studies in a wide range of areas, including the beneficial effects of biostimulants, new formulations for sustainable agriculture under different climate change-associated abiotic stresses, and the application of biostimulants for plant growth promotion and protection. The compilation of research weaves together a tapestry of data that illuminate novel pathways toward enhancing crop productivity, resilience, and sustainability in the face of changing climatic conditions. Through a synthesis of diverse approaches and findings, this Editorial aims to contextualize the contributions of the included articles and underscore their collective implications for advancing climate-resilient agriculture.

Several studies within this Research Topic investigate the application of plant growth-promoting rhizobacteria (PGPR) to enhance plant growth, performance, and stress tolerance. Chamkhi et al. and Cheto et al. highlight the efficacy of PGPR consortia in mitigating phosphorus deficiency stress and water deficit, respectively. Chamkhi et al. delve into the realm of PGPR to enhance faba bean growth under P deficiency stress, advocating for a multi-strain consortium approach. Their findings underscore the potential of synergistic bacterial interactions in improving plant growth parameters under challenging conditions. The study by Cheto et al. explores the synergistic effects of rhizobacterial consortia and intercropping on faba bean and wheat plants under water deficit and low P availability stress. Additionally, Lobato-Ureche et al. shed light on the application of PGPR strains to enhance pepper plant productivity, showcasing the potential of specific bacteria in improving plant growth parameters and reducing reliance on chemical fertilizers. These studies underscore the importance of microbial inoculants in improving plant nutrition, health, productivity, and soil fertility, paving the way for sustainable agricultural management practices.

Nazari and Smith's research delves into the impact of Thuricin 17 on canola plants under drought and heat stress conditions, highlighting the compound's potential as a growth regulator to enhance crop resilience in challenging environments. Their findings illuminate the intricate mechanisms by which microbial symbionts enhance plant adaptation, thereby opening new avenues for enhancing plant tolerance to adverse environmental conditions through innovative biostimulants. Similarly, Sati et al. and Ait-El-Mokhtar et al. delve into the interplay between drought stress and the plant microbiome, emphasizing the multifaceted interactions that underpin plant resilience and adaptation strategies. Sati et al. underscore the importance of utilizing drought-tolerant PGPR strains to mitigate drought stress in wheat plants, showcasing the potential of native bacteria in enhancing agricultural sustainability in regions vulnerable to water scarcity. Ait-El-Mokhtar et al.'s review delves into the complex dynamics of plant-microbiome interactions under drought conditions, emphasizing the significance of understanding the molecular mechanisms driving these relationships. Their insights illuminate the potential of harnessing the crop microbiome as a strategy for improving plant resilience and agricultural sustainability amidst changing environmental conditions. Their work underscores the significance of harnessing microbial allies to bolster crop resilience in the face of environmental challenges.

Fite et al. provide a comprehensive review of endophytic fungi, highlighting their diverse roles in pest biocontrol, growth promotion, and climate change resilience. These studies underscore the importance of harnessing natural resources and microbial symbionts to foster sustainable agricultural systems. In a similar vein, Suriani et al. discussed the use of *Brevibacillus agri* and compost to enhance the growth and phytochemical compounds of *Piper caninum* plants. They demonstrated that the dual combination of both biostimulants, with 1% *B. agri* showing the greatest effect, effectively enhanced growth, nutrient uptake, and phytochemical production in these herbal plants. This strain exhibits traits such as auxin production, protease enzyme activity, and nitrogen fixation. Furthermore, Tajdinian et al. concluded that the foliar application of brown alga (*Sargassum angustifolium*) extract can elicit defense responses in strawberry plants challenged by *Macrophomina phaseolina* (Tassi) Goid, leading to improved growth indices and reduced disease severity. Building on this, Sheffield et al. investigated the impact of biochar on plant growth parameters, shedding light on its potential as a soil amendment to enhance crop productivity.

Addressing the challenges and opportunities in sustainable agriculture, the review article by Lumactud et al. underscores the complexities of soil microbiology and plant-microbiome interactions in agricultural ecosystems. They emphasize the need for interdisciplinary research and holistic approaches to harnessing biostimulants for sustainable agriculture. These insights provide a roadmap for future endeavors aimed at optimizing the use of biostimulants to enhance agricultural resilience and mitigate the impacts of climate change.

Collectively, the articles compiled in this Research Topic present a mosaic of innovative approaches and findings that underscore the transformative potential of biostimulants, microbial interactions, and climate resilience in shaping the future of climate-smart and sustainable agriculture. As we navigate the complexities of a changing climate, the insights gleaned from this Research Topic offer valuable guidance for fostering resilience, sustainability, and food security in agricultural systems worldwide. This Research Topic exemplifies interdisciplinary studies that have applied principles from multi-omics, experimental biology, and eco-physiology to demonstrate the role of biostimulants research in alleviating stresses and improving food safety. The articles also provide insights into future challenges and opportunities in the field of biostimulants. They discuss the need for additional strengthening of biostimulant products and specific crop-based research and development under changing climate contexts.

We trust that these innovative contributions will provide insights, address longstanding inquiries (while also sparking new ones), and motivate researchers in vital fields related to plant (a)biotic stress, climate change, and molecular biology. We invite authors to persist in submitting their high-quality research in Crop Biology and Sustainability to Frontiers in sustainable Food Systems.

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Impacts of humic-based products on the microbial community structure and functions toward sustainable agriculture

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Humic-based products (HPs) are carbon-rich organic amendments in the forms of extracted humic substances from manure, compost, and raw and extracted forms of lignites, coals and peats. HPs are widely used in agriculture and have beneficial effects on plants. While the agronomic benefits of HPs have been widely reported, information on their impact on the soil microbial community composition and functions is lacking, despite claims made by companies of humic substances as biostimulants. In this review, we explored published research on microbial responses with HPs application in an agronomic context. Although research data are sparse, current results suggest indirect impacts of HPs on microbial community composition and activities. HPs application changes the physico-chemical properties of the soil and influence root exudation, which in turn impact the microbial structure and function of the soil and rhizosphere. Application of HPs to the soil as biostimulants seemed to favor plant/soil beneficial bacterial community composition. HPs impacts on microbial activities that influence soil biogeochemical functioning remain unclear; existing data are also inconsistent and contradictory. The structural properties of HPs caused inconsistencies in their reported impacts on soil properties and plants. The sources of HPs and forms (whether extracted or raw), soil type, geographic location, crop species, and management strategies, among others, affect microbial communities affecting HPs efficacy as biostimulants. A more holistic approach to research encompassing multiple influential factors and leveraging the next-generation sequencing technology is needed to unravel the impacts of HPs on the soil microbiome. Addressing these knowledge gaps facilitates sustainable and efficient use of HPs as organic agricultural amendments reducing the use of chemical fertilizers.

KEYWORDS

humic-based products, humic acids, soil amendments, biostimulants, microbial communities, microbial activities

Introduction

The increasing demand for food production has intensified crop production, leading to the depletion of agricultural lands and soil and water pollution due to the intensive use of fertilizer and pesticide (Glibert et al., 2006; Galloway et al., 2008; Silva et al., 2019). Other main challenges that current agricultural practices face with are high inorganic fertilizer prices and low nutrient use efficiency by crops (e.g., nitrogen) due to the loss of nutrients by leaching, denitrification and volatilization (Malhi et al., 2001; Cassman et al., 2002). These environmental and economic concerns necessitate environmentally friendly agricultural management strategies that improve soil health and resilience. One strategy for sustainable farming practice is using less agrochemical input and more soil organic amendments, such as humic-based products (HPs).

Humic-based products are carbon-rich organic amendments in the forms of extracted humic substances from manure, compost, and raw and extracted forms of lignites, coals and peats. For decades, HPs have been widely reported to improve soil health and crop productivity (Guo et al., 2019; Nardi et al., 2021; Ampong et al., 2022). HPs can be synthetic, recycled wastes, or natural and have wide industrial applications (El-shazly et al., 2015; Dong et al., 2017; Bezuglova et al., 2019; Guo et al., 2019; Zhou et al., 2019). Natural HPs are geological deposits formed out of the natural decomposition of animal and plant material from millions of years ago that make up to 80% of soil organic matter (Olk et al., 2018). Humic acids (HAs) have been widely used in the world, with a market value exceeding USD 532.7 million in 2021 and a projected growth rate of 11.8% from 2022 to 2028 (Pulidindi and Prakash, 2021).

Numerous research studies have demonstrated positive crop responses to HPs from various sources in fields, greenhouse, and controlled environmental studies (Rose et al., 2014; Nardi et al., 2021; Yang et al., 2021; Bezuglova and Klimenko, 2022). Ampong et al. (2022) recently provided a comprehensive review on the significant positive impacts of humic acid (HA) on crop growth, yield, and soil physical and chemical properties. Plants are more stress-tolerant, productive, healthier, and yield better quality in soil with high humic acid content (Nardi et al., 2021; Ampong et al., 2022). Although the effect of HPs on the soil's physical and chemical properties and crop responses are well-studied, their effects on microbial structure and functions are poorly understood.

Soil is the most diverse and complex habitat containing more than 1,000 kg of microbial biomass carbon per hectare (Suttle, 2007; Paez-Espino et al., 2016). Soil microorganisms play vital roles in soil ecosystem functions and services, such as nutrient cycling, biogeochemical cycles, soil fertility and resilience, thereby affecting plant growth and health (Berg, 2009; Madsen, 2011; Chaparro et al., 2012). Soil microbial community structure and functions are well-known as soil health indicators. Parameters like basal respiration, nitrogen (N) mineralization, N_2 fixation and microbial enzyme activities are used to determine microbial functional activities in the soil. Biochar-based humic products induced changes in microbial biomass, community composition and diversity, and biogeochemical processes (Xu et al., 2016). While HPs are known to stimulate soil microbial communities that drive vital soil processes needed for plant growth and health, we have yet to unravel these key microbiome players. The interactive effects of HPs with crop and soil types, various agricultural practices, and the soil microbiome structure and function are poorly understood.

Recent advances in marker genes, genomic and metagenomic research have significantly increased our ability to differentiate the microbial activities under different agricultural management practices. These advances enhance our knowledge of microbial metabolic capabilities and their influence on soil fertility, plant growth and health. The reliability of humic-based products as biostimulants/biofertilizers will improve as we learn and understand more about their impact on the microbiome community and functions across ranges of biotic and abiotic factors.

This review aims to provide key insights into the impact of HPs on soil microbial structure and activities and gain further insights into opportunities for future research with a specific emphasis on agriculture. We have summarized what has been learned from recent work on soil microbial communities and functions under humic-based amendments. This review is focused on HPs as an amendment to soil microbial community structure and functions. HPs have been used as biocontrol, but that is outside the scope of this review. While we acknowledge the controversies surrounding humic substances (Lehmann and Kleber, 2015; Kleber and Lehmann, 2019), the research work presented in the following sections are based on all forms of humic products, including the raw and extracted forms of lignite-based HPs and those that were extracted from humic-rich materials (i.e., vermicompost, sewage sludge, etc.) (Figure 1). Challenges and opportunities associated with managing soil microbial communities to maximize agricultural productivity and sustainability using naturally sourced humic-based products are discussed. This review also highlights key research directions that could shape the future of humic-based products.

Humic-based products in agriculture: A historical perspective

In agricultural systems, humic-based products are organic amendments in the forms of extracted humic substances from manure, compost, and extracted or raw naturally-sourced humic substances from earth's soils and sediments derived from decayed biomatter through the humification process. HPs are

FIGURE 1
Different forms of raw and extracted humic-based products (Humalite).

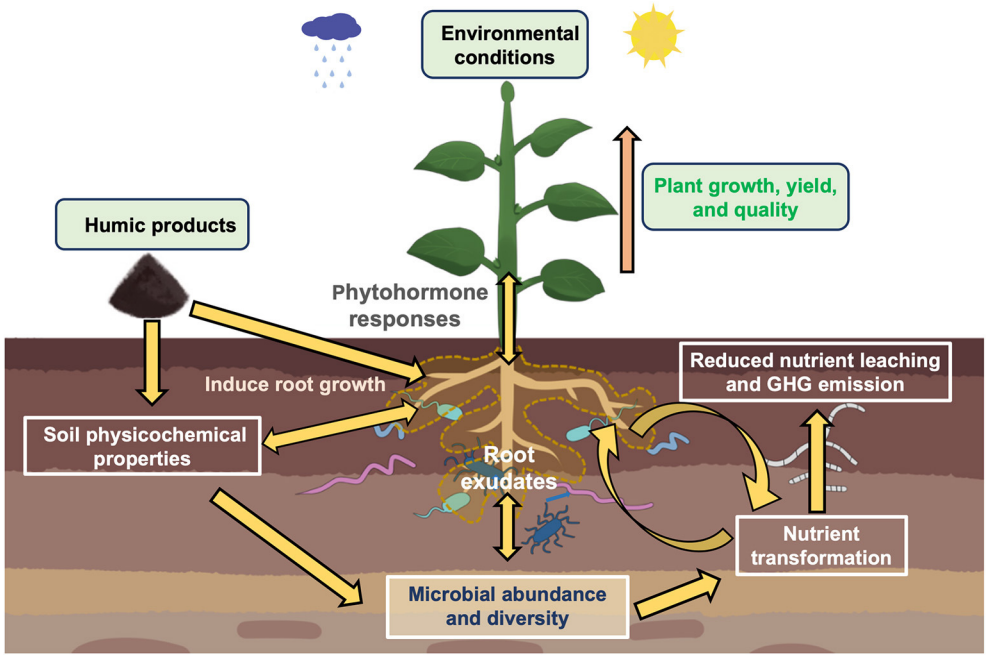

FIGURE 2
An illustration showing complex interactions determining impacts of humic-based products (HPs) application in agricultural soil. Multiple factors shape the microbial community assembly and functioning, such as sources of humic-based products and various biotic and abiotic factors (crop species, soil environmental parameters, microclimatic conditions, etc.). HPs application impacts the soil (physicochemical properties) and plant (root growth, root exudates, phytohormonal responses, shoot growth) and subsequent effects on microbial community composition and functions, such as soil nutrient transformations, a crucial soil ecosystem process for plant growth and health.

thought to stimulate soil microbiota that are beneficial to plants. Plant-beneficial microbiota have been shown to improve soil nutrient availability and thus plant growth and productivity.

The earliest account where humans used soil organic amendment was in Brazil's Amazon basin more than 2,000 years ago, where the ancient soil practice was using Terra Preta, also known as black soil (Neves et al., 2004). Terra Preta is the conceptual basis for the use of biochar to improve the fertility of soils. The native Indians in the Amazon basin would generate charcoal, mix it with organic matter, and apply it to their agricultural lands. This Amazonian black earth is characterized by its rich black color. It has been known that the black soil is due to high plant carbons that accumulated in the soil for thousands of years (Neves et al., 2004). Since the discovery of black soil, researchers around the world have explored the potential of charcoal and partially combusted organic waste or biochar to mimic the soil organic matter of the Amazonian black soil (Novotny et al., 2009).

There have been constant explorations of soil amendments for carbon addition to the soil. The agronomic potential

of humic-based products has been investigated. Humic acid, as a part of humic substances in general, affects the soil physicochemical characteristics, nutrient availability, and plant growth and physiological characteristics (Khaled and Fawy, 2011). Most humic acids that are currently being used today come from lignite and sub-bituminous coal. These low-grade coals form mineable humic substances when oxidized. They are known as humalite in Alberta and leonardite in other parts of the world (Leonard, 2012). Leonardite deposits have been produced in North Dakota (since the 1920's) and Mexico.

Over 200 years ago, components of humic substances (HS)–humic acid, fulvic acid (FA), and humin were extracted as three distinct fractions. However, the alkali extraction process of HA is still hounded by controversy as the HA that is synthesized is not really an acid (Kleber and Lehmann, 2019). The mechanisms of the humic substances' formation, as well as their structure, are still a subject of discussion and controversy. While the agronomic benefits of humic substances have been well-studied, the debatable nature of HS slogs the flow of scientific data as regards humic products or humic substances' impact on the microbial communities in the plant-soil interface.

Impact of HPs on soil microbial communities

HPs serve as the sources of carbon and energy for the soil microbes. HPs alter the soil physicochemical characteristics and physiological responses of plants which in turn affect the soil microbial community composition (Figure 2) (Puglisi and Trevisan, 2013; Puglisi et al., 2013). Table 1 presents selected studies on the impact of HPs on soil microbial communities.

Previous studies show that the application of HPs into the soil strongly affects the bacterial community composition and abundance and to a lesser extent the bacterial group actinomycetes, soil fungi, and microalgae (Dong et al., 2009; Puglisi et al., 2009). A combined application of diverse microbial consortium and humic substances significantly changed the bacterial community structure, but not the fungal communities in the blueberry rhizosphere (Schoebitz et al., 2016). Application of vermicompost enriched with commercial alkaline-extracted HAs to the soil increased the diversity of rhizosphere bacteria and fungi as revealed by denaturing gradient gel electrophoresis (DGGE) profiles and enhanced nodulation of *Pisum sativum* by nodule-forming nitrogen-fixing bacteria was also found with HPs applications (Maji et al., 2017).

Studies using 16S rRNA gene-based phylogenetic microarrays revealed the impact of commercial HPs on the resident bacterial community in differing soil profiles (van Trump et al., 2011; Puglisi et al., 2013). The most probable number (MPN) enumeration of agricultural soils amended with HPs revealed large populations of nitrate-reducing bacterial communities. These nitrate-reducing bacterial communities were phylogenetically diverse and included members of Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria (van Trump et al., 2011).

A leonardite-derived HA changed the microbiome structure of soil grown with potato plants under greenhouse conditions (Akimbekov et al., 2020). HA-treated plants showed higher microbial diversity and richness compared to the control and observed predominance in Proteobacteria. This study also demonstrated the beneficial impacts of HAs on potato plant growth (Akimbekov et al., 2020). Hita et al. (2020) isolated the cultivable bacteria from sedimentary humic acid-treated cucumber plants and recovered isolates from the phyla–Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. The isolates were also assayed for plant growth-promoting traits and found that most of the isolates were able to fix N_2 and produce plant hormones–indole-3-acetic acid and several cytokinins (Hita et al., 2020).

The effects of humic acid extracted from vermicompost on rice plants have been investigated under growth chamber conditions, where the microbial groups (e.g., *Chitinophaga* and *Pseudomonas*) that were enriched in the presence of HA were known to be related to plant defense against pathogens (da Silva et al., 2021). It was noted that in stressful environments, such as pathogen attacks, HA may modulate the plants' physiological mechanisms, triggering the plants to recruit microbes with biocontrol potential (da Silva et al., 2021). Inoculation of plant growth-promoting bacteria and HA regulates genes related to plant protection, oxidative stress, and chitin metabolism even under non-stressful conditions in tomato plants (Galambos et al., 2020). These adaptive metabolic changes may alleviate plant stress in biotic and abiotic stress conditions (Galambos et al., 2020). It was found that secondary and plant defense metabolisms were stimulated in tomato plants when plant growth-promoting bacteria and HPs were applied together (Olivares et al., 2015).

In a long-term field experiment with peanuts amended with HA and inorganic fertilizers, changes in soil enzyme activity alter the soil microbial community structure: the number of beneficial bacteria increased while harmful bacteria decreased (Li et al., 2019a). Furthermore, taxonomic groups that were reported to be beneficial to plants, including Firmicutes (bacteria), Basidiomycota (fungi) and Mortierellomycota (fungi), increased following HA application (Li et al., 2019a).

The microbial community data involving HPs applications that are currently available are based on culture-dependent and molecular fingerprint methods. While these are informative, these data are unable to decipher microbial taxonomic compositions and the identification of potential key species. In addition, the culturable microbial community is largely underestimated as only a very small percentage can be cultivated. The next generation sequencing has been around in the last decade and has been pivotal in unraveling microbial community composition and function. These high throughput technologies

TABLE 1 The effects of humic-based products on microbial communities and activities.

Humic-based products source	Crop type	Experimental condition and microbial niche	Microbial responses		References
			Microbial communities	Microbial activities	
Sedimentary humic acid from leonardite	Cucumber (*Cucumis sativus* L. var. Ashley)	Hydroponic system; plant tissues	Cultivable isolates Phyla: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes Promising isolates were from genera: *Pseudomonas Sphingomonas* *Stenotrophomonas* *Arthrobacter Microbacterium*	Plant growth-promoting traits: - fix nitrogen - produce plant hormones (indole-3-acetic acid and several cytokinins) - some isolates were able to solubilize phosphate and/or produce siderophores	Hita et al., 2020
Humic acid (from Sigma Aldrich, USA)- rich vermicompost	Pea (*Pisum sativum* cv. Bonneville)	Pot soil experiment; rhizosphere	Increased fungal and bacterial populations	- Promoted soil CO_2 respiration, microbial biomass carbon and nitrogen	Maji et al., 2017
Lignohumate	–	*In vitro*	Oxalobacteriaceae correlated with increased N fixation	- Increase in soil microbial respiration rate by 10–30% proportional to humic application rate - Increase in nitrogen fixation - Increase in methane formation - Decreased denitrification processes	Pozdnyakov et al., 2020
Vermicompost-derived humates by alkaline extraction	Winter wheat (*Triticum aestivum* cv. Zelenogradskaya-11)	Field; rhizosphere	- Indirect effects on the rhizosphere microbial communities - Treatments with both humates and herbicides showed reduced herbicide chemical stress on plants, increased plant growth and root exudation thereby affecting the dynamics of microbes in the rhizosphere - Less sharp change of dominant species - Higher resistance of *K*-strategists species to the negative effect of herbicides in the presence of humates		Bezuglova et al., 2019
Leonardite-derived humic acid	Potato (*Solanum tuberosum* L. cv. *Agata*)	Greenhouse; bulk soil	Predominance in Proteobacteria		Akimbekov et al., 2020
Parental leonardite			Predominance in Actinobacteria		
Lignite-derived HA	Oat (*Avena sativa*, L.)	Field; bulk soil	Increased soil enzyme urease (up to 89%), invertase, and catalase with increasing years of annual HA application over 2 growing seasons		Ma et al., 2022

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will enable us to systematically identify microbial interactions and functions toward identifying the major players in nutrient cycling processes. While indirect effects of HPs on microbial communities have been demonstrated through their effects on rhizodeposition (Puglisi et al., 2013), much research still needs to be completed to investigate responses under differing biotic and abiotic stress conditions. Addressing these knowledge gaps facilitates a clear understanding in developing an optimal use of HPs toward reduction of chemical fertilizer use.

Impact of humic-based products on microbe-mediated biochemical activities in the soil

As discussed above, HPs alter microbial communities in the soil. Plants treated with HPs trigger phytohormone production in plants, causing changes in root and shoot growth, and rhizodeposition that will then influence the microbial community composition in the plant-soil system (Figure 2) (Puglisi et al., 2009, 2013; Canellas and Olivares, 2014; Olivares et al., 2017). This change in microbial communities influences microbial activities, particularly the soil enzyme activities that catalyze various key biogeochemical processes (Zhang et al., 2018; Dai et al., 2020). Table 1 provides a list of research work showing the impacts of HPs to soil microbial activities and functions.

Microbial nitrogen cycling

Mineralization and nitrification are the most crucial processes in the soil N cycle as these steps influence plant N uptake, nitrate leaching, and reactive N gas emissions (Philippot et al., 2007; Norton and Stark, 2011). Microbial-mediated mineralization of organic N into ammonium (NH_4^+) and its subsequent nitrification processes– nitrite (NO_2^-) or nitrate (NO_3^-), is of major importance of N availability (Jackson et al., 2008). Understanding the effects of HPs on the microbial N cycle in the soil is key toward modern sustainable agriculture. Humic-based input affects the soil physical and chemical structure of the soil, which in turn affects the microbial N cycling in the soil. Furthermore, microbial N cycling in the soil is also controlled by environmental factors. Specifically, oxygen availability is one of the most important parameters; high oxygen soil concentrations promote nitrification whereas anoxic conditions stimulate microbial denitrification (Braker and Conrad, 2011; Lam and Kuypers, 2011). Air-filled and water-filled pores affect the moisture content in the soil, which will then directly affect the oxygen supply. High water-filled soil pores result in the depletion of dissolved oxygen concentrations due to increased microbial respiration facilitating anaerobic processes (Drenovsky et al., 2004). Besides oxygen availability and soil moisture, pH, soil organic carbon contents, and N availability control the microbial N cycling processes (Nicol et al., 2008; Hsu and Buckley, 2009; Saarnio et al., 2013). As the impacts of HPs on various soil physical and chemical structures, such as soil pores, soil moisture, pH, soil organic carbon, etc. of the soil remain inconclusive (Ampong et al., 2022), and so their effects on microbial N cycling remain unknown.

Soil amended with HPs will affect the availability of many nutrients, trace elements, electron acceptors, and other compounds such as carbon substrates that determine microbial growth and activity (Figure 2). The addition of HPs (humic acids) as seed coating was reported to improve root growth (Gorim and Asch, 2012), resulting in increased root exudates, stimulating microbial activity in the rhizosphere of cereal plants. In addition, enhancement in plant growth also increases plant nutrient uptake, thus decreasing the bioavailable N in the soil affecting microbial activity (Saarnio et al., 2013).

While research is scarce on the impact of HS on microbially-mediated N cycling processes in agricultural systems, several research works have been completed in landfill leachate/wastewater treatments that will be incorporated in the subsections below. Humic acids, the main component in the endogenous substances in the landfill leachate system, usually account for ∼4 to 44% of the total organic carbon (Liu et al., 2022). Humic acids play a dual role as both an organic pollutant and natural electron shuttle in the landfill leachate systems' concurrent nitrification, annamox, and denitrification technology (Liu et al., 2022). The system facilitates efficient HA biodegradation and efficient removal of N resulting from faster electron transfer efficiency that enhances enzymatic activity (Liu et al., 2022).

Nitrification

Many of the beneficial N-fixing bacteria live in root nodules of leguminous plants, while others are free-living in the soil (a.k.a diazotrophs). In the landfill leachate nitrification process, HA could enhance nitrifying bacteria's cellular permeability and act as an electron shuttle to reduce membrane transporter genes (Luo et al., 2019). Increasing HA concentration subsequently enriches *Nitrospira* abundance, which can oxidize nitrite to nitrate under aerobic conditions (Luo et al., 2019). In anaerobic conditions, the mechanism of redox action of HS is complex, as it participates in redox reactions as both an electron acceptor and electron donor for microbial respiration (Li Y. et al., 2019).

Several studies have demonstrated the positive effects of soil organic amendments, specifically biochar– another carbon-rich soil amendment product, on microbial N fixation. A combination of isotopic analysis and molecular techniques revealed increases in nitrogenase activities, proportion of soil N input originated from microbial N fixation, and abundance of N-fixing microorganisms in soil amended with biochar (Quilliam

et al., 2013; Harter et al., 2014; Mia et al., 2014). Increased *nifH* gene copy numbers were also observed in controlled soil microcosm experiments (Ducey et al., 2013; Harter et al., 2014). Increase in the relative abundance of many known N-fixing families: Bradyrhizobiaceae, Frankiaceae, and Rhizobiaceae were observed in biochar-amended pot experiments and rice paddy study (Anderson et al., 2011; Chen et al., 2015). In a greenhouse experiment using a broad range of biochars from different feedstocks, Güereña et al. (2015) observed increased plant biomass, nodule number and biomass, and the proportion of N derived from symbiotic N fixation in common beans (*Phaseolus vulgaris* L.).

Fulvic acid induced the growth of *Sinorhizobium meliloti*, and this combination showed an increase in active nodules and yield in *Medicago sativa* (Capstaff et al., 2020). These FA-treated plants showed up-regulation in not only early nodulating genes, but also in various processes, such as defense, oxidoreduction, and carbon and nitrogen metabolism (Capstaff et al., 2020).

Denitrification

Denitrification is a key microbial process resulting in N loss to the atmosphere, where nitrate or nitrite is reduced to gaseous NO (nitric oxide), N_2O (nitrous oxide), and N_2 (dinitrogen), by denitrifying microbes (Conrad, 1996). NO and N_2O are important greenhouse gases, with the latter holding the warming capacity that is 296 times higher than CO_2 (Zumft, 1997). Thus, understanding the microbiome underpinning the biogeochemical processes is crucial due to the economic loss of N and greenhouse gas emissions resulting from incomplete denitrification.

Many of the denitrification processes were driven by heterotrophic denitrifying microorganisms. Fulvic acid can serve as electron shuttles between bacteria and electron acceptors (Li et al., 2016). These electrons are consumed by four key denitrifying enzymes (NAR, NIR, NOR, and NOS) that are mainly encoded by the genes *nar*G, *nir*K/S, *nor*B, and *nos*Z (Li et al., 2016). The authors observed that the addition of FA into denitrification bacterial culture accelerated the metabolism of carbon sources to generate ATP and NADH through microbial glycolysis metabolism. Increased dosage of FA (from 0.5 to 1 mM) improved anammox (conversion of ammonium and nitrite to N_2 gas) bacterial activity, thereby increasing the production of extracellular polymeric substances, implying its potential role in quorum sensing performance, and enhanced N removal efficiency (Liu et al., 2020).

HPs have significant impacts in the N removal process in industrial sewage/wastewater treatment facilities. The data from this system would shift microbial composition and functional genes in relation to the soil N cycle. Concentration of FA affects microbial community, N conversion efficiency, and abundance of functional genes responsible for ammonia-N oxidation. The presence of low FA concentration (0–50 mg/L) enhances NADH generation that favors denitrification and nitrite reduction (Zhang et al., 2023).

The effect of HA on the simultaneous nitrification, anammox and denitrification (SNAD) treatment method in the landfill leachate system was investigated (Liu et al., 2022). The electron transfer efficiency of the SNAD system was enhanced due to the redox properties of HA. This efficient transfer enhanced the electron transport system activity resulting in an increase in adenosine triphosphate (ATP) that plays an essential role in microbial N metabolism. The enhanced metabolic activity by the SNAD method increased the enzymatic activities of ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, and heterotrophic denitrifying bacteria, thereby facilitating efficient N removal (Liu et al., 2022).

Phosphorus solubilization

Phosphorous (P) is an essential element that can be solubilized by root exudates or microbes and is an essential macro nutrient for crop growth (Holford, 1997). Although soils generally contain a large amount of total P, only a small proportion is immediately available for plant uptake. Plants obtain P as orthophosphate anions (predominantly as HPO_4^{2-} and $H_2PO_4^{1-}$) from the soil solution. In most soils, the concentration of orthophosphate is low (Richardson et al., 2009).

HPs affect the phosphorus cycle by altering the P uptake of plants by (1) providing a source of organic P, (2) enriching the microbial activity of P-solubilizing microbes, (3) increasing organic acid levels in soil, thereby stimulating P solubilization, and (4) increasing P availability in soil by forming a complex linkage with humic substances and metal ions (Jindo et al., 2020). Microbes involve in P cycling activities of the soil-rhizosphere interface are classified into two groups based on their P solubilizing strategies. The first group is microbes that produce nuclease enzymes, phospholipases, and phytases that hydrolyze P-organic compounds. Phytases are key enzymes in P cycling as 50% of the soil organic P is in phytate form. The second group is P-solubilizing microbes that convert sources of inorganic P into soluble orthophosphate ions.

The relationship between HPs and bioavailable P mediated by microbes is yet complex, wherein HPs are itself sources of organic P (Jindo et al., 2020). Interactions between HPs and rhizosphere microbiomes reportedly involve the central component in the auxin hormonal pathway. HPs affect the formation of lateral roots and root hair length and density, thereby stimulating the release of root exudates (O'donnell, 1973; Gorim and Asch, 2012). Some of these root exudates are precursors of IAA and other AUX-like compounds, including the amino acid L-Tryptophan (Jindo et al., 2020). Increased IAA in the rhizosphere results in acidification and consequent release of inorganic P into the soil (Chaiharn and Lumyong, 2011).

Application of the humic acid-rich vermicompost to plants resulted in increasing available P due to the promotion of phosphatase secretion and activity, microbial diversity, and abundance compared to fertilizer-only treatment (Maji et al., 2017). The authors observed increase in diversity and abundance of P-solubilizing microorganisms following humic acid-rich vermicompost treatments (Maji et al., 2017). Bezuglova et al. (2019) observed positive effect of HPs on P mobilization and crop productivity, possibly due to the activation of the rhizosphere microbiota through root exudates. Research on the interaction of HPs and soil/rhizosphere microbiomes in relation to P cycling is sparse; more research needs to be conducted.

Soil microbial biomass and enzyme activities

Microbial activities drive soil organic matter transformation and nutrient cycling processes. Understanding the impact of HPs on the soil microbial-mediated biogeochemical processes has important implications on soil health management and sustaining agricultural productivity. Microbial extracellular enzymes regulate the carbon degradation and nutrient release. Thus, enzymatic activities are sensitive indicators of soil health and can be used to determine soil responses to agricultural management practices and environmental changes (Xiao et al., 2018; Li et al., 2019b; Nannipieri et al., 2020). The key soil enzymes involved in soil C, N and P cycles are glucosidases, cellulases, hydrolases, ureases, invertases, laccases, peroxidases, proteases, nitrate reductases, and phosphatases (Nannipieri et al., 2012).

The complexity of humic substances deters the complete understanding of their impact on soil microbial activity. Previous studies show that the application of HPs had substantial impacts on soil enzyme activity (Mato et al., 1971, 1972; Malcolm and Vaughan, 1979; Pflug, 1980; Pflug and Ziechmann, 1981; Allison, 2006; Dou et al., 2018). A research study with nuclear magnetic resonance (NMR) spectroscopy showed that soil enzymes are encapsulated in humic substances, thereby causing a reduced or inhibited catalytic activity (Tomaszewski et al., 2011). Humic substances can alter bacterial enzymes causing the inactivation of extracellular enzymatic activity (van Trump et al., 2006; Mazzei et al., 2013). Humic acid fractions have been shown to inhibit phosphatase activities in several agricultural crops, and thus may have implications in relation to the humic acids' role in P nutrient cycling processes (Malcolm and Vaughan, 1979). The role of soil extracellular enzymes may be reduced because of contact with HS (Allison, 2006). Nuclear magnetic resonance revealed that β-glucosidase enzyme activity was substantially reduced by increasing fulvic acid concentration (Mazzei and Piccolo, 2013), possibly due to fulvic molecules covering the β-glucosidase active sites or the modification of the enzyme conformational structure during the humic-enzyme complex formation.

The inhibitory effects of HS on enzyme activity may be offset by increased stability and/or resistance to enzyme degradation (Marzadori et al., 2000a,b; Dong et al., 2008). The effects of humic substances on enzymes are dependent on pH, ionic strength, and mass ratio of HS/enzyme (Li et al., 2013). In particular, the enzyme activity in the HS-enzyme complexes was suppressed when ureases, the enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamates, were oppositely charged to the humic substances (Li et al., 2013). Conversely, no enzyme complexes were formed when both ureases and HS were both negatively charged (Li et al., 2013). Structural characteristics of HAs affect urease activity. The high molecular weight HA fraction significantly improved urease activity and stability especially in alkaline soil (Dong et al., 2008).

Up to now, very limited research has been conducted on the impact of HPs on the microbial enzyme in the soil, and the results are contradictory. A combined application of NPK and humic acid in sugarcane rhizosphere significantly increased catalase, dehydrogenase, phosphatase activities, and microbial biomass (Sellamuthu and Govindaswamy, 2003). This enhanced soil enzymes activities and increased microbial biomass in the presence of HPs may be due to a greater carbon-rich substrates availability to microbes, thereby promoting heterotrophic growth (Blagodatskaya et al., 2014). A combined application of inorganic fertilizer and HA changed the soil microbial community structure that consequently changed the soil enzyme activities– sucrase, urease, and phosphatase (Li et al., 2019a). Changes in the C/N ratio of the soil due to the addition of HA favor conditions for microbial growth (Griffiths et al., 2012). A study by Dong et al. (2017) in landfill leachate system showed that HA shape bacterial community, increase enzyme activities and upregulate microbial genes related to denitrification. HA positively affected the denitrification process in landfill leachate that promoted the growth of denitrifying bacteria with the predominance of *Thauera* (Dong et al., 2017), thereby facilitating removal of N from leachate.

Biochar and lignite-based amendments showed contradictory effects on microbial activities and greenhouse gas emissions in laboratory-incubated microcosms using agricultural soil (Li et al., 2021). This study demonstrated that biochar-amended treatment increased microbial community growth and ability to utilize diverse carbon sources, which contrasted with the lignite-based amended treatment. Biochar-amended treatment also showed increased carbon dioxide emission, which was inhibited in the lignite-based setup. In contrast, nitrous oxide emission decreased in the biochar-amended soil but increased in the lignite-amended treatment (Li et al., 2021).

Increased application of lignite-based HA displayed an increasing trend in soil enzyme activities (Ma et al., 2022).

TABLE 2 The effect of humic-based products with other inputs on microbial community and activities.

Humic-based products with other input	Humic-based product source	Crop type	Experimental setup and microbial niche	Microbial responses	References
Microbial inoculants					
Pseudomonas spp. and *Bacillus amyloliquefaciens* Arbuscular mycorrhizal fungi (AMF)	Humic acid extracted from green compost	Maize (*Zea mays* cv. Aphoteos, Limagrain S.p.a.)	Greenhouse pot experiment; rhizosphere	- Increased P absorption - Significant changes in bacterial and fungal diversity	Cozzolino et al., 2021
Herbaspirillium seropedicae	Humates from vermicomposted cattle manure	Maize (*Zea mays* L. cv. UENF 506-8)	Greenhouse pots and field; plant tissues	- Promoted microbial enzymes associated with N assimilation	Canellas et al., 2013
Microbial consortium (bacteria and fungi)	Leonard-derived	Blueberry (*Vaccinium corymbosum* L.)	Field experiment; rhizosphere	- Significantly changed rhizosphere bacterial community structure - No change in the fungal structure	Schoebitz et al., 2016
Agrochemicals					
Inorganic fertilizer	Commercial humic acid	Peanut (*Arachis hypogaea* L.)	Long-term field experiment; bulk soil	- Increased abundance of Firmicutes in bacteria Basidiomcycota and Mortierellomycota in fungi - Increased urease, sucrase, and phosphatase activities - Decreased bacterial abundance - Increased fungal abundance	Li et al., 2019a

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Repeated annual application of HA (1,500 kg/ha) at a dryland experimental farm in North Central China planted with oats found increased urease, invertase and catalase activities. The authors explained that these increased enzyme activities might be the result of increased soil organic carbon and improved soil physicochemical properties that provided a condition conducive to the growth and activities of soil microorganisms (Ma et al., 2022).

Tolerance to abiotic stress

The utilization of biostimulants, such as HPs was reported to potentially mitigate the adverse effects of environmental stresses on plants (Canellas and Olivares, 2014). Previous reports indicated that HPs induce changes in the root morphology to facilitate colonization of plant beneficial microbes (Canellas et al., 2013; Canellas and Olivares, 2017). Canellas and Olivares (2017) demonstrated that the exogenous application of humic acid in maize facilitated the release of border cells from root tips resulting in increased colonization density of the diazotrophic bacteria, *Herbaspirillum seropedicae* in the roots. Borders cells exuded from the roots are the first living partition at the plant-soil interface in the root cap (Driouich et al., 2012). Borders cells are sources of biologically active chemicals that can modulate the division of root tip cells, expansion and gravitropism in plants (Zhu et al., 1997). Borders cells were also reported to neutralize toxic chemicals near the roots and possibly inhibit or promote the growth of other rhizosphere organisms (Bais et al., 2006).

The soil is a complex habitat with intricate interacting biotic and abiotic components. The application of HPs on the soil affects its physicochemical properties, and on the plants its growth and rhizodeposition, that in turn impact the microbial community composition and function. The plant-soil feedbacks affecting microbial communities are crucial in soil nutrient cycling and plant uptake processes. Up to now, data are scarce, and results are inconclusive on how HPs impact the soil. Specifically, it remains unknown how HPs and their different forms and sources affect the soil pH (Ampong et al., 2022), which is a key predictor of microbial structure and composition at field and continental scales (Lauber et al., 2009; Chu et al., 2010; Banerjee and van der Heijden, 2022). For instance, the complex nature of HPs caused inconsistencies regarding whether HPs form an enzyme complex that stabilizes urease activity, or whether HPs negatively affect urease activity. A stable urease activity in an enzyme complex facilitates the gradual release of plant-available form nitrogen that will then promote plant uptake and lessen nutrient leaching. It also remains unclear whether HP application will have stimulating effects on plant beneficial bacteria. Thus, more research work needs to be done on how the application of HPs and their various forms from different sources in different crop species covering various soil types and environmental conditions impact the soil microbial community composition, structure, and function, which consequently impact plant growth and health (Figure 2).

Interactive effects of humic-based products with other biologicals and amendments on microbial community and function

Microbial inoculants

Inoculants with organic amendments alter soil microbiota and promote plant growth (Olivares et al., 2017). The combined use of humic acids with *Pseudomonas* spp. and *Bacillus amyloliquefaciens* in maize demonstrated superior effects on P absorption compared with the inoculation of each bacterial strain alone. The greatest P uptake was observed when *B. amyloliquefaciens* was combined with HA and AMF and *Pseudomonas* spp. with HA were applied to soil. This work also noted significant changes in bacterial and fungal diversity with HA application (Cozzolino et al., 2021). Selected studies showing combined effects of HPs with other inputs on microbial community structure and functions are presented in Table 2.

Microbial enzymes associated with N assimilation were promoted when HA and *Herbaspirillium seropedicae* were applied to maize plants (Canellas et al., 2013). Similarly, HA application combined with *Enterobacter* sp. 32 A inoculant induced genes related to N assimilation in tomatoes (Galambos et al., 2020). Moreover, the combined application of beneficial bacteria and humate form HPs increased productivity in tomatoes and stimulated secondary metabolism and plant defense (Olivares et al., 2017). Galambos et al. (2020) also observed gene expression related to plant hormones, such as jasmonic acid, auxins, gibberellins, and cytokinins with the combined application of HA and plant growth-promoting bacteria.

Application of sodium humate to soybean inoculated with *Bradyrhizobium* in the presence of molybdate improved soybean yield, nodule number, and biological nitrogen fixation (Til'ba and Sinegovskaya, 2013). The greater efficiency of nodulation in the presence of HPs may be linked to the ability of these substances to regulate quorum sensing in rhizobia. Quorum sensing plays an essential role in the growth and development of legume-rhizobia symbiosis (Bogino et al., 2015). In the presence of water-soluble HPs, a greater increase in N fixation was observed in soybean inoculated with *Bradyrhizobium liaoningense* in greenhouse experiments (Guo Gao et al., 2015). These results revealed the direct effect of HPs on bacteria and their potential to improve microbial symbiosis with the host plant.

It was found that HPs (at a rate of ~ 800 mg/L) and saprophytic microorganisms as biofertilizers enhanced the growth of mycelium and mycorrhizal fungus formation by

Glomus claroideum BEG23 in a hydroponic system, compared with control treatments with either only biofertilizer or no HS (Gryndler et al., 2005). Conversely, mycorrhizal colonization and hyphal length in laurel roots (*Laurus nobilis* L.) were inhibited by the presence of HS at a concentration of >800 mg/kg (Vallini et al., 1993).

Agrochemical fertilizers

HPs can increase the soil nutrient holding capacity from agrochemical inputs by enhancing the nutrient cycling within different compartments of the soil organic matters as well as the exchange of dissolved nutrients in the soil pore water (Ampong et al., 2022). Consequently, the amount of dissolved nutrients in the pore water can be reduced but is replenished over time. Since organic amendments could act as slow-release fertilizers, this beneficial situation could be maintained over long periods of time, in contrast to the mineral fertilizers alone. The combined application of humic acid and inorganic fertilizers increased peanut yield and quality in a long-term experiment (Li et al., 2019a). HA alleviate problems adherent to continuous cropping systems. In the presence of HA, plant-available NPK, and soil organic matter increase, resulting in increased plant NPK uptake (Li et al., 2019a). Additionally, improvements in soil physico-chemical properties and plant growth leads to enhanced soil microbial diversity and soil enzymatic activities (Li et al., 2019a).

Conclusions, future directions, and perspectives

Environmental problems and increasing production costs of chemical fertilizer use have driven interest in finding sustainable methods to maintain agricultural soil health. Biostimulants, such as humic substances from a raw or extracted form of oxidized lignite and extracted humic products (also popularly known as humic acids) from other organic materials, are being explored for their potential to reduce chemical fertilizer use. Despite extensive research on the agronomic benefits of HPs, their impacts on microbial communities are not fully explored. Interactions of HPs and microbial communities remain unknown, even though HPs are widely sold as biostimulants that purportedly stimulate beneficial bacteria in the rhizosphere. Until substantial research works have been done on the impact of various HPs on the soil across varying ecological zones, it is difficult to draw conclusions about the mechanisms and interactions of HPs regulating the microbial community structure and functions in the plant-soil interface. Current research results are contradictory due to the complex nature of HPs.

HPs are sold in various forms: humic acid, fulvic acid, humate and the properties vary depending on the source or origin. While microbial data on the effects of HPs are scarce, current results on their impacts on biogeochemical enzyme activities have also been contradictory. In addition, most research studies were performed under controlled environmental conditions, thus there is a need for field research across soil types covering various crop species and rotations. The effectiveness of HPs depends on the nature of humic materials and where they have been sourced (Rose et al., 2014). Different HPs properties, experimental setups, and biotic and abiotic conditions will have differing effects on the soil microbial structure and composition (Figure 2). Humic-based products can promote root growth and root exudation, which can also have indirect effects on the soil microbiome. The direct effects of HPs on microbial communities have yet to be unraveled. To decipher the impact of HPs, a more holistic approach to research should be done considering interactions among different crop species/genotype under varying ecosystems and environments.

Knowledge of the long-term effects of HP application on soil physical, chemical, and biological (microbial) properties remains unclear. We hope that the current advancements in sequencing technologies will accelerate more research on investigating humic-based products on microbial community structure and functions. Addressing the fundamental knowledge gaps will facilitate the successful application of HPs in agricultural crop production.

Author contributions

RL wrote the manuscript and contributed to the concept and outline of the manuscript. MT and LG contributed to the concept and outline of the manuscript, provided comments/suggestions, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Macroalgal treatment to alleviate the strawberry yield loss caused by *Macrophomina phaseolina* (Tassi) Goid. in greenhouse cultivation system

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The application of algae has been considered a key element for integrated disease management in sustainable agriculture. These organisms can act as a bio-stimulant for induction of resistance against a variety of abiotic and biotic agents that cause economical loss to crop production globally. Charcoal rot disease caused by *Macrophomina phaseolina* (Tassi) Goid. is one of the biotic agents restricting strawberry (*Fragaria* × *ananassa* Duch.) yield in many cultivation sites. Herein, the foliar application of brown alga (*Sargassum angustifolium*) was investigated for the reduction of the disease symptoms and improvement of vegetative and reproductive indices in strawberries under greenhouse conditions. The results showed that alga-treated infected plants showed symptom remission. Moreover, vegetative and reproductive indices of alga-treated plants were significantly improved. Biochemical analysis showed that in alga-treated infected plants the total phenol, flavonoids, and total antioxidant activity were significantly increased compared to non-treated infected plants. Furthermore, the content of defense-related enzymes, viz. phenylalanine ammonia-lyase and polyphenol oxidase, were significantly increased in the infected plants pre-treated with the alga extract. Foliar application of *S. angustifolium* extract can induce defense responses in strawberry plants infected by *M. phaseolina* leading to improved growth indices of the plants. It can be concluded that *S. angustifolium* extract is a promising source of bio-stimulants for induction of disease resistance against charcoal rot disease in strawberry cultivations.

KEYWORDS

seaweed extract, charcoal rot disease, induced resistance, growth index, greenhouse cultivation system

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) belonging to the family Rosaceae, sub-family Rosoidea, and the genus *Fragaria*, is one of the most important small fruits of temperate regions, which is widely consumed in the world (Guttridge, 2019). Strawberry is an herbaceous and perennial plant with creeping stems (stolon), which is usually propagated at the commercial level through vegetative methods (production of stolon) (Caleb et al., 2016). Due to the presence of desired conditions for strawberry cultivation, Iran is one of the major producers of strawberries in the Middle East region (Tehranifar and Sarsaefi, 2002).

Strawberry fruit is non-perishable with a limited shelf life and is highly perishable against physical damage and fungal invasion (Figueroa et al., 2008). The charcoal rot disease caused by the fungus *Macrophomina phaseolina* (Tassi) Goid. is one of the most devastating diseases of strawberries globally (Zveibil and Freeman, 2005; Avilés et al., 2008; Baino et al., 2011; Hutton et al., 2013; Sánchez et al., 2013; Hajlaoui et al., 2015; Baggio et al., 2019). Damage caused by strawberry charcoal rot disease has been estimated to be 15–20% of yield reduction or 3–4 million dollars per year in the states located in the southeastern United States (Baggio et al., 2019). Every year, this disease destroys 2–5% of strawberry plants in the state of Florida, and if the climatic conditions are favorable, it destroys 80% of the field (Baggio et al., 2019). In Iran, *M. phaseolina* has been isolated from the crown and roots of strawberry plants resembling wilting and rotting symptoms in Kurdistan, Mazandaran, and Golestan provinces (Sharifi and Mahdavi, 2012). The fungus produces resistant structures called microsclerotia that can survive for long periods in soil and strawberry debris (Zveibil et al., 2012). These microsclerotia are usually the main source of new infections and their numbers increase in susceptible hosts in the soil and grow continuously for several seasons (Baggio et al., 2019).

The use of alga as fertilizer in agriculture has been common since ancient times in the Roman Empire (Pereira and Cotas, 2019). Alga extract is more useful than chemical fertilizer due to its biodegradable, non-toxic, and environmentally friendly properties. These are the most important reasons for using alga extract in recent years for sustainable agriculture in organic and integrated agriculture (Mukherjee and Patel, 2020). In the last decade, the use of natural plant biostimulants has become widespread (Drobek et al., 2019). Induction of plant defense mechanisms using polysaccharide or oligosaccharide extracted from the alga, which have a promising protective and strategic strategy (Benhamou and Rey, 2012). Alga extract has been used directly or mixed with soil as compost, which ultimately increases soil fertility (Khan et al., 2009; Craigie, 2011). The positive effects of alga extract on improving yield and improving the resistance level of garden and agricultural plants to biotic and abiotic stresses have been reported by several researchers (Park et al., 2005; Erulan et al., 2009; Mansori et al., 2015; Shukla et al., 2017, 2018; El-Sheekh et al., 2020, 2021; Mostafa et al., 2022). It was hypothesized that the use of macroalgal extract can reduce the damage of charcoal rot disease in strawberry plants. The goal of this study was to use the extract of brown alga (*Sargassum angustifolium*) for the reduction of *M. phaseolina* effects and improvement of growth indices in strawberry plants under greenhouse conditions.

Abbreviations: TAA, Total antioxidant activity; PAL, Phenylalanine ammonia-lyase; PPO, Polyphenol oxidase activity; *S. angustifolium*, *Sargassum angustifolium*; *M. phaseolina*, *Macrophomina phaseolina*; °C, Celsius Degree; g, Gram; cm, Centimeter; mm, Millimeter; mg, Milligram; min, Minute; h, Hour; µL, Microliter; l, Liter; ddH_2O, Deionized distilled water; mL, Milliliter; *g*, Gravity; rpm, Centrifugal rotation speed; DPPH, Diphenylpicrylhydrazyl; mM, Millimolar; nm, Nanometer; *A*, Absorbance; CA, Control absorbance; SA, Sample absorbance; U, Unite; dpi, Days post inoculation; C, Control; P, Pathogen; A, Alga.

Methods

Plant material and cultivation system

The certified Strawberry plantlets, cultivar Paros, were obtained from a commercial nursery in Sanandaj (Kurdistan province, western Iran). The plantlets were transferred into cylindrical plastic pots (20 cm × 18 cm) containing sterilized soil with a texture consisted of sand (37%), silt (39%), clay (20%), and organic matter (4.0%). The soil texture class was loam. No fertilization was applied to the pots during the experiment. A uonset greenhouse (height: 3 m, diameter: 6 m), with polyethylene cover and pad & fan cooling system located in Mollasani city [longitude (λ): 48.8648334, latitude (ϕ): 31.6242601], was used. The conditions of the greenhouse (temperature: 22 ± 3°C, relative humidity: 45 ± 5%, 12-h photoperiod) were regularly checked in terms of temperature and humidity. After 30 days, the first flowers were removed for better plant growth, and then the treatments were applied. The treatments were applied before the emergence of flowers, and after the fruits reached commercial maturity, they were harvested manually, wrapped in foil, instant frozen in liquid nitrogen, and stored at −70°C for further experiments.

Preparation of alga extract

The brown alga (*S. angustifolium*) was collected from the shores of Chabahar, southeastern Iran (25.300278°N 60.612778°E), and transferred to the laboratory. To remove impurities, the alga was first washed with deionized distilled water (ddH_2O) and impurities were removed. After air-drying

for 10 days, the tissues were then ground using a grinder. To prepare the aqueous solution, 50 g of alga powder and 500 mL of ddH_2O were shacked at room temperature. Then it was boiled for 60 min and filtrated. A stock solution of the extract with a concentration of 5 mg/mL was prepared and used for the assay (Sivasankari et al., 2006; Ramarajan et al., 2012).

Fungal inoculation

A virulent isolate (MP) of *M. phaseolina* was prepared from the Fungal Culture Collection of Shiraz University (Shiraz, Iran). Fungal microsclerotia were obtained without culture, by placing a block of agar culture containing active fungal mycelium in a flask containing potato extract and dextrose (Short and Wyllie, 1978). The flask was incubated for 3 months at room temperature until thick tangled strands of microsclerotia were formed. These thick tangled strands were removed and washed 3 times with ddH_2O and dried at 35°C and then it was ground gently with a mortar. Microsclerotia was mixed with 1,000 g of sterile sand and stored at 4°C. Before inoculation, this mixture was added to the soil required to be contaminated by the pathogen (Goudarzi et al., 2011).

Treatments

The treatments were divided into four groups: control, pathogen (*M. phaseolina*), alga (*S. angustifolium*), and alga-pathogen. Control included non-treated pathogen-free plants irrigated with 180 mL of ddH_2O every 2 days. In the pathogen treatment, the soil next to the crown was replaced with 1,000 g of infested sand containing 100 viable of the fungus microsclerotia/g soil that was prepared in advance, and irrigation was applied. In case of non-inoculated plants, 1,000 g of pathogen-free sterilized sand was placed around their crown. In the alga-pathogen and alga treatment, 7.0 mL the algae extract (5 mg/mL concentration) was applied as a foliar spray every 3 days, and the application of this treatment continued until the appearance of flowers, and after the appearance of flowers and fruits, irrigation was conducted. The experiment was terminated 120 days post incubation in the greenhouse. A completely randomized design with total number of 4 replications per treatment was used and the experiments were performed two times.

Evaluation of disease severity

The disease severity caused by *M. phaseolina* in strawberry plants was visually evaluated for the inoculated plants 92 days post inoculation. To this end, each plant was inspected and scored from 0 to 5 according the developed symptoms (Table 1). The percentage of disease severity index (DSI) was then measured using Equation (1) which has been previously described by Camara et al. (2013):

TABLE 1 Symptom severity classes on the pathogen-inoculated strawberry plants.

Class	Symptom
0	No symptom
1	Partial root blackening
2	Total root blackening
3	Total root blackening and leaf necrosis
4	Total root blackening, leaf necrosis and reduced fruit size
5	Overall wilting

$$DSI\ (\%) = \sum_{e=0}^{4} \frac{eRe\ \times 100}{5N} \quad (1)$$

In which DSI = disease severity index; e = class; Re = number of plants in class €; N = total number of plants.

Vegetative and reproductive indices

The vegetative and reproductive growth of strawberry plants were mainly determined according to Breen and Martin (1981). Vegetative traits included plant height, root length, aerial length, fresh weight of aerial organs, and dry weight of root. Also, reproductive indices included fruit number, fruit weight, fruit length and diameter, and fruit volume. Water displacement technique was used to measure the fruit size. The indices were measured at the end of the experiment.

Total phenol

To measure the amount of total phenol in the leaf, the Folin-Cicalito reagent was used (Singleton and Rossi, 1965). For this purpose, 1,500 μL of Folin-Cicalito reagent diluted with a ratio of one to ten (1:10) was added to 300 μL of the leaf homogenate (1 g) and placed at room temperature (25°C) for 5 min. 1,200 μL of 7.5% sodium carbonate were added to the resulting mixture and placed on a shaker in a dark place for 90 min. Six mL of ddH_2O were added to the solution and finally, the absorbance number at 765 nm wavelength was recorded with a spectrophotometer (made in the USA—model 2100-UV). The amount of total phenol was calculated using the standard curve of gallic acid and the results were expressed in terms of mg of gallic acid per 100 g of fresh fruit weight. To prepare gallic acid standard solutions, 0.1 g of gallic acid was dissolved in ddH_2O and the final volume was brought to 100 mL using

distilled water. Then, to prepare 0, 10, 20, 30, 40, 80, 160, 320, and 480 mg/100 mL standards, 0, 0.1, 0.2, 0.3, 0.4, 8 0.0, 1.6, 3.2, and 4.8 mL of gallic acid solution were removed and made up to 10 mL using ddH_2O. Then, 300 μL were taken from the standards, and the rest of the steps were carried out like the samples.

Flavonoids

For extraction, one g of strawberry leaf was kept with 8 mL of 80% methanol for 12 h at 4°C. Then it was centrifuged at 8,000 *g* for 25 min at 4°C. To measure the flavonoids, one mL of supernatant was mixed with 0.5 mL of 5% sodium nitrite. After 6 min, 0.5 mL of 10% aluminum chloride and after 6 min, 2 mL of one molar sodium hydroxide were added to the mixture and kept for 15 min. The absorption number at 510 nm wavelength was recorded with a spectrophotometer and the results were reported in terms of mg per 100 g of fresh fruit weight (Li et al., 2014).

Total antioxidant activity

The total antioxidant activity (TAA) was determined using the free radical reduction method (diphenylpicrylhydrazyl [DPPH]) with the method of Sanchez-Moreno (2002). First, DPPH solution with a concentration of 0.1 mM was obtained by dissolving 6 mg of DPPH in 100 mL of 80% methanol. This solution was prepared daily in a dark container to measure the inhibition percentage. To prepare the alcoholic extract, 0.3 g of leaf sample was pounded with 3 mL of solvent in the dark and shaken at 100°C for 30 min. After that, it was centrifuged for 10 min at 4°C at 5,000 rpm and the supernatant extract was used to measure TAA. For this purpose, 75 μL of the alcoholic extract was taken and 2,925 μL of DPPH solution was added to it. The samples were placed in the dark chamber for 30 min. The absorbance of the samples was measured at a wavelength of 517 nm (A_{517}) using a spectrophotometer. The control sample was read using DPPH solution without adding the extract. Finally, using Equation (2), the inhibition percentage of the samples was calculated:

$$Inhibition\ (\%) = \frac{CA - SA}{CA} \times 100 \quad (2)$$

In which CA is the absorbance of the control and SA is the absorbance of the sample.

Polyphenol oxidase activity

To measure the activity of polyphenol oxidase (PPO) according to the proposed method (Worthington, 1988), one mL of 50 mM sodium phosphate buffer, one mL of 1 mM tyrosine, and 900 μL of ddH_2O were placed into the cuvette and 100 μL of enzyme extract was added to the cuvette. It was added and the increase in absorbance at 280 nm wavelength was recorded using a spectrophotometer at 2, 4, 6, 8, 10, and 12 min. By placing the absorbance changes (the difference between the highest and the lowest number read during 12 min, which is linear) in Equation (3), the amount of enzyme activity was calculated in mg/g/min.

$$Enzyme\ activity\ (mg/g/min) = A280/min \times 50 \quad (3)$$

Phenylalanine ammonia-lyase activity

Phenylalanine ammonia-lyase (PAL) activity was measured using the method of Zucker (1968). First, one g of leaf tissue was crushed with 5 mL of sodium borate buffer [pH 8.8]. Then the prepared solution was centrifuged at 12,000 rpm for 10 min. Five hundred microliter of the centrifuged extract, two mL of sodium borate buffer with 500 μL of 20 mM phenylalanine solution were poured into a tube and placed in a hot water bath for one h at 37°C was placed. Enzyme activity was determined by measuring the absorbance of the solution at a wavelength of 290 nm for one h with an interval of 15 min and was reported as units per mg of enzyme extract protein (U/mg protein).

Data analysis

All data collected during the experiment were transferred into Excel software and prepared for statistical analysis. Data analysis was performed using SAS software (v. 9.4) (Yuan, 2011). The statistical design used in this study was a completely randomized design with 4 replications and the means were compared based on Duncan's multiple range test with an alpha error level of 5%. In the case of biochemical experiments, technical (sample) and biological (plant) replications were considered per treatment. To analyze the data from disease severity index, Kruskal–Wallis H test was used and the significance at $P = 0.01$ level was determined.

Results

Disease severity

The symptoms of the disease including blackening of root and crown, reduction of leaf area, leaf necrosis, reduction of fruit size, and decreased number of fruits were observed on *M. phaseolina*-inoculated plants 92 days post inoculation (dpi) (Figure 1a). Strawberry plants inoculated with the fungus were treated with the alga extract. In these plants, there are fewer symptoms compared to the non-treated plants (Figure 1b).

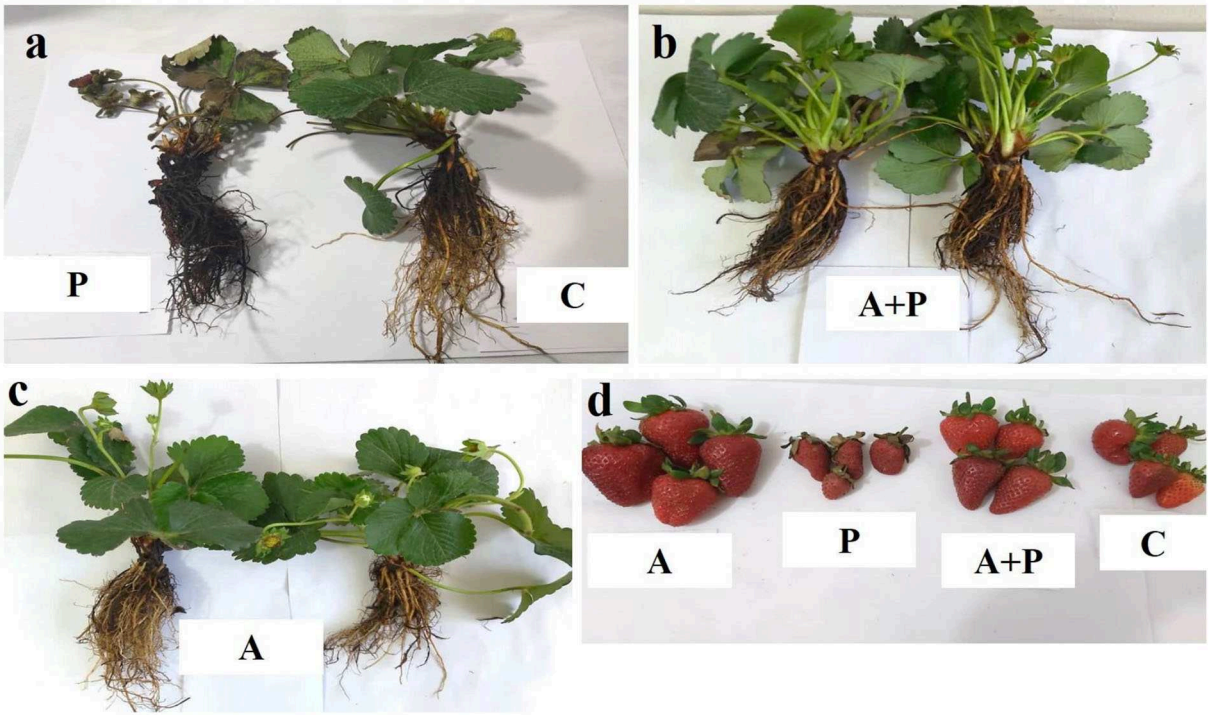

FIGURE 1
Phenotypic responses of strawberry plant to the fungal pathogen (*M. phaseolina*) among different treatments 92 days post inoculation. **(a)** Symptoms of charcoal rot in the pathogen-inoculated plant (P) compared to non-inoculated plant (C); **(b)** vegetative growth of infected plants pre-treated with the alga (*S. angustifolium*) extract (A + P); **(c)** vegetative growth of non-inoculated plants pre-treated with the alga extract (A); **(d)** comparison of fruits from plants under the different treatments.

Healthy strawberry plants treated with the alga extract showed improved vegetative growth (Figure 1c). Figure 1D shows the comparison of strawberry fruits among different treatments at 92 dpi. Accordingly, in plants treated with the alga extract, the size of the fruits was larger than those of non-treated plants (Figure 1d). In contrast, the infected plants which had not been treated with the alga extract produced the smallest fruits. Also, infected plants treated with the alga extract produced larger fruits compared to infected non-treated plants. Evaluation of disease severity showed that alga-treated infected plants had a significantly lower disease severity index (20%) than non-treated infected plants (80%). These results showed that foliar application of *S. angustifolium* extract to strawberry plants can reduce the disease severity caused by *M. phaseolina*.

Vegetative indices

Statistical analysis of the data from the effect of different treatments on strawberry plant length showed that the highest plant length (29.75 cm) was related to the plants under alga extract treatment, while the lowest plant length was found in non-treated infected plants (17 cm) (Table 2). Also, the length of the plant treated with alga extract was significantly higher than the other treatments, and the length of the infected plants was significantly lower than other treatments. The difference between the length values of non-infected alga-treated plants and infected alga-treated plants was not significant.

Similarly, the highest root length (18 cm) was found in non-infected alga-treated plants, while non-treated infected plants showed the lowest root length (8 cm) (Table 2). The root length of infected alga-treated plants was significantly higher than infected non-treated plants.

The highest value of aerial length (14 cm) was recorded for infected alga-treated plants (Table 2). The second highest value of aerial length (12 cm) belonged to non-inoculated alga treatment plants. The remaining plants did not show any significant difference in aerial length.

The non-inoculated alga-treated plants showed the highest fresh weight of the aerial organs (12.025 g), while non-treated infected plants exhibited the lowest fresh weight of aerial organs (5.84 g) (Table 2). Furthermore, this index in infected alga-treated plants was significantly higher than in non-treated infected plants.

The dry weight of roots in control plants was determined as the highest value (10.97 g), while non-treated infected plants

TABLE 2 Effect of different treatments on vegetative indices of the strawberry plant.

Treatment*	Vegetative index†				
	Height (cm)	Root length (cm)	Aerial length (cm)	Fresh weight of aerial organs (g)	Dry weight of root (g)
A	29.75^{a}	18.00^{a}	12.00^{b}	12.025^{a}	10.04^{ab}
A + P	27.00^{b}	13.00^{b}	14.00^{a}	10.34^{b}	9.00^{b}
P	17.00^{c}	8.00^{d}	10.75^{c}	5.84^{d}	5.58^{c}
C	20.50^{c}	10.75^{c}	10.50^{c}	7.71^{c}	10.97^{a}

*A, alga (S. angustifolium) extract; P, pathogen (M. phaseolina); A + P, alga-treated pathogen-infected; C, control.
†Letters show the significant difference between the treatments according to the results of Duncan's multiple range test.

TABLE 3 Effect of different treatments on reproductive indices of the strawberry plant.

Treatment*	Reproductive index†				
	Fruit number	Fruit weight (g)	Fruit volume (g/mL)	Fruit diameter (mm)	Fruit length (mm)
A	5.50^{a}	8.86^{a}	10.37^{a}	16.87^{a}	27.87^{a}
A + P	4.25^{b}	7.38^{b}	7.93^{b}	16.56^{a}	22.79^{b}
P	3.00^{d}	4.11^{d}	3.87^{d}	12.50^{c}	20.02^{c}
C	3.50^{c}	5.48^{c}	5.68^{c}	14.00^{b}	22.65^{b}

*A, alga (S. angustifolium) extract; P, pathogen (M. phaseolina); A + P, alga-treated pathogen-infected; C, control.
†Letters show the significant difference between the treatments according to the results of Duncan's multiple range test.

showed the lowest value (5.58 g) (Table 2). Also, this index in alga-treated infected plants was significantly higher than in non-treated infected plants. The difference between the dry weight of roots of non-infected alga-treated plants and infected alga-treated plants was not significant.

Reproductive indices

Table 3 shows the effect of alga treatment on infected and non-infected strawberry plants. The mean number of fruits (5.50) in the alga-treated plants was significantly larger than that of other plants. Also, the significantly smallest mean number of fruits (3.00) was found in the non-treated infected plants compared to other treatments. Similarly, the highest value of fruit weight (8.86 g) and fruit volume (10.37 g/mL) were observed in alga-treated plants. These indices in non-treated infected plants were significantly lower than in plants under other treatments. Also, the highest fruit diameter was found in alga-treated plants and alga-treated infected plants (16.87 mm and 16.56 mm, respectively). The non-treated infected plants showed the lowest fruit diameter (12.5 mm) compared to other plants. The highest fruit length (27.287 mm) belonged to the alga-treated plants. The non-treated infected plants showed the lowest fruit diameter (12.50 mm) compared to plants under other treatments. There was no significant difference between the fruit diameter of alga-treated infected plants and control plants.

Biochemical factors

The results showed that alga-treated plants had a significant effect on the total phenol, flavonoids, and TAA of strawberries (Figure 2). Alga-treated infected plants had significantly more phenol content (1.60 mg/g) and flavonoids (1.44 mg/g) than plants under other treatments (Figures 2A,B). The lowest phenol content and flavonoids were found in control plants (0.93 mg/g and 0.51 mg/g, respectively). Phenol content and flavonoids were not significantly different between alga-treated and non-treated infected plants (Figures 2A,B). The highest level of TAA (0.2) was recorded for alga-treated infected plants (Figure 2C). In contrast, non-treated infected plants showed the lowest level of TAA (0.06).

Enzyme activity

The results from the effect of different treatments on the PAL content in strawberry plants showed that the alga-treated infected plants had the highest enzyme content (0.89 U/mg) found while the lowest amount of PAL enzyme was recorded for alga-treated non-infected plants (0.54 U/mg) (Figure 3A). The content of the PAL enzyme was not significantly different between non-treated infected and control plants (Figure 3A).

In the alga-treated infected plants, the highest amount (0.65 mg/g/min) of PPO enzyme was found while the lowest value was determined in the alga-treated

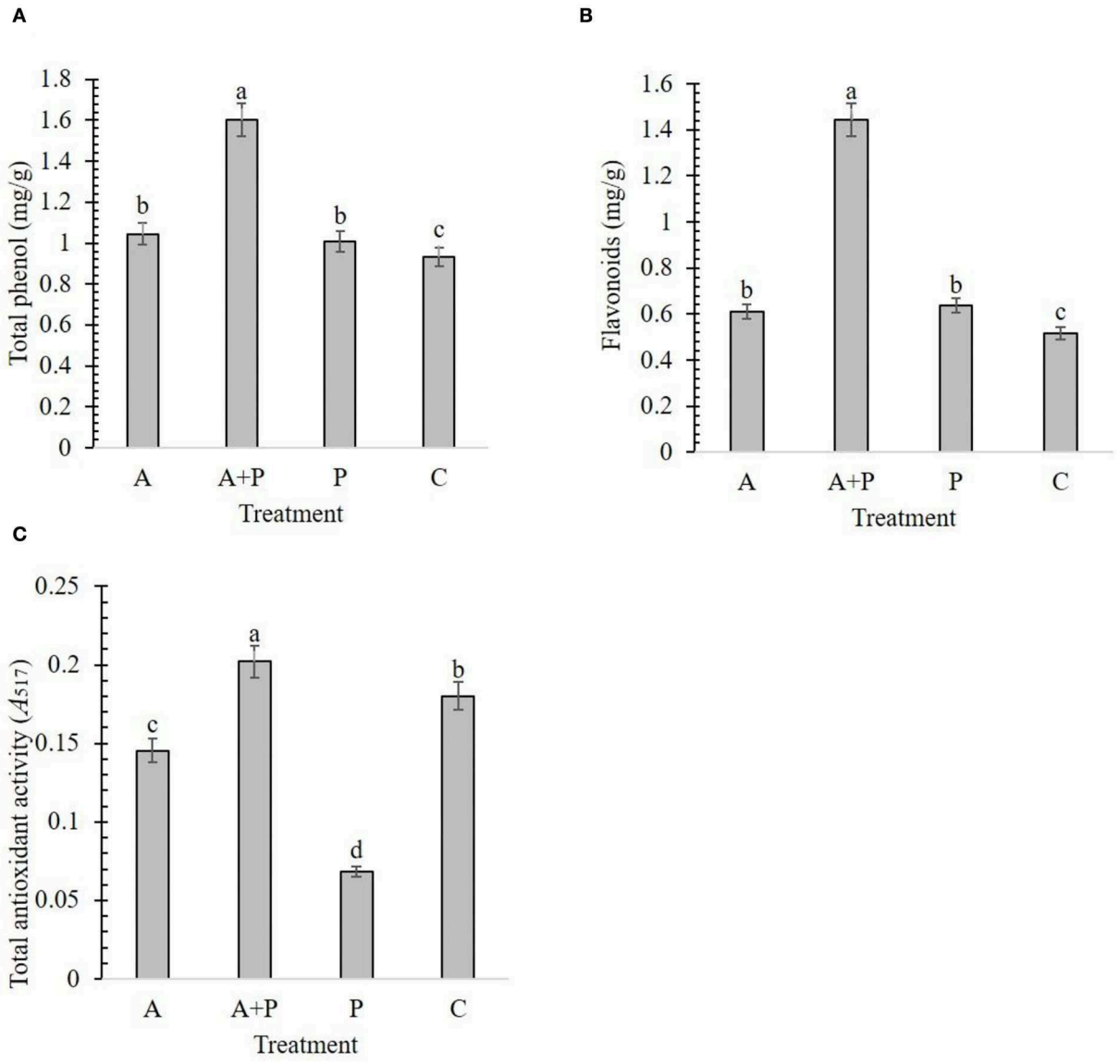

FIGURE 2
Effect of different treatments [A, alga (*S. angustifolium*) extract; A + P, pathogen (*M. phaseolina*)-inoculated pre-treated with the alga extract; P, pathogen-inoculated; C, control] and on some biochemical factors of the strawberry plant including total phenol **(A)**, flavonoids **(B)**, and total antioxidant activity **(C)**. Letters on bars show the significant difference between the treatments according to the results of Duncan's multiple range test.

non-infected plants (0.34 mg/g/min) (Figure 3B). These results showed that foliar application of alga extract can significantly increase the content of PAL and PPO in infected plants.

Discussion

Algae affect crops to increase plant growth, plantlet growth, and secondary root growth. They can also improve nutrient composition, fruit set, pest and disease resistance, improve stress response (drought, salinity, and temperature) (Mukherjee and Patel, 2020). It has been shown that foliar spray of alga extract increases the absorption of nutrients, promoting growth and root development in various crops, such as corn (Jeannin et al., 1991), tomato (Crouch and van Staden, 1992), Arabidopsis (Rayorath et al., 2008), grape (Mugnai et al., 2008), strawberry (Alam et al., 2013), spinach (Fan et al., 2013), okra (Zodape et al., 2008), olive (Chouliaras et al., 2009) and broccoli (Mattner et al., 2013). It has also increased the consumption of nutrients such as nitrogen, phosphorus, potassium, calcium, sulfur, and micronutrients such as magnesium, zinc, manganese,

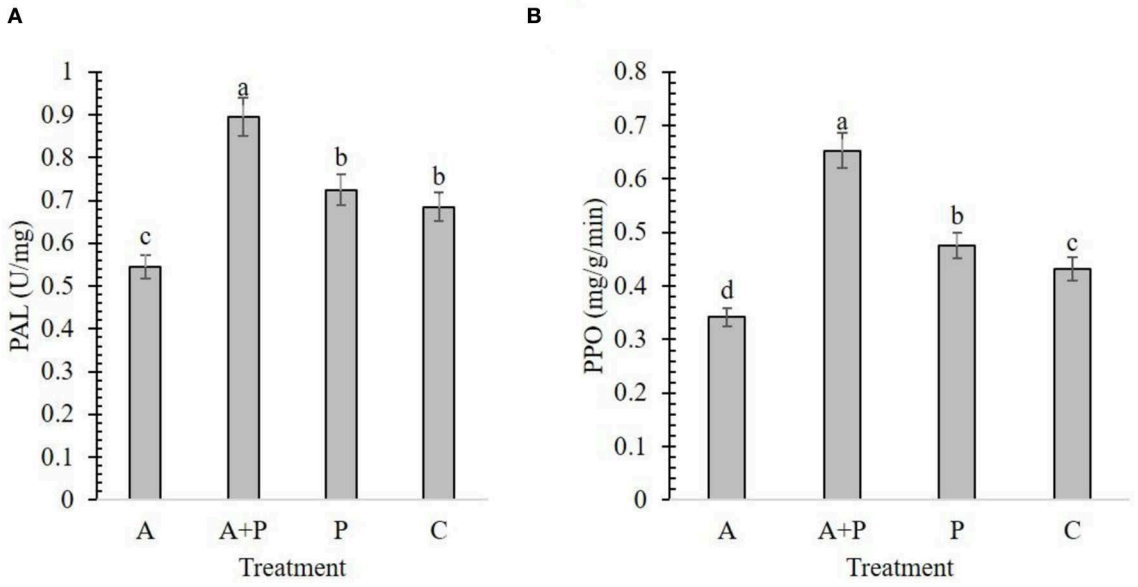

FIGURE 3
Effect of different treatments [A, alga (*S. angustifolium*) extract; A + P, pathogen (*M. phaseolina*)-inoculated pre-treated with the alga extract; P, pathogen-inoculated; C, control] and on the activity of strawberry enzymes including PAL **(A)** and PPO **(B)**. Letters on bars show the significant difference between the treatments according to the results of Duncan's multiple range test.

and iron (Crouch and van Staden, 1992; Mancuso et al., 2006; Rathore et al., 2009; Zodape et al., 2011). Similarly, our results showed that the foliar application of a brown alga improved the vegetative indices of strawberry plants including height, root length and fresh weight of aerial organs (Table 2). Additionally, strawberry fruit indices including number, weight, diameter and length showed a significant increase in alga-treated plants (Table 3) demonstrating the positive effect of macroalgal treatment on growth and yield of strawberry plants. Although the positive effect of an alga (*Ascophyllum* sp.) extract on strawberry growth has been reported previously by Alam et al. (2013), here we reported a new alga species (*S. angustifolium*) with a beneficial effect. The antifungal activity of seaweeds has been reported against phytopathogenic fungi including *Fusarium oxysporum* (El-Sheekh et al., 2020), *F. solani* (El-Sheekh et al., 2021) and *F. oxysporum* f. sp. *lycopersici* (Mostafa et al., 2022). Particularly, El-Sheekh et al. (2021) demonstrated that the mycelial growth of *M. phaseolina* was inhibited when cucumber plants were treated with the extract of green seaweeds, *Ulva fasciata*, and *Enteromorpha flexuosa*. Herein, however, the anti-fungal effect of brown alga, *S. angustifolium,* was shown indirectly as foliar treatment of the algal extract could reduce the disease severity caused by *M. phaseolina* in strawberry plants. Marine algae metabolites contain bioactive molecules with anti-fungal, anti-viral, anti-bacterial, and anti-protozoal properties. Usually, metabolites isolated from brown, red, and green algae are stronger than antimicrobial chemicals (Ben Salah et al., 2018). In our experiment, the brown alga and the pathogen were applied in different sites (shoot and crown, respectively), therefore, the reduced disease severity of *M. phaseolina* in alga-treated strawberry plants is not probably due to the anti-fungi metabolites within *S. angustifolium* extract. Alternatively, induced resistance might be the main mechanism by which the alga-treated strawberry plants encounter *M. phaseolina* invasion.

Moreover, algae are a source of stimulants due to the presence of several different polysaccharide compounds. These polysaccharides are involved in primary signaling processes through the activation of plant secondary metabolic pathways and the mobilization of messenger molecules to activate the defense response in the host plant (Paulert et al., 2009; Sharma et al., 2014). Alga polysaccharides make plants resistant to plant pathogens (Mercier et al., 2001; Sangha et al., 2015). The increase in total phenol and flavonoids are general defense responses against biotic agents such as plant pathogens (Wallis and Galarneau, 2020). Similarly, our results demonstrated that foliar application of *S. angustifolium* extract can significantly increase total phenol and flavonoids within strawberry plants (Figure 2) which probably contributes to induced resistance against *M. phaseolina* leading to symptom remission of infected plants (Figure 1). Generally, free radicals are produced as a defensive response to pathogen infection in plant cells which trigger the systemic acquired resistance (SAR) within plant (Agrios, 2005; Wendehenne et al., 2014). The antioxidant activity, however, suppress the oxidation by scavenging the free radicals (Larson, 1995). In our experiment, the alga-treated infected plants exhibited a significant increase in total antioxidant activity

(Figure 2C) suggesting that a non-SAR pathway, i.e., induced systemic resistance (ISR) (Heil and Bostock, 2002), is involved in strawberry plant defense against the fungal pathogen. Enzymes such as PAL and PPO are major biomolecules that have a key role in the induced resistance of plants challenged by a pathogen (Ngadze et al., 2012). Herein, the alga-treated infected plants showed a significant increase in the content of two defense-related enzymes, viz. PAL and PPO (Figure 3), suggesting the role of induced resistance in the reduction of disease severity caused by *M. phaseolina*. Taken together, foliar application of *S. angustifolium* extract can elicit the defense responses of strawberry plants challenged by *M. phaseolina* leading to improved growth indices of the plants. Further experiments are required to examine the other application ways to find the optimum method for the alga usage in strawberry greenhouses facing charcoal rot disease.

Conclusions

Algal metabolites are a valuable source of nutrients and elicitors for the improvement of plant growth and encountering biotic agents. Brown alga (*S. angustifolium*) is one of the beneficial macroalgae that has been used in disease management programs. Foliar application of *S. angustifolium* extract can improve both vegetative and reproductive indices of strawberry plants. Furthermore, it can reduce the charcoal rot severity caused by *M. phaseolina* through an increase in the level of total phenol, flavonoids, TAA, and defense-related enzymes (PAL and PPO) within the plants. It is recommended that the macroalga is used against charcoal rot disease in strawberry greenhouses.

Data availability statement

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

Author contributions

ST mainly conducted this work, performed the experiments, and collected the data. MR-J conceived the study. MG analyzed the data. MR-J and MG mainly wrote the manuscript. All authors edited and approved the final version of the manuscript.

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Conflict of interest

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

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Pseudomonas 42P4 and *Cellulosimicrobium* 60I1 as a sustainable approach to increase growth, development, and productivity in pepper plants

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The production of pepper plants for industrial use is not enough to satisfy the demand of consumers and agrochemicals are frequently used to increase production. In this study four native plant growth promoting rhizobacteria (PGPR) was tested as an alternative to select the most effective to enhance growth, development, and productivity of pepper plants. Seedlings were inoculated with *Pseudomonas* 42P4, *Cellulosimicrobium* 60I1, *Ochrobactrum* 53F, *Enterobacter* 64S1 and cultivated on pots in the greenhouse and the morphological, biochemical, and physiological parameters were determined. In addition, the phenolic compound profiles were evaluated. All four strains increased the different parameters evaluated but *Pseudomonas* 42P4 and *Cellulosimicrobium* 60I1 were the most effective strains, improving leaf and root dry weight, stem diameter, nitrogen level, stomatal conductance, chlorophyll quantum efficiency, chlorophyll SPAD index, total chlorophyll and carotenoid levels, number of flowers and fruits per plant, and the length, diameter and dry weight of the fruit. Also, these strains modified the phenolic compound profiles, and 18 compounds were quantified. *Pseudomonas* 42P4 inoculation modified the phenolic compound profile similarly to the Fertilized treatment and induced the synthesis of different endogenous compounds in the flavonoid family, also increasing catechin, naringin, naringenin, myricetin, procyanidin B1, epigallocatechin-gallate, cinnamic, and ferulic acids related to antioxidant activity and catechin, cinnamic, and ferulic acids related to the induced systemic response. *Pseudomonas* 42P4 can be used as a bioinoculant in pepper plants to enable better agronomic management, decreasing the use of chemical fertilizer to contribute to sustainable agriculture.

KEYWORDS

pepper plants, sustainable agriculture, PGPR, *Pseudomonas*, *Cellulosimicrobium*, bioinoculants, phenolic compounds

Highlights

- Bioinoculants formulated with PGPR are a sustainable alternative to increase crop production.
- *Pseudomonas* 42P4 and *Cellulosimicrobium* 60I1 are two promising strains for preparing a bioinoculant.
- The strains used have the ability to increase growth, development, and production of pepper plants.
- The phenolic compound profiles (eighteen compounds identified and quantified) are modified in plants inoculated with the strains under study.
- *Pseudomonas* 42P4 mainly increases compounds grouped in the flavonoid and phenolic acid families.

Introduction

Pepper (*Capsicum annuum*) is one of the most important horticultural crops worldwide. It is an essential food ingredient and the fourth major crop produced globally. Approximately 400 varieties of pepper are cultivated worldwide (Saxena et al., 2016). In the province of Mendoza, Argentina, it is calculated that around 1246 ha of peppers are grown for the packaging industry (FAOSTAT, 2021). Pepper plants need a sufficient amount of nutrients to grow and develop fruit and so the farmers apply fertilizer and agrochemicals to improve the production. However, the overuse of some chemical products affects human health and deteriorates the quality of the environment.

Bioinoculants formulated with microorganisms are currently considered as a promising alternative to inorganic fertilizers, constituting a powerful tool in organic agriculture and for the restoration of degraded soils (De-Bashan et al., 2012; Gouda et al., 2018). Some bioinoculants are formulated with plant growth promoting rhizobacteria (PGPR). These strains have beneficial properties that improve plant growth and development through different mechanisms, such as atmospheric nitrogen fixation, siderophore production, phosphate solubilization, and production of plant growth regulators (Basu et al., 2021; Mohanty et al., 2021). In addition, native PGPR can alleviate the environmental stresses to which plants are often exposed, since they tend to adapt easily to the local conditions of the soils from which they were isolated, withstanding environmental stress caused by extreme gradients of temperature, high concentrations of salts in the soil and hydric stress (Grover et al., 2011; Cordero et al., 2018).

In the rhizosphere there are a series of organic molecules that regulate chemotaxis between plant roots and PGPR. The phenolic compounds stand out within this group of molecules, which are part of the secondary metabolism in plants, and their levels in plants may be regulated by interactions that occur in the rhizosphere (Feng et al., 2018). Simple phenolic alcohols, flavonoids, phenolic acids, and stilbenoids represent the main families of phenolic compounds. In each family the number of phenolic compounds and their concentrations are different depending on the plant tissue studied (Vuolo et al., 2019; Alara et al., 2021). Phenolic compounds have a key role as defense compounds under environmental stress conditions, such as inadequate light (excess or deficiency), saline stress or heavy metals, low temperatures, unfavorable pH, pathogen infection, herbivory, and nutrient deficiency, which can induce a higher production of oxidative species in plants (Lattanzio, 2013; Tanase et al., 2019).

Some studies based on colorimetric techniques have reported an increase in phenolic compounds in plants inoculated with PGPR (Del Rosario Cappellari et al., 2017; Pagnani et al., 2018; Rahimi et al., 2020). However, the mechanism of modification of phenolic compound profiles in the plant-PGPR interaction is limited and the specific role of the different families is unknown (Sarma et al., 2002). For this purpose, it is necessary to use liquid chromatography to individualize each compound.

In Mendoza province, Argentina, our research group isolated and characterized native PGPR strains from roots and the rhizosphere of a tomato crop (Pérez-Rodriguez et al., 2020). We showed that inoculation with these native strains was effective in promoting the germination percentage, the vigor index and modifying the profile of phenolic compounds, suggesting an elicitation of phenylpropanoid pathways related to induced systemic response (IRS) in pepper seeds (Lobato-Ureche et al., 2021). We also reported that these native PGPR promote the growth and development of tomato plants and reduce the negative effects of salt stress by NaCl in tomato plants cultivated in the greenhouse (Pérez-Rodriguez et al., 2020, 2022).

In the present study, we were interested in the impact of these native strains, known as *Enterobacter* 64S1, *Pseudomonas* 42P4, *Cellulosimicrobium* 60I1, and *Ochrobactrum* 53F, on the morphological, biochemical, physiological, and on the phenolic compounds profile changes of pepper plants. Also we evaluated the effect of inoculation on the fruit production. Our hypothesis was that inoculation of pepper plants with native PGPR modifies the profile of phenolic compounds, favoring growth, development, and yield, similar to a chemical fertilizer.

Materials and methods

Plant materials

Seeds of *Capsicum annuum* cv Calafyuco INTA were kindly supplied by Dr. C. Galmarini (National Institute of Agricultural Technology, INTA-EEA, La Consulta, Mendoza, Argentina).

Bacterial cultures

The strains used were *Cellulosimicrobium* 60I1 (60I1), *Ochrobactrum* 53F (53F), *Enterobacter* 64S1 (64S1), and *Pseudomonas* 42P4 (42P4). These strains belong to the Microbiology and Agricultural Physiology Lab (IBAM-FCA, CONICET-UNCuyo, Mendoza, Argentina) and have been deposited in the GenBank (NCBI) under accession numbers MT047266, MT047264, MT047267, MT045593. These strains were isolated from the rhizosphere and roots of tomato plants from productive farms in Mendoza, Argentina and were characterized as PGPR (Pérez-Rodriguez et al., 2020).

The pre-inoculum was prepared in 50 mL of a rich medium of LB (Luria Broth, Sigma Chem. Co.) from one colony of the *Cellulosimicrobium* 60I1, *Ochrobactrum* 53F, *Enterobacter* 64S1, and *Pseudomonas* 42P4 strains. Every culture was grown for 24 h at 28°C and 120 rpm until reaching an $OD_{530} = 1.2$. To prepare the inoculum, 500 μL of pre-inoculum were grown in 50 mL of LB for 24 h at 28°C and 120 rpm until reaching 10^8 CFU mL^{-1}. The seedlings were inoculated with 1,000 μL of each culture as detailed below.

Seed germination, growth conditions, and plant inoculation

The seeds were surface disinfected with 20% sodium hypochlorite for 1 min, then washed three times with sterile distilled water to remove the rest of the disinfectant. The seeds were sown in sterile trays containing sterilized substrate Kekkilä DSM 1 W (Kekkilä professional, Vantaa, Finland). The substrate contained 70% brown and 30% dark *Sphagnum fuscum* dominant peat, N–P_2O_5-K_2O 15–12–29 and microelements 0.6 kg m^{-3}, pH 5.9, electrical conductivity 0.2 dS m^{-1}. The trays were placed in a greenhouse at a temperature of (24–26°C average daily temperature, 10–14 light and dark periods, and 700–750 μmol m^{-2} s^{-1} of light intensity at solar noon). The seedlings with two fully expanded leaves were inoculated with 1,000 μL of PGPR containing 10^8 CFU mL^{-1} of the corresponding bacterial culture (inoculum). Thus, the treatments were: (1) seedlings inoculated with *Cellulosimicrobium* 60I1, (2) seedlings inoculated with 53F, (3) seedlings inoculated with *Enterobacter* 64S1, (4) seedlings inoculated with *Pseudomonas* 42P4, (5) seedlings treated with fertilizer Hakaphos® 18-18-18 (N-P-K), (6) Control: seedlings without bacterial inoculum (inoculated with LB medium). All treatments were applied on the soil surface near the root collar. The seedlings were then transplanted into plastic pots (30 L) containing a mixture of Kekkila DSM 1 W substrate:sand (50:50) and placed in a greenhouse with the same condition described previously. The Fertilized treatment was prepared at the rate of 0.7 g L^{-1} with Hakaphos® (a completely water soluble fertilizer for fruit and vegetable crops, free of chlorine and urea with EDTA-chelated trace elements). The treatment was applied at the rate of 1 mL per seedlings, and followed by subsequent treatments at 10 mL on 45 days-old and 20 mL on 70 days-old plants. A completely randomized design of six treatments (1–6) was established, with three repetitions of 10 seeds in each one (with a total of 60 seedlings). The experiment was repeated three times.

The growth parameters were measured in 75 day-old plants and data were collected to evaluate the morphological aspects including: plant height and stem diameter, while leaf area was measured using the Micrometrics SE premium software. Physiological parameters were also measured at 90 day-old plants: stomatal conductance was determined with a porometer (Decagon Devices, Model SC-1, USA), maximum quantum yield of PSII (Fv/Fm) was determined with a fluorometer (Hansatech Instruments LTD). **Fv/Fm** is the maximum efficiency at which light is absorbed by PSII and is used as a sensitive indicator of plant photosynthetic performance (Asghari et al., 2020), where Fm: maximum fluorescence and Fv: the variable fluorescence. The Chlorophyll index (SPAD), was determined using a portable chlorophyll meter (SPAD-502, Konica Minolta Sensing). At the time of flowering, the number of flowers per plant and the days of flowering were counted. Yield parameters were measured at the end of the assay after 110 days (number of fruits per plant, weight, length and diameter of the fruits). Finally, the aerial and root dry weights were determined.

Nitrogen determination

Leaves of 55 day-old plants were dried in an oven at 70°C for 72 h until reaching a constant dry weight. Later, they were ground and the nitrogen content was determined by Micro Kjeldahl, as described by Guebel et al. (1991).

Photosynthetic and photoprotective pigments

Determinations were carried out spectrophotometrically in a UV–Vis spectrophotometer Cary 50 (Varian Inc.) as described by Chappelle et al. (1992), with modifications of Cohen et al. (2015), using leaf samples (55 days old). Total chlorophyll (Chl; Chl a + Chl b), carotenoid and anthocyanin levels were measured from 1 cm diameter disc samples and expressed in mg^{-1} of leaves.

Extraction and quantification of phenolic compounds

The phenolic compounds were extracted using a liquid-solid extraction according to a previously reported procedure (Moussi et al., 2015), which can be briefly described as follows: 0.5 g of lyophilized material (leaves) were weighed in a conical centrifuge tube and mixed with 5 mL of ethanol. Then, the tube was left in an ultrasonic bath for 10 min and the supernatant obtained by centrifugation (2,500 *g* for 10 min) was evaporated to dryness using a rotary evaporator at 40°C. The residue was redissolved in 1 mL of 0.1% (v/v) formic acid.

Each individual phenolic compound in the pepper leaf extracts was separated using high-performance liquid chromatography, coupled to diode array and fluorescence detectors (HPLC-DAD-FLD). Dionex UltiMate 3000 HPLC system (California, USA). Chromatographic separations were carried out in reversed-phase Kinetex C_{18} column (3.0 mm × 100 mm, 2.6 μm). Phenomenex (Torrance, CA, USA) at 35°C. The mobile phases were ultrapure water with 0.1% (v/v) formic acid (phase A) and acetonitrile (phase B). Separation of the analytes was performed using the following gradient: 0–1.7 min, 5% B; 1.7–10 min, 30% B; 10–13.5 min, 95% B; 13.5–15 min, 95% B; 15–16 min, 5% B; 16–19, 5% B. The flow rate was set constant at 0.8 mL min^{-1} during the whole process, and the injection volume was 5 μL.

The identification and quantification of the target phenolic compounds in the extracts were based on the comparison of the retention times (t_R) and the maximum absorbance value of detected peaks in samples of interest with those obtained by the injection of pure standards. The working wavelengths for DAD of the different families of analytes were 254, 280, 320, and 370 nm, while an excitation wavelength (Ex) of 290 nm and monitored emission (Em) responses of 315 and 400 nm were used depending on the targeted analytes for FLD. The Chromeleon 7.1 software was used to control all the acquisition parameters of the HPLC-DAD-FLD system and also to process the data obtained.

Statistical analysis

Data were processed by analysis of variance followed by a Duncan test to discriminate between the averages by the minimum difference with a significance level of $P \leq 0.01$. The InfoStat statistical software (InfoStat version 2018v. Grupo InfoStat, Argentina) was used.

Results

Effect of inoculation with PGPR on the development of pepper plants grown in pots under greenhouse conditions

Inoculation with PGPR increased the leaf and aerial (leaves plus stems) dry weight of the plants, as shown in Figure 1. The Fertilized, *Pseudomonas* 42P4, and *Cellulosimicrobium* 60I1 treatments increased the dry weight of the leaves (34, 21, and 15%, respectively), with respect to the Control. A similar trend was observed in the dry weight of the aerial part.

Inoculation with PGPR increased root dry weight and stem diameter, as shown in Figures 2A, B. The increase in the root dry weight was the same between the inoculated treatments with respect to the Control. The Fertilized, *Enterobacter* 64S1, *Cellulosimicrobium* 60I1, and *Pseudomonas* 42P4 treatments increased stem diameter by 16%; 13%; 11 and 6%, respectively.

The highest nitrogen content was found in the inoculated and fertilized treatments with respect to the Control, as shown in Figure 3. The *Pseudomonas* 42P4 and *Cellulosimicrobium* 60I1 treatments increased the nitrogen content (48 and 41%, respectively), with respect to the Control, showing similar behavior to the Fertilized treatment, while *Enterobacter* 64S1 and *Ochrobactrum* 53F exceeded the control (29 and 18%, respectively).

Effect of inoculation on the accumulation of photosynthetic and photoprotective pigments of pepper plants

Inoculation with PGPR modifies the physiological parameters in pepper plants. The *Enterobacter* 64S1, *Pseudomonas* 42P4, fertilized, *Ochrobactrum* 53F and *Cellulosimicrobium* 60I1 treatments increased stomatal conductance by 24%; 21, 21%; 18 and 15%, respectively, with respect to the Control (Figure 4A). Similarly, the Fv/Fm increased in the inoculated and Fertilized treatments, exceeding the control by as much as 11% (Figure 4B).

The contents of photosynthetic pigments (Chl a and total), as well as carotenoids, were higher in the inoculated and Fertilized treatments, with respect to the Control, as shown in Table 1 and Figure 5.

Effect of inoculation on the yield of pepper plants grown in pots

Inoculation did not modify the days to flowering. However, the number of flowers per plant increased, and all treatments

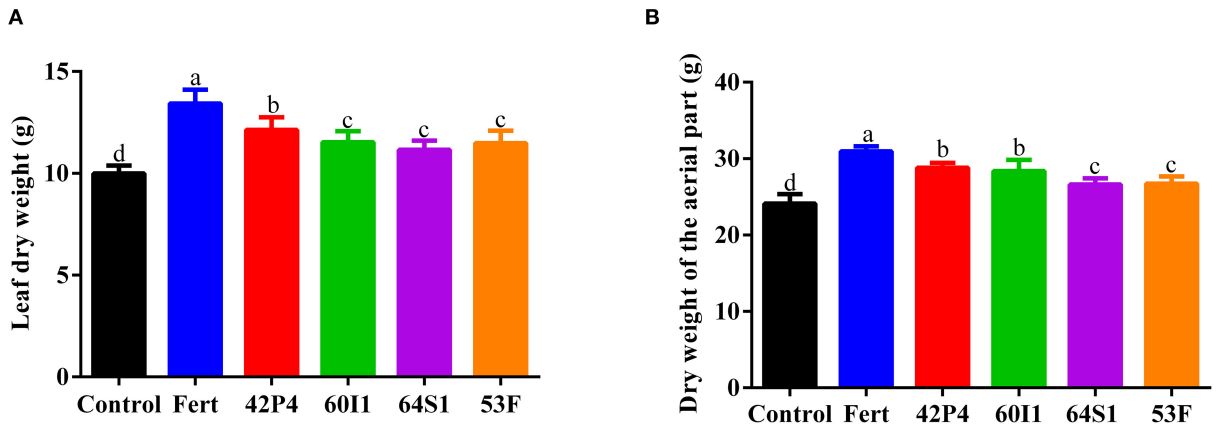

FIGURE 1
(A) Dry weight of the leaves and **(B)** dry weight of the aerial parts (leaves and stems) of pepper plants of 110 day-old grown in pots and treated with: Fert: Fertilized; 42P4: *Pseudomonas* 42P4; 60I1: *Cellulosimicrobium* 60I1; 64S1: *Enterobacter* 64S1; 53F: *Ochrobactrum* 53F and Control (without bacteria). Data are presented as mean ± SEM of seven independent biological replicates. Different letters indicate significant differences according to one-way ANOVA with Duncan's multiple range test. *P* **(A)** = 0.0034 and *P* **(B)** = 0.0005.

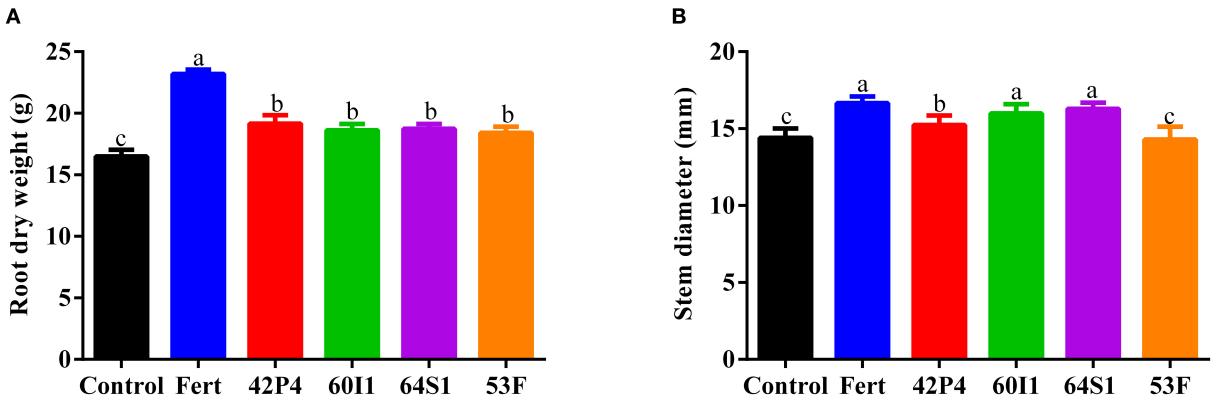

FIGURE 2
(A) Root dry weight and **(B)** stem diameter, of pepper plants of 110 and 75 day-old (respectively), grown in pots and treated with: Fert: Fertilized; 42P4: *Pseudomonas* 42P4; 60I1: *Cellulosimicrobium* 60I1; 64S1: *Enterobacter* 64S1; 53F: *Ochrobactrum* 53F and Control (without bacteria). Data are presented as mean ± SEM of 10 independent biological replicates. Different letters indicate significant differences according to one-way ANOVA with Duncan's multiple range test. *P* **(A)** = 0.0001 and *P* **(B)** = 0.0028.

differed from the Control except the 64S1 treatment, as shown in Table 2.

The Fertilized, *Pseudomonas* 42P4, *Cellulosimicrobium* 60I1, and *Enterobacter* 64S1 treatments had the highest fruit production with respect to the Control. The Fertilized treatments, 42P4 and 60I1 had the longest fruits (9; 8 and 8%, respectively), with respect to the Control. The *Enterobacter* 64S1, *Pseudomonas* 42P4 and *Ochrobactrum* 53F treatments increased the fruit diameter and were different to the Fertilized and Control treatments. The Fertilized, *Pseudomonas* 42P4, *Cellulosimicrobium* 60I1, and *Ochrobactrum* 53F treatments increased the fruit dry weight (67; 45; 42 and 38%, respectively) with respect to the Control (Table 2).

Figure 6 shows the biplot graph for 16 variables (morphological, physiological, and yield component) of the pepper plants cultivated in pots. PC1 explained 76% of the variance and the variables associated with this component were fruit dry weight, total chlorophyll, carotenoids, Fv/Fm, total Chl, Chl a, aerial dry weight, fruit length, root dry weight, leaf dry weight, number of fruits and SPAD index. PC2 explained 12% of the variance and the variables associated with this component were fruit diameter, stomatal conductance, and number of flowers. Principal components analysis separated the *Pseudomonas* 42P4, *Cellulosimicrobium* 60I1 and Fertilized treatments in a first group and the second group consisted of the *Ochrobactrum* 53F3 and

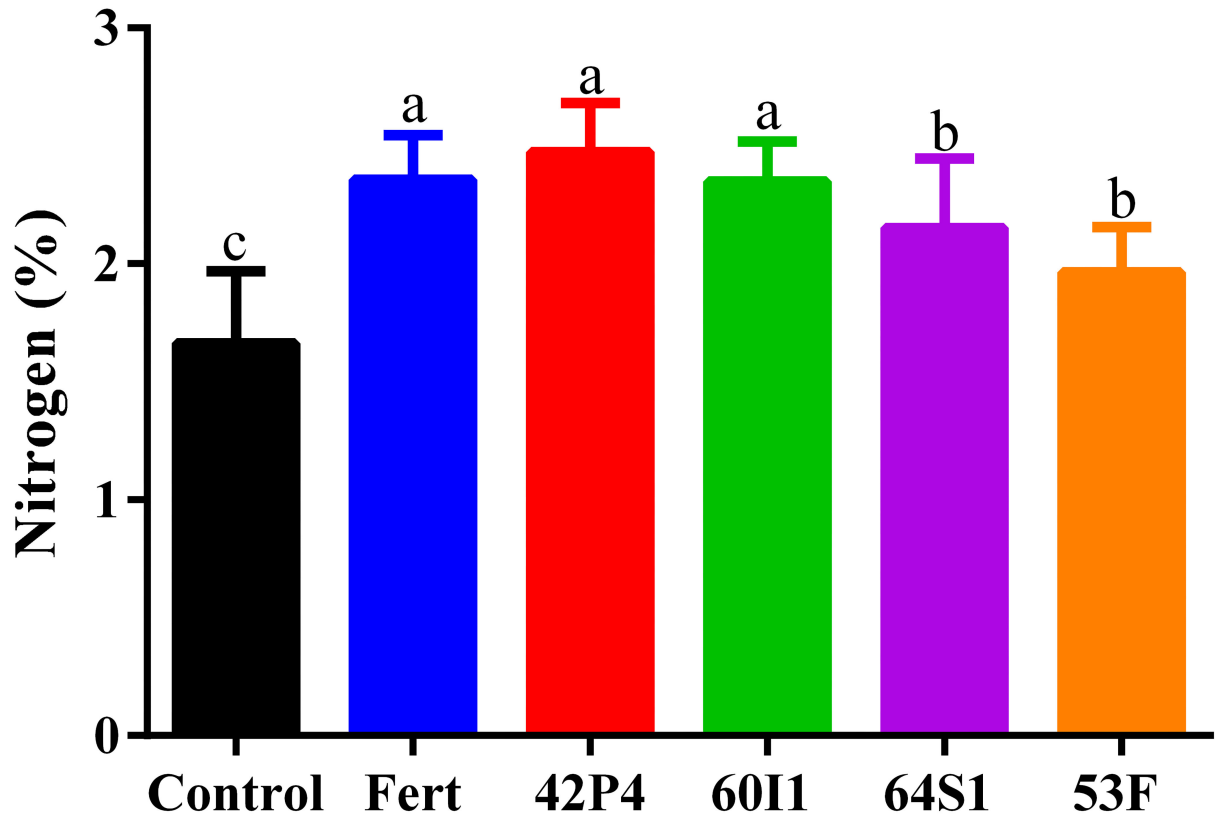

FIGURE 3
Nitrogen content in leaves of pepper plants of 55 day-old after treatment with Fert: Fertilized; 42P4: *Pseudomonas* 42P4; 60I1: *Cellulosimicrobium* 60I1; 64S1: *Enterobacter* 64S1; 53F: *Ochrobactrum* 53F and Control (without bacteria). Data are presented as mean ± SEM of three independent biological replicates. Different letters indicate significant differences ($P < 0.0085$) according to one-way ANOVA with Duncan's multiple range test.

Enterobacter 64S1 treatments, and the third group, the Control treatment.

The constitution of the first group shows that inoculation with *Pseudomonas* 42P4 and *Cellulosimicrobium* 60I1 obtained results similar to those found with the chemical fertilizer treatment. Based on these results, these treatments were selected for evaluating the phenolic compound profiles and to compare with the Control.

Effect of inoculation on the profile of phenolic compounds in leaves of Calafyuco pepper plants

The phenolic compounds present in the leaves of uninoculated (Control), inoculated with *Pseudomonas* 42P4 and *Cellulosimicrobium* 60I1 strains, and Fertilized pepper plants are shown in Table 3. A total of 18 phenolic compounds, grouped in four families based on their chemical structure, were identified and quantified: phenolic alcohols, flavonoids, phenolic acids, and stilbenoids. This study revealed that the sum of the phenolic compounds was higher in plants inoculated with the *Pseudomonas* 42P4 strain and Fertilized treatments, whereas plants inoculated with the *Cellulosimicrobium* 60I1 strain were similar to the Control treatment.

As phenolic alcohols we identified hydroxytyrosol and tyrosol. The concentration of both compounds was highest in the Fertilized treatment (4.92 mg g^{-1}), followed by the Control (2.17 mg g^{-1}), but the inoculated treatments had lower concentration.

In the flavonoid family, the following compounds were identified and quantified: (+)-catechin, rutin, (-)-gallocatechin gallate, (-)-epigallocatechin gallate, quercetin, astilbin, naringin, naringenin, myricetin, and procyanidin B1. The Fertilized treatment had the highest concentration of total flavonoids (152.71 mg g^{-1}), followed by *Pseudomonas* 42P4 (140.55 mg g^{-1}), *Cellulosimicrobium* 60I1 (52.38 mg g^{-1}), and the Control (45.58 mg g^{-1}). The (+)-catechin and procyanidin B1 were only detected in the *Pseudomonas* 42P4 treatment. The 42P4

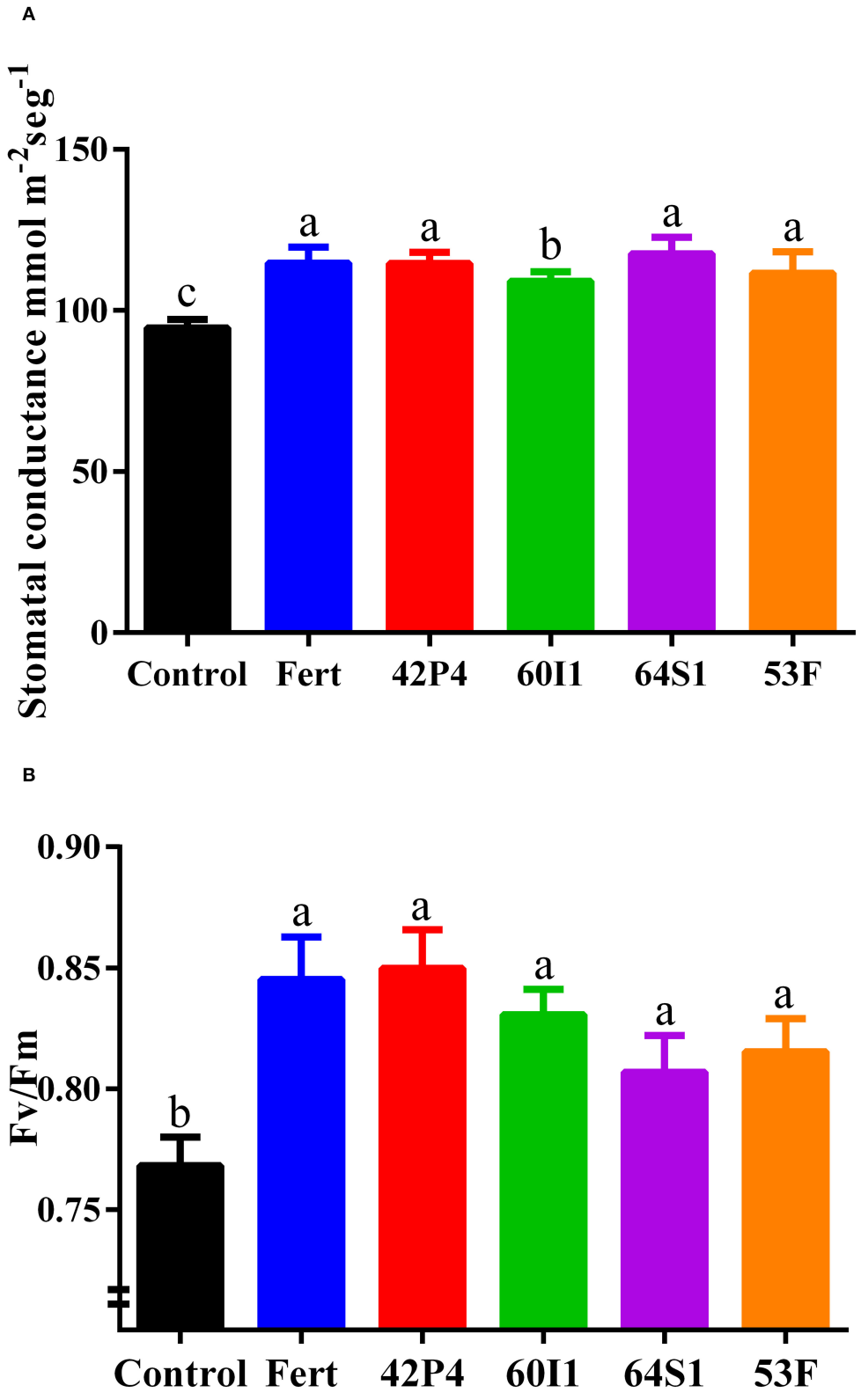

FIGURE 4
(A) Stomatal conductance and **(B)** Chlorophyll quantum efficiency (Fv/Fm) of pepper plants of 90 day-old grown in pots and treated with: Fert: Fertilized; 42P4: *Pseudomonas* 42P4; 60I1: *Cellulosimicrobium* 60I1; 64S1: *Enterobacter* 64S1; 53F: *Ochrobactrum* 53F and Control (without bacteria). Data are presented as mean ± SEM of 10 independent biological replicates. Different letters indicate significant differences according to one-way ANOVA with Duncan's multiple range test. *P* **(A)** = 0.0025 and *P* **(B)** = 0.0009.

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TABLE 1 Biochemical parameters determined in 90 days-old pepper plants.

Treatments	Chlorophyll a (μg mg^{-1} leaf)	Chlorophyll b (μg mg^{-1} leaf)	Total chlorophyll (μg mg^{-1} leaf)	Carotenoids (μg mg^{-1} leaf)	Anthocyanins (mg^{-1} leaf)
Control	4.32 ± 1.21b	2.00 ± 0.30a	6.32 ± 1.24b	1.17 ± 0.14b	0.32 < 0.01a
Fertilized	5.03 ± 0.58a	2.10 ± 0.25a	7.13 ± 0.63a	1.58 ± 0.19a	0.34 < 0.01a
Pseudomonas 42P4	5.00 ± 0.36a	2.08 ± 0.23a	7.08 ± 0.42a	1.36 ± 0.11a	0.32 < 0.01a
Cellulosimicrobium 60I1	4.98 ± 0.65a	2.01 ± 0.32a	7.11 ± 0.72a	1.42 ± 0.08a	0.29 < 0.01a
Enterobacter 64S1	5.01 ± 0.47a	2.04 ± 0.18a	7.05 ± 0.50a	1.45 ± 0.10a	0.30 < 0.01a
Ochrobactrum 53F	5.01 ± 0.56a	2.07 ± 0.28a	7.08 ± 0.62a	1.38 ± 0.16ab	0.33 < 0.01a

Data are presented as mean ± SEM of six independent biological replicates. Different letters indicate significant differences ($P < 0.0001$) according to one-way ANOVA with Duncan's multiple range test.

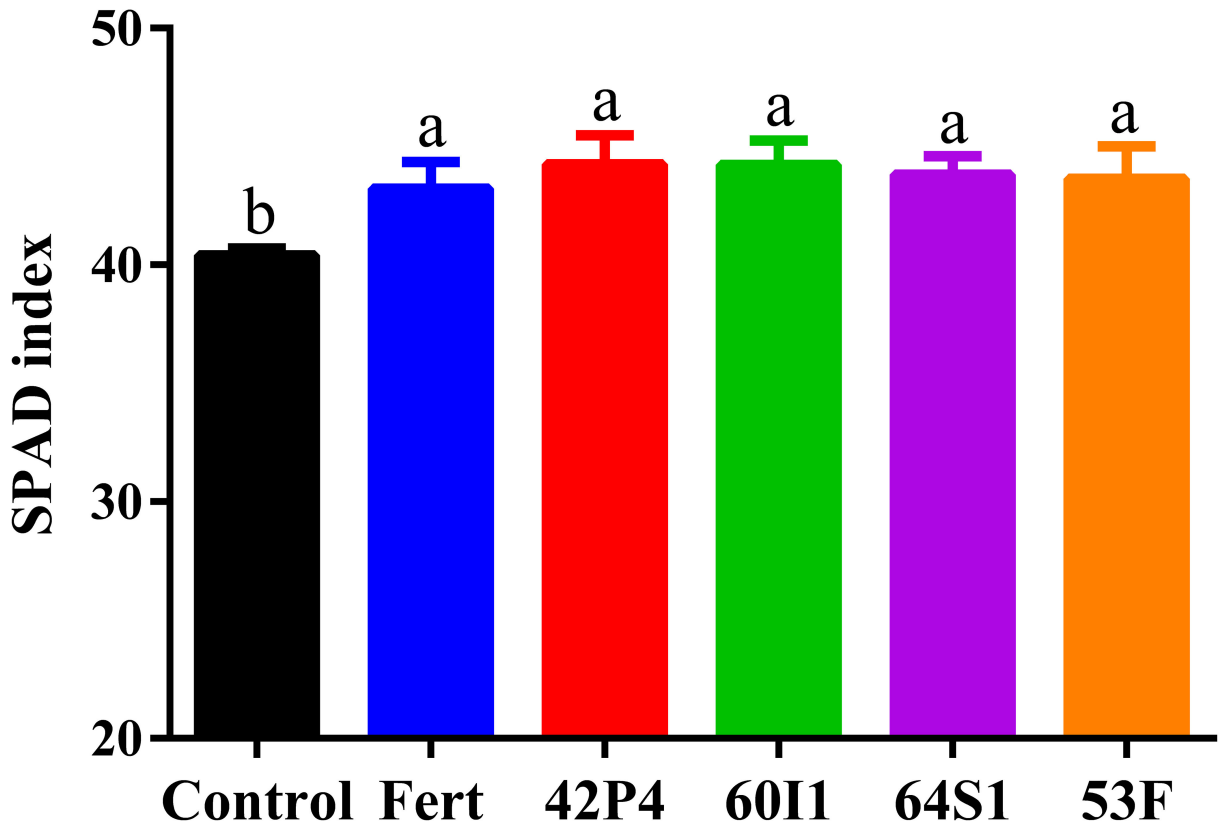

FIGURE 5
Chlorophyll index (SPAD) of pepper plants of 90 day-old grown in pots and treated with: Fert: Fertilized; 42P4: *Pseudomonas* 42P4; 60I1: *Cellulosimicrobium* 60I1; 64S1: *Enterobacter* 64S1; 53F: *Ochrobactrum* 53F and Control (without bacteria). Data are presented as mean ± SEM of 10 independent biological replicates. Different letters indicate significant differences ($P < 0.0099$) according to one-way ANOVA with Duncan's multiple range test.

had the highest concentration of (-)-gallocatechin gallate (35.98 mg g^{-1}), followed by Fertilized and *Cellulosimicrobium* 60I1 (30.95 and 15.61 mg g^{-1}, respectively) and they differed from the Control (12.15 mg g^{-1}). The Fertilized had the highest concentration of naringin (68.68 mg g^{-1}), followed by *Pseudomonas* 42P4 and *Cellulosimicrobium* 60I1 (54.93 and 32.91 mg g^{-1}, respectively) and they differed from the Control (24.66 mg g^{-1}).

As phenolic acids, cinnamic, p-coumaric, and ferulic acids were quantified. The 42P4 had high concentrations

TABLE 2 Yield component parameters of pepper plants of 110 days-old.

Treatments	Days to flowering	N° flowers/plant	N° fruits/plant	Fruit length (mm)	Fruit diameter (mm)	Fruit dry weight (g)
Control	91	10.76 ± 1.23b	5.67 ± 0.36b	86.32 ± 3.20b	58.49 ± 1.90c	5.28 ± 0.97c
Fertilized	90	15.20 ± 1.67a	7.96 ± 0.77a	94.10 ± 4.09a	60.98 ± 2.22b	8.84 ± 1.06a
Pseudomonas 42P4	90	14.36 ± 1.62a	7.20 ± 0.48a	93.57 ± 3.46a	62.86 ± 2.25a	7.65 ± 0.99a
Cellulosimicrob. 60I1	90	14.53 ± 1.54a	7.42 ± 0.53a	93.28 ± 3.85a	60.35 ± 2.20b	7.52 ± 1.04a
Enterobacter 64S1	91	11.18 ± 1.49b	7.00 ± 0.51a	89.13 ± 2.54ab	63.84 ± 2.91a	6.97 ± 0.98b
Ochrobactrum 53F	90	13.15 ± 1.28a	5.98 ± 0.39b	92.56 ± 4.24a	62.11 ± 2.23a	7.26 ± 1.02a

Data are presented as the mean ± SE of a total of 10 pepper plants for each treatment. Different letters indicate significant differences ($P < 0.0001$) according to one-way ANOVA with Duncan's multiple range test.

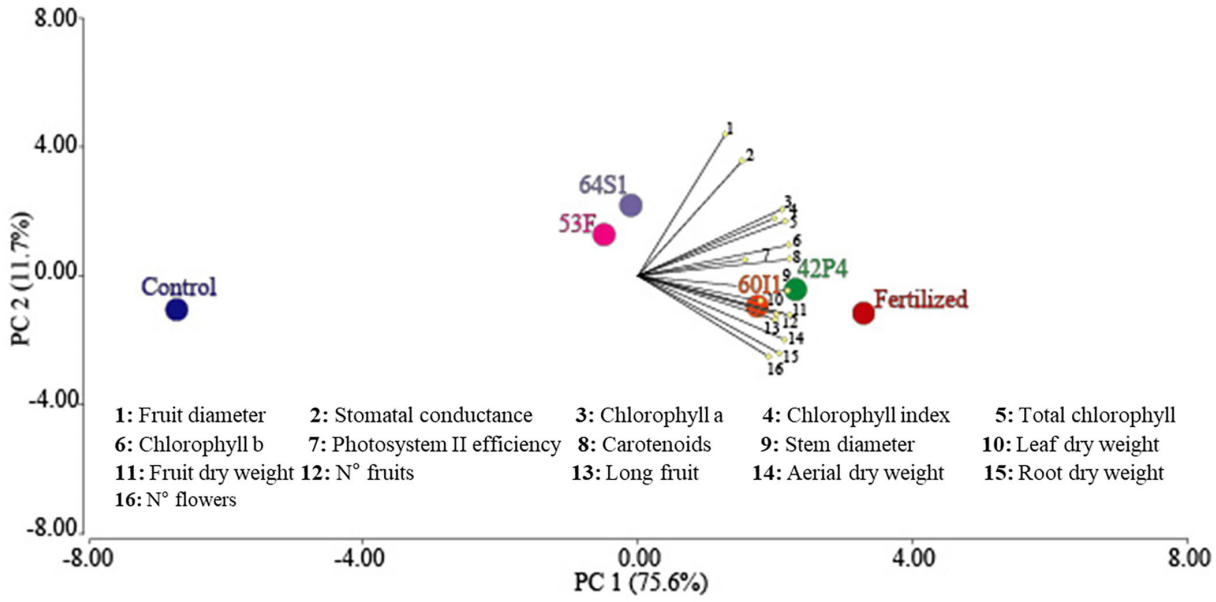

FIGURE 6
Biplot of principal components analysis of the effect of PGPR strains on the parameters evaluated in pepper plants grown in pots under greenhouse conditions.

of total phenolic acids (39.63 mg g^{-1}), followed by Fertilized, Control, and 60I1 (35.96, 9.14, and 5.50 mg g^{-1}, respectively). The *Pseudomonas* 42P4 and Fertilized treatments had high concentrations of cinnamic acid (32.07 and 28.92 mg g^{-1}, respectively). The *Pseudomonas* 42P4 had the highest concentration of ferulic acid (7.24 mg g^{-1}), followed by Fertilized and *Cellulosimicrobium* 60I1 (6.60 and 1.95 mg g^{-1}), but it was not detected in the Control. The Control had high concentrations of p-coumaric acid.

The stilbenoid group includes polydatin, trans-resveratrol and pterostilbene. The Control had high concentrations of total stilbenoid (16.16 mg g^{-1}) followed by *Pseudomonas* 42P4, Fertilized and *Cellulosimicrobium* 60I1 (11.97; 9.24 and 6.06 mg g^{-1}). The Control had the highest concentration of polydatin, triple the content of the other treatments; while *Pseudomonas* 42P4 had higher concentrations of trans-resveratrol.

Discussion

Capsicum annuum is an important commercial horticultural crop that has high nutritional value (Shiragaki et al., 2020). Bioinoculants formulated with PGPR are an alternative for increasing production while reducing adverse effects on the environment. In our study, the inoculation with PGPR stimulated the growth and nitrogen uptake of pepper plants. The greater absorption of nitrogen in the inoculated plants

TABLE 3 Phenolic compounds quantified in pepper leaves of plants subjected to different treatments.

Compound	Control	*Cellulosimicrobium* 60I1	*Pseudomonas* 42P4	Fertilized
Phenolic compounds				
Simple phenolic alcohols				
Hydroxytyrosol	1.69 ± 0.26b	0.75 ± 0.08c	0.15 ± 0.01c	4.37 ± 0.26a
Tyrosol	0.48 ± 0.10a	0.46 ± 0.08a	0.56 ± 0.08a	0.55 ± 0.02a
∑ Simple phenolic alcohols	2.17 ± 0.27b	1.21 ± 0.11c	0.71 ± 0.08c	4.92 ± 0.26a
Flavonoids				
(+)-Catechin	n.d.*	n.d.*	0.82 ± 0.28	n.d.*
Rutin	0.92 ± 0.28b	0.15 ± 0.01c	2.93 ± 0.03a	2.95 ± 0.08a
(-)-Gallocatechin gallate	12.15 ± 1.07c	15.61 ± 2.34b	35.98 ± 2.42a	30.95 ± 2.69a
(-)-Epigallocatechin gallate	4.14 ± 0.25b	0.65 ± 0.06c	8.25 ± 0.23a	0.69 ± 0.06c
Quercetin	0.68 ± 0.16c	0.78 ± 0.09c	15.97 ± 0.31a	11.13 ± 0.36b
Astilbine	n.d.*	n.d.*	8.85 ± 0.96b	25.80 ± 1.69a
Naringin	24.66 ± 0.57c	32.91 ± 1.80b	54.93 ± 3.37a	68.68 ± 3.24a
Naringenin	n.d.*	n.d.*	0.39 ± 0.04a	0.36 ± 0.06a
Myricetin	3.03 ± 0.23b	2.28 ± 0.19b	10.31 ± 0.96a	11.43 ± 1.28a
Procyanidin B1	n.d.*	n.d.*	2.14 ± 1.02	n.d.*
∑ Flavonoids	45.58 ± 2.07c	52.38 ± 4.48c	140.55 ± 2.31b	151.99 ± 0.53a
Phenolic acids				
Cinnamic acid	8.31 ± 0.30b	3.35 ± 0.66c	32.07 ± 4.45a	28.92 ± 2.62a
P-coumaric acid	0.83 ± 0.57a	0.20 ± 0.02c	0.32 ± 0.10c	0.44 ± 0.00b
Ferulic acid	n.d.*	1.95 ± 0.23c	7.24 ± 0.45a	6.60 ± 0.21b
∑ Phenolic acids	9.14 ± 0.64b	5.5 ± 0.69c	39.63 ± 4.47a	35.96 ± 2.62a
Stilbenoids				
Polydatin	10.83 ± 1.77a	0.81 ± 0.08c	2.68 ± 0.19b	3.62 ± 0.25b
Trans-resveratrol	3.28 ± 0.94b	3.36 ± 0.25b	7.89 ± 2.37a	3.99 ± 0.64b
Pterostilbene	2.05 ± 0.25a	1.89 ± 0.51a	1.40 ± 0.21b	1.63 ± 0.12b
∑ Stilbenoids	16.16 ± 2.01a	6.06 ± 0.57c	11.97 ± 2.38b	9.24 ± 0.69b
∑ Total phenolic compounds	73.02 ± 5.58b	65.13 ± 5.22b	192.83 ± 0.63a	202.81 ± 3.02a

Data are presented as mean ± SEM of three independent biological replicates. Different letters indicate significant differences ($P < 0.0001$) according to one-way ANOVA with Duncan's multiple range test.

*n.d., non-detectable. The results are expressed in mg g^{-1} of dry material.

favored an increase in the physiological parameters, such as the content of total Chl, Chl index, and maximum quantum yield of PSII (Fv/Fm). The increase in Chl and the stability of the photosynthetic rate allow us to explain the increase in morphological variables, such as the stem diameter and the aerial and root dry weights. Pérez-Rodriguez et al. (2020) showed that the strains used in this study produce indole acetic acid and siderophores, fix nitrogen and solubilize phosphate. In the present study, we suggest that these mechanisms could be involved in the higher dry matter of the inoculated plants. Similar results have been reported in other plant species inoculated with PGPR (Samaniego-Gámez et al., 2016; Lopes et al., 2018; Hafez et al., 2019; Anbi et al., 2020).

The greater vegetative development in the inoculated plants improved the parameters associated with the yield components. The inoculated plants had a greater number of flowers associated with a greater probability of fruit set, which correlated with an increase in yield. The higher fruit dry weight of the inoculated plants could be a consequence of the higher photosynthetic rate, which induced a higher fixation of photo-assimilates that favor the filling of the fruits.

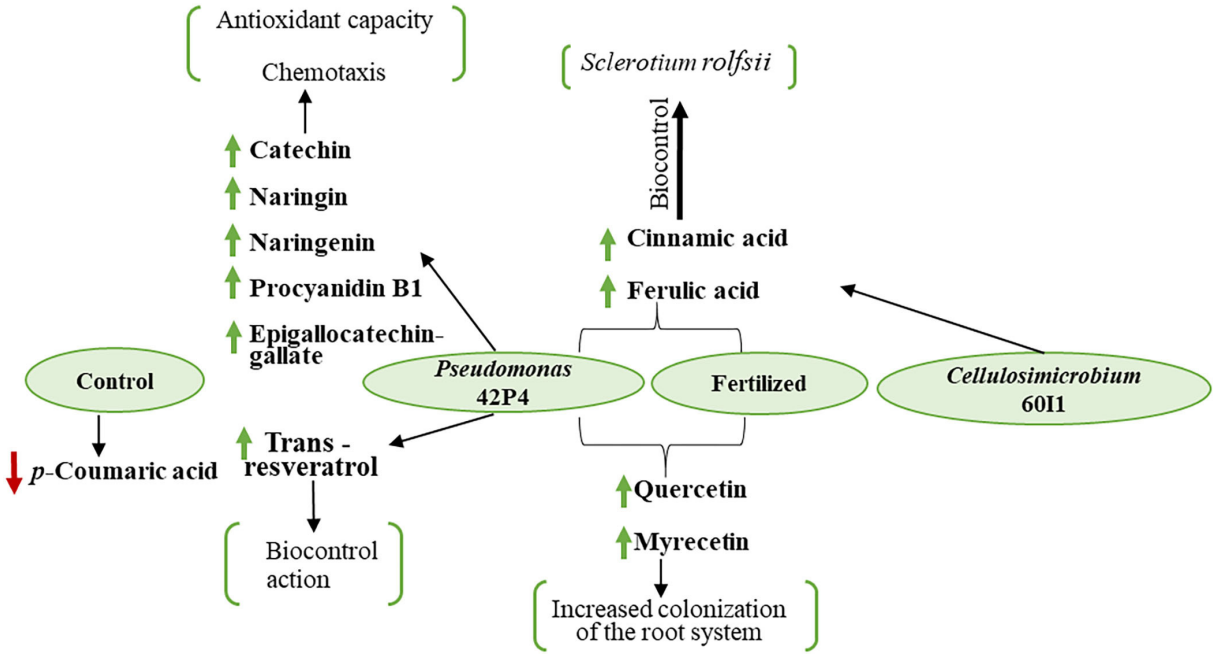

FIGURE 7
Summary diagram of the main phenolic compounds in pepper plants grown in pots and treated with: Fert: Fertilized; 42P4: *Pseudomonas* 42P4; 60I1: *Cellulosimicrobium* 60I1 and Control (without bacteria).

The principal component analysis showed that the treatments inoculated with *Cellulosimicrobium* 60I1 and *Pseudomonas* 42P4 behaved similarly to the Fertilized treatment and these strains were the most effective inoculation treatments. We suggest that the strains have displayed various growth promotion mechanisms, inducing an improvement in the growth and yield parameters with respect to the Control treatment. Similar results have been reported after inoculation with different PGPR in chrysanthemum (Kumari et al., 2016), blackberry (Robledo-Buriticá et al., 2018), strawberry (Ipek et al., 2014; Kurokura et al., 2017), and cherry tomato plants (Aini et al., 2019).

An increase in the total phenolic compounds has frequently been reported in different plant species inoculated with PGPR. However, to deepen the topic, we studied four families of phenolic compounds. In this way, the individualization of each compound helped us to clarify their possible role in the inoculated pepper plants. So, the abundance of flavonoids in plants inoculated with the *Pseudomonas* 42P4 strain may be related to the effective colonization of the root system by bacteria. The role of flavonoids in the signaling that mediates root colonization by PGPR is well-documented (Mierziak et al., 2014).

The compound (+)- catechin was only detected in the treatment inoculated with *Pseudomonas* 42P4. The role of catechin has not been clearly defined in plant physiology (Bais et al., 2010). Rani et al. (2011) indicated that the exogenous application of this compound in *Arabidopsis thaliana* plants improved the net photosynthetic rate, stomatal conductance, and indole acetic acid (IAA), also enhancing biomass accumulation, leaf area and leaf thickness. Other authors reported that catechin is associated with tolerance to oxidative stress and cold acclimatization in plants (Yiu et al., 2011; Ding et al., 2019). These antecedents led us to suggest that inoculation with strain *Pseudomonas* 42P4 stimulates the synthesis endogenous of (+)-catechin, which might induce a positive response in growth, nitrogen accumulation, enhanced Chl and photosynthetic rate, which leads to a greater accumulation of biomass and an increased yield. Similar results were reported by Chakraborty et al. (2015) in tea plants inoculated with *Bacillus megaterium*.

The inoculated and Fertilized treatments had the highest concentration of endogenous naringin and myricetin. These compounds are considered as a non-enzymatic antioxidant mechanism effective in the elimination of reactive oxygen species (Csepregi and Hideg, 2018; Liu et al., 2021). According to our results, we suggest that the metabolic balance observed in the photosynthetic rates and physiological parameters in inoculated plants pepper could be related to the antioxidant capacity conferred by antioxidant compounds, such as naringin and myricetin.

Naringenin was only detected in the *Pseudomonas* 42P4 and Fertilized treatments. Naringenin is a precursor for the synthesis of other flavonoids (Liu et al., 2021). It is feasible to suggest

that both treatments induce the production of naringenin, stimulating the production of other flavonoids in pepper plants. This hypothesis is confirmed by the fact that procyanidin B1 concentrations were also higher in the *Pseudomonas* 42P4 treatment. However, the results allow us to suggest an alternative hypothesis based on the accumulation of (+)-catechin for the production of procyanidin B1 in plants inoculated with *Pseudomonas* 42P4. A recent study indicated that (+)-catechin is closely linked to the synthesis of procyanidin B1 in *Camellia sinensis* (Wang et al., 2020).

The flavonoid (-)-epigallocatechin gallate was only quantified in the *Pseudomonas* 42P4 treatment. Therefore, it can be suggested that the 42P4 strain acts by modifying or modulating the levels of endogenous (-)-epigallocatechin gallate, favoring the accumulation of biomass and increasing the contents of photosynthetic pigments of plants. This compound increased seed germination and the growth of tomato seedlings. In addition, (-)-epigallocatechin gallate is a known protective agent against different types of stress such as salt stress, heat, cold, and drought. The beneficial effect of (-)-epigallocatechin gallate is based on the protection of the photosynthetic apparatus through the reduction of ROS (Ahammed et al., 2018; Li et al., 2019). Hong et al. (2015) reported that exogenous (-)-epigallocatechin gallate induces antifungal defense in *Arabidopsis thaliana*.

The *Pseudomonas* 42P4 treatment had the highest concentration of phenolic acids. The most abundant compounds quantified in this treatment were cinnamic and ferulic acids that have antioxidant activity and are effective in controlling pathogens. Therefore, *Pseudomonas* 42P4 may promote induced systemic resistance by stimulating the synthesis of these compounds. These family compounds can act as powerful antifungals, improving the response of pepper plants against the attack of phytopathogens. These results agree with studies carried out under *in vitro* conditions where an increase in endogenous phenolic acids was demonstrated when confronting strains of *P. aeruginosa* and *P. fluorescens* in the presence of *Sclerotium rolfsii*, which is an important phytopathogen in pepper crops (Singh et al., 2012). In this sense, other authors reported that inoculation with *P. fluorescens* and *Micrococucuce yunnanensis* increased the endogenous levels of cinnamic acid (Sarma et al., 2002; Sarma and Singh, 2003; Singh et al., 2012; Rahimi et al., 2020).

The higher concentration of trans-resveratrol was quantified in the *Pseudomonas* 42P4 treatment. This compound may benefit the health of pepper plants because it intervenes in the response of plants against fungal attacks. Similar results were reported in another study where grapevine plants inoculated with *Paraburkholderia phytofirmans* modulated the levels of stilbenoids (Miotto-Vilanova et al., 2019).

In summary, this study demonstrated that *Pseudomonas* 42P4 and *Cellulosimicrobium* 60I1 are two promising native strains that can be used to improve the growth, development, and production of pepper plants. However, the response of *Pseudomonas* 42P4 was more effective than *Cellulosimicrobium* 60I1, and *Pseudomonas* 42P4 inoculation modified the phenolic compound profile similarly to the Fertilized treatment. Figure 7 shows a schematic representation of the main changes produced by the different treatments. *Pseudomonas* 42P4 induced the synthesis of different endogenous polyphenolic compounds mainly related to chemotaxis (flavonoid family), antioxidant capacity (catechin, naringin, naringenin, myricetin, procyanidin B1, and epigallocatechin-gallate), and the induction of resistance to pathogens because trans-cinnamic acid and benzoic acid are precursor of salicylic acid, a hormone that mediates host response upon pathogen infection. Also cinnamic and ferulic acid participate in the response to pathogen attack. These results contribute to understanding the changes in the endogenous levels of the phenolic compound profile. However, studies with exogenous application of these compounds are necessary to corroborate the proposed hypothesis. Finally, *Pseudomonas* 42P4 can be used as a bioinoculant in pepper plants to allow better agronomic management, decreasing the use of chemical fertilizers to contribute to climate-smart and sustainable agriculture, improving productivity and contributing efficiently to the country's economy and the conservation of natural resources.

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 at: https://www.ncbi.nlm.nih.gov/ MT047266, MT047264, MT047267, and MT045593.

Author contributions

Conception and design of the experiments: RM, AC, and ML-U. Performance of the experiments and data analysis: ML-U with collaboration of MP-R (bacteria and morphological determination) and DS (nitrogen determination). Contribution of reagents/materials/analysis tools: AC and RM. Writing the initial draft: ML-U. All authors critically reviewed and modified the paper.

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Conflict of interest

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

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Plant-beneficial *Bacillus*, *Pseudomonas*, and *Staphylococcus* spp. from Kumaon Himalayas and their drought tolerance response

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Plant growth-promoting rhizobacteria (PGPR) have been shown to augment plant responses against drought and other abiotic stresses. In the present study, we isolated 27 bacteria from the rhizosphere of various plants cultivated in the Kumaon Himalayas., and to measure their abiotic stress tolerance, these 27 isolates were subjected to variations in pH, temperature, and drought. All 27 isolates were also screened for various plant growth-promoting traits. Among these, the four isolates RR1, ASC1, AFS3, and NG4 demonstrated various plant growth promotion activities including the synthesis of indole-3-acetic acid (IAA), siderophores, ammonia, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production, and concomitantly high tolerance to abiotic stresses. Moreover, 16S rRNA sequencing of these four isolates validated their identities as *Bacillus*, *Pseudomonas*, and *Staphylococcus* sp. Finally, to assess the *in-vivo* drought tolerance potential of these four isolates, a pot-trial experiment was undertaken in wheat cultivar VL-892. The results demonstrated that inoculating wheat plants with these four PGPR isolates greatly improved plant growth under drought circumstances by increasing root and shoot length and both fresh and dry weight of root and shoot. This study endeavors to discover the biochemical and molecular diversity of cultivable PGPR in six remotely located districts of Uttarakhand. In conclusion, the drought-tolerant PGPR strains described in this study are plant-beneficial and can effectively mobilize nutrients under drought conditions. Consequently, they could be used as bioinoculants to alleviate drought stress in wheat plants, in a sustainable manner. To the best of our knowledge, this is the first report of exploring the diversity and characterization of PGPR from the Kumaon Himalayas and their drought evaluation.

KEYWORDS

plant growth-promoting rhizobacteria (PGPR), drought stress, PEG 6000, inoculation, wheat

1. Introduction

Global warming, rising population, and declining agricultural land are all going to exacerbate global food insecurity. The expected growth in the world's population will place significant pressure on food security and constitutes a substantial threat to sustainable agriculture. Similarly, chemical fertilizers and pesticides were employed to meet a major portion of this increased

requirement in crop yields (Alori and Babalola, 2018). The extensive utilization of pesticides and fertilizers has already caused serious repercussions, including loss of soil quality and pollution in the agroecosystem (Meena et al., 2017). Due to this unexpected shift in ecological parameters, the plant's ability to adapt to fluctuating climates has been compromised. Plants are exposed to a variety of abiotic stresses present in their environment. Some of the most significant abiotic challenges that plants endure include desiccation, flooding, extreme temperature, salinity, and heavy metal contaminants (Gontia-Mishra et al., 2016). Among these, the most prominent abiotic factor that inhibits plant production is drought (Sati et al., 2022b). Drought is among the most widespread abiotic stress encountered by plants, and it impacts root-water dynamics and other general responses, further lowering the growth and nutrients of the plant and, consequently, global agricultural production (Ma et al., 2020). Drought exerts its effects by altering the morpho-physiological characteristics of plants by changing the water potential and turgor pressure of plants (Mukarram et al., 2021). Wheat (*Triticum aestivum* L.) is a key cereal crop grown worldwide. For about 35% of the global population, wheat serves as the primary food source (Poursarebani et al., 2014). In comparison to any other cereal e.g., maize, rice, etc., wheat offers greater proteins and calories (Kumar et al., 2017). Unfortunately, wheat is also among the top key cereal crops, where high temperatures and drought impede plant growth and harvest. The wheat crop is primarily grown in non-irrigated settings, having typical precipitation lower than 900 mm (Zhang et al., 2022). Wheat grown in non-irrigated circumstances in many emerging economies is vulnerable to drought in every stage of development (Rockström et al., 2009). Conventional plant breeding and genetic engineering approaches have been utilized to enhance drought resistance in crops. Using genetic engineering for making drought-tolerant cultivars is difficult since drought tolerance is a composite and multigenic character (Kumar et al., 2018). As a result, new solutions that offer higher crop yields while still ensuring ecological protection are urgently needed. One of the approaches to help plants cope with drought is by using PGPR, which has been documented to boost the nutritional, biochemical, physiological, and morphological properties of several plants in an environment-friendly and cost-effective manner (Sati et al., 2020). PGPR promotes plant growth directly by delivering the plant with substances synthesized by the bacteria or assisting the plant's absorption of soil nutrients, i.e., by phytohormone and siderophore formation, dissolution of mineral (P, Zn, K), and reducing ethylene levels in plants (Lugtenberg and Kamilova, 2009). Indirect PGP traits involve the exclusion of harmful aspects of a phytopathogen by diverse means, e.g., imparting resistance from the pathogen, production of enzymes, antibiotics, and anti-fungal chemicals (Goswami et al., 2016; Sati et al., 2022a). Plants treated with PGPR strains maintained better water stature than non-PGPR control, leading to greater production under drought (Shivakumar and Bhaktavatchalu, 2017). The Kumaon region experiences frequent extreme climatic disturbances each year. The monsoon season accounts for 60–85% of the annual precipitation rate, which ranges from 260 to 3,955 nm. Likewise, the average temperature fluctuates between below zero to −43°C due to the diverse topography of the region (Malik and Kumar, 2020). Keeping in mind the recurrent extreme climatic events and the significance of PGPR in growth enhancement, the current study was conducted to evaluate the diversity of native PGPR from the rhizosphere of various crop plants in the Kumaon region.

2. Materials and methods

2.1. Site description

The bacterial isolates were collected from all six districts of the Kumaon region of Uttarakhand. The Kumaon is one of the two administrative provinces in Uttarakhand, and it encompasses six districts: Almora, Bageshwar, Champawat, Nainital, Udham Singh Nagar, and Pithoragarh (Figure 1). The Kumaon region has a geographical size of 21,313 km^2 and an elevation range of 223–3,669 m above MSL. Kumaon is ensconced in the highlands of North India, bordering Chinese Tibet and Nepal. It encompasses the Himalayan Mountain range and is one of India's most secluded, scarcely inhabited, and undeveloped areas, with a static agricultural economy. Along the elevational gradient, the Kumaon region is divided into three agro-climatic zones: (i) lower elevation (up to 1,200m), (ii) intermediate elevation (between 1,200 and 2,300 m), and (iii) higher elevations (above 2,300 m). This region is excellent for the growth of multiple varieties of plants owing to its diverse climate (subtropical to alpine), height, elevation, soil types, valleys, rivers, watersheds, and forest resources.

2.2. Isolation and screening of soil bacteria

Soil samples were randomly obtained from the rhizosphere of diverse plants across six districts of the Kumaon region of Uttarakhand. Samples were taken from four separate places at each location. To generate the composite sample, five samples from each site were taken and mixed. The obtained soil samples were diluted serially for up to 10^{-6} dilutions and a 0.1 mL aliquot of this diluted soil solution was spread onto the Nutrient Agar (NA) plates. Microbial colonies began to develop on the plates during an overnight incubation at 28 ± 2°C. For further purifications, only colonies with clearly distinguishable morphologies were chosen. The pure culture was stored in a petri dish and maintained at 4°C for regular use. A solution of 20% glycerol was used to briefly store the pure isolates at −20°C.

2.3. Screening of isolates for tolerance to drought, pH, and salinity

A nutrient broth (NB) medium was prepared by amending polyethylene glycol (PEG 6000) with varying water potentials to test the drought endurance of isolated bacteria (−0.05, −0.15, −0.30, −0.49, and −0.73 MPa) (Sarma and Saikia, 2014; Gontia-Mishra et al., 2016). A 100 µL of each bacterial isolate having a concentration of 1×10^7 colony-forming unit (CFU)/mL, calculated by taking optical density (OD) at 600 nm, was added to the test tubes containing 5 ml of NB. The OD at 600 nm was measured spectrophotometrically after overnight incubation in an orbital

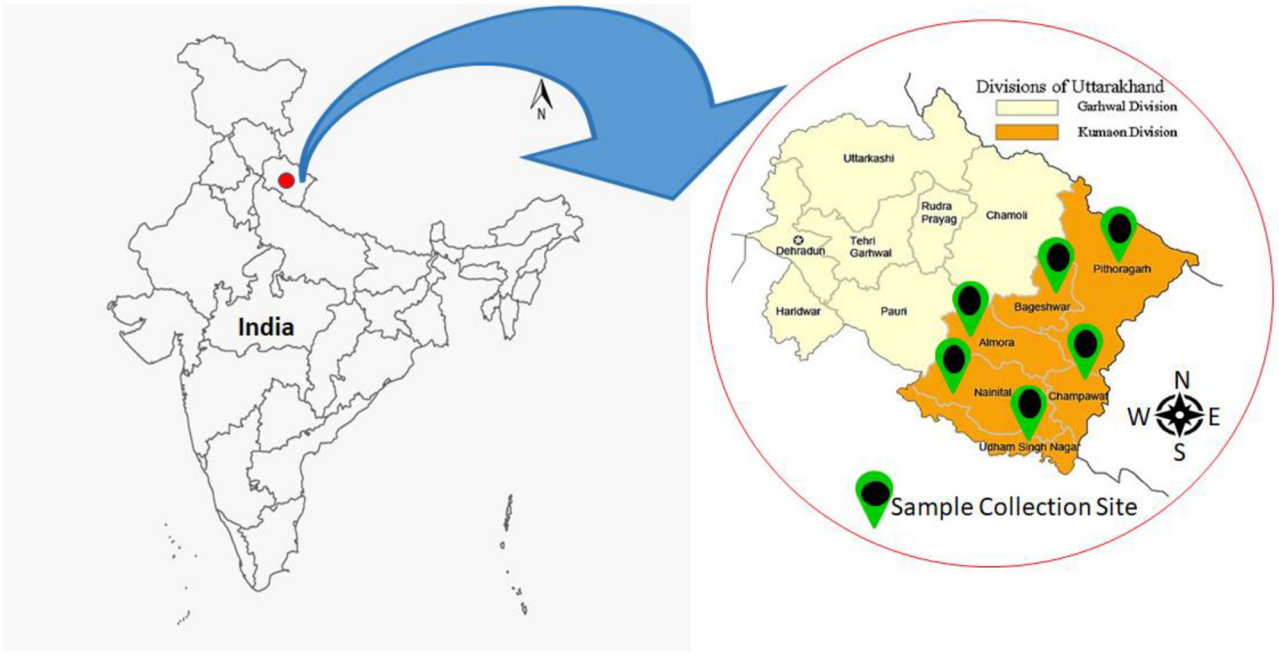

FIGURE 1
Map depicting the various geographical locations in the Kumaon region where samples were collected.

shaker (200 rpm) at 30°C. Under varying levels of PEG, the growth of bacterial cultures was evaluated. By growing the test isolates in the nutrient broth with varying pH i.e., 4, 6, 8, and 10 with either 1N HCl or NaOH their pH tolerance was determined (Küçük et al., 2006). Isolated cultures were evaluated for salinity endurance in nutrient broth (NB) supplemented with varying percentages of NaCl (0, 2.5, 5, 7.5, and 10%) (ben Romdhane et al., 2009). A 50 µl of each bacterial inoculum having culture turbidity of 1×10^7 CFU/ml, accessed spectrophotometrically, was injected per 5 ml of media and overnight incubated in an orbital shaker (200 rpm) at 30°C. The absorbance at 600 nm was calculated with a UV-Vis spectrophotometer to quantify growth (Eppendorf) (Gontia-Mishra et al., 2016).

2.4. Assessment for plant growth-promoting (PGP) traits

All the 27 isolates were tested for plant beneficial attributes e.g., indole acetic acid (IAA) generation, siderophore formation, ACC deaminase production, phosphate dissolution, HCN, and NH_3 generation, were evaluated as discussed by Bakker and Schippers (1987), Schwyn and Neilands (1987), Bric et al. (1991), Cappucino and Sherman (1992), Nautiyal et al. (2000), Penrose and Glick (2003), respectively. The Salkowski colorimetric test was used to identify and estimate IAA biosynthesis using Nutrient Broth supplemented with L-tryptophan (2 mgL^{-1}; Sigma-Aldrich, Milan, Italy). To analyze the potential of bacterial isolates to form siderophores, the isolates were spot inoculated on agar plates supplemented with Chrome-azurol S (CAS) dye, as defined by Schwyn and Neilands (1987). The appearance of an orange-yellow zone surrounding the colony after 5–7 days of incubation at 28°C revealed the siderophore synthesis by microbes. The presence of enzyme ACC deaminase (ACCd) in bacterial cultures was calculated by monitoring growth on nitrogen-free minimum medium (MM) agar (Dworkin and Foster, 1958) enriched with 3 mM ACC (Sigma-Aldrich) after incubating at 28°C in darkness for 5–7 days of incubation at, as defined by Jaemsaeng et al. (2018). 2 gL^{-1} $(NH_4)_2SO_4$ enriched MM agar plates were employed for control. Phosphate solubilization was checked using a modified NBRIP medium defined by Nautiyal et al. (2000). The isolated strains were examined for exopolysaccharide (EPS) synthesis and biofilm production (Kavita et al., 2011). The indirect plant beneficial traits e.g., hydrogen cyanide (HCN) formation were confirmed as per (Bakker and Schippers, 1987).

2.5. Seed germination assay

Seeds of late sown wheat cultivar VL-892 were procured from VPKAS Hawalbagh, Uttarakhand, India. Robust seeds were hand-picked and surface disinfected by immersing them in 70% ethanol for 3 min and then by dipping them in 0.2 % (v/v) $HgCl_2$. The seeds were rinsed five times in deionized water to eliminate residual ethanol. For each bacterial isolate, a 0.8 OD bacterial solution was prepared in which sterilized seeds were soaked for around 60 min. Seeds serving as control were kept in 0.5% (w/v) saline solution and the immersed seeds were laid on Petri plates coated with damp sterilized filter paper and cultured in triplicate at 25°C and under dark conditions. After incubating for 72 h, the germination percent, root, and shoot length were calculated, and an average score was determined for ten seedlings. For pot assay, cultures displaying greater impacts on

seedlings (in terms of root/shoot or vitality index) were selected. In the current work, the bacterial cultures were chosen depending on their tolerance to drought, and various plant-beneficial traits e.g., ACCd synthesis, siderophore formation, IAA production, P and Zn dissolution, HCN production, and seed germination rate. Based on the above-mentioned properties, four isolates namely RR1, ASC1, AFS3, and NG4 were selected. Discrete PGPR isolates were chosen for their high drought endurance, and potential to promote root proliferation and vitality index.

2.6. Inoculation of PGPR and drought stress induction

The surface-sterilized, untreated (control), and PGPR-treated seeds of wheat cultivar VL-892 were grown in separate $12'' \times 9''$ pots, filled with three kilograms of sterile soil, sand, and peat. The pot assay experiment involved 10 treatments as follows:

Control: Unstressed, uninoculated
T1: Unstressed, inoculated with AFS3
T2: Unstressed, inoculated with ASC1
T3: Unstressed, inoculated with NG4
T4: Unstressed, inoculated with RR1
D: Drought-stressed, uninoculated
DT1: Drought-stressed, inoculated with AFS3
DT2: Drought-stressed, inoculated with ASC1
DT3: Drought-stressed, inoculated with NG4
DT4: Drought-stressed, inoculated with RR1.

The experiment was done in triplicate and ten plants were maintained per pot. Seven days old PGPR-treated seedlings were again supplemented with 1% bacterial solution ($\sim 10^5$ CFU ml^{-1}), while untreated control plants were given an equal amount of MS media. Both the PGPR-treated and control plants were watered every alternate day with 70% of field capacity for 3 weeks. Further, these plants were subjected to 7 days of drought stress by not providing water. The non-stressed plants were watered normally. For drought recovery purposes, plants were watered again subsequently for 3 days. Water-stressed saplings without any PGPR treatment were taken as the negative control, while regularly watered plants were taken as the positive control. The saplings were treated with PGPR strains RR1, ASC1, NG4, and AFS3 to evaluate their growth promotion potential in both non-stressed (watered every alternate day) and drought-stressed (watered after 7 days) conditions.

2.7. Plant analysis

After the 7 days of induced drought and 3 days of drought recovery, the growth characteristics of each plant, e.g., root length, shoot length, fresh and dry weight of root and shoot were measured. To calculate the total dry weight (DW) plants were oven dried at 70°C for 72 h. The fraction of root adhering soil to root tissue (RAS/RT) was calculated by Sandhya et al. (2009). The relative water content (RWC) was determined according to Zhang and Blumwald (2001).

2.8. Phylogenetic analysis

For PCR amplification of the 16S ribosomal DNA, the primer sets 27f (5′AGAGTTTGATCCTGGCTCAG-3′) and 1378r (5′-CGGTGTGTACAAGGCCCGGGAACG-3′) targeting the rDNA of about 1,500 bp were utilized (Gontia-Mishra et al., 2016). The PCR reaction steps were early denaturation for 5 min at 94°C, afterward 35 rounds for 1 min at 55°C, extension for 3 min at 72°C, and final extension for 10 min at 72°C. The 16S rRNA gene sequence was obtained by Biologia Pvt. Ltd. (India) *via* genomic sequencing of the PCR sample. The basic sequence alignment BLAST Program was used to compare the sequences, scanned against the database given on the National Center for Biotechnology Information's website (http://www.ncbi.nlm.nih.gov/BLAST). MEGA version 6 was used to perform a phylogenetic assessment of the 16S rRNA gene sequences (Tamura et al., 2013). The neighbor-joining technique (Saitou and Nei, 1987) was used to construct the phylogenetic tree.

2.9. Statistical analysis

The data from all 10 different wheat saplings was computed. All the experiments were carried out in triplicates and the results were shown as mean ± SD. The results for, bacterial drought tolerance and bacterial treatments were examined by one-way ANOVA using GraphPad Prism (version 5) software. To calculate the significant differences between the means bacterial treatment under control and stress conditions. The critical difference (C.D.) values were calculated at the $p = 0.05$ level. The significantly different mean values are indicated by different letters.

3. Results

3.1. Isolation and characterization of rhizobacteria

A total of twenty-seven bacteria were isolated from the rhizosphere of Finger millet, Linseed, Wheat, Rice, Potato, Red kidney bean, Maize, Sugarcane, Nettle grass, Barley, Tomato, Chili, Horse gram, Black soybeans, and, Hemp from all the six districts of Kumaon region of Uttarakhand, India (Table 1).

3.2. Abiotic stress tolerance of isolates

The drought endurance of these PGPR isolates was tested by culturing them in varying concentrations of PEG 6000 in NB medium, and nine of them showed luxuriant growth in 15% PEG. Among these nine, only five demonstrated growth in 25% PEG. Lastly, only four isolates exhibited growth up to 35% PEG (Figure 2). These isolates were further tested for pH tolerance, and five of them demonstrated tolerance in NB medium up to pH 4.5 and only two demonstrated tolerance up to pH 9.5 (Figure 3). However, salt tolerance remains lacking in all the selected isolates.

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TABLE 1 Geographical location, type of soil, and the number of isolates collected from various districts of the Kumaon region.

Sample collection site	Location	Soil type	CFU-1	Number of isolates
Someshwar, Almora	29°46′ 56.9″ N 79°36′ 04.2″ E, elevation 1,545 m	Entisols	4×10^4-2×10^6	6
Kafligair, Bageshwar	29°45′ 09.9″ N 79°44′ 37.9″ E, elevation 1,555 m	Mollisols	4×10^4-3.2×10^6	9
Devidhura, Champawat	29°24′ 36.0″ N 79°51′ 57.1″ E, elevation 1,767 m	Inceptisols	5×10^4-2×10^6	3
Ramgarh, Nainital	29°26′ 55.7″ N 79°33′ 48.2″ E, elevation 1,797 m	Inceptisols	4×10^4-1.2×10^6	2
Pantnagar, Udham Singh Nagar	29°00′ 28.4″ N 79°28′ 11.5″ E, elevation 231 m	Inceptisols	4×10^4-2×10^6	3
Munsyari, Pithoragarh	30°05′ 53.4″ N 80°14′ 34.4″ E, elevation 2,502 m	Mollisols	4×10^4-2×10^6	4

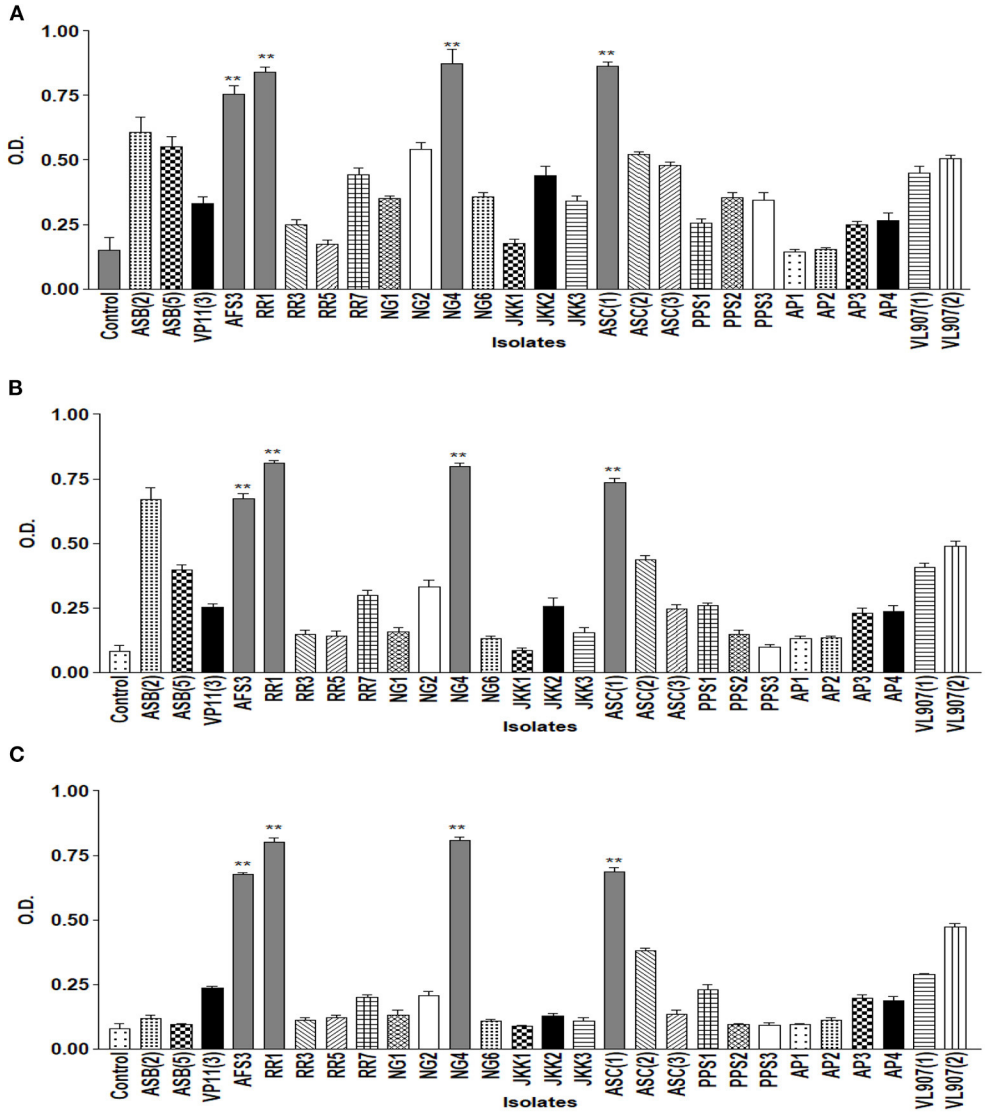

FIGURE 2
Graph showing growth curve at different concentrations of PEG **(A)** 15%; **(B)** 25%; **(C)** 35% of all 27 isolates. ** = p value < 0.01.

3.3. Plant growth-promoting traits of the isolates

All twenty-seven isolates were analyzed for IAA generation, phosphate solubilization, and ACCd activity among other direct PGP characteristics. Out of 27 isolates, 18 isolates were IAA producers. Isolate NG 4 registered the highest IAA production (0.9 µg/ml of culture medium). Similarly, except for nine isolates (ASB5, RR5, RR7, NG1, JKK2, JKK3, PPS3, AP1, and AP3), all the other isolates showed *in vitro* phosphate solubilization. Isolate ASC2 showed maximum phosphate solubilization (0.83 µg/ml). The PGPR isolates were assessed for ACC deaminase activity and except for 5, all 22

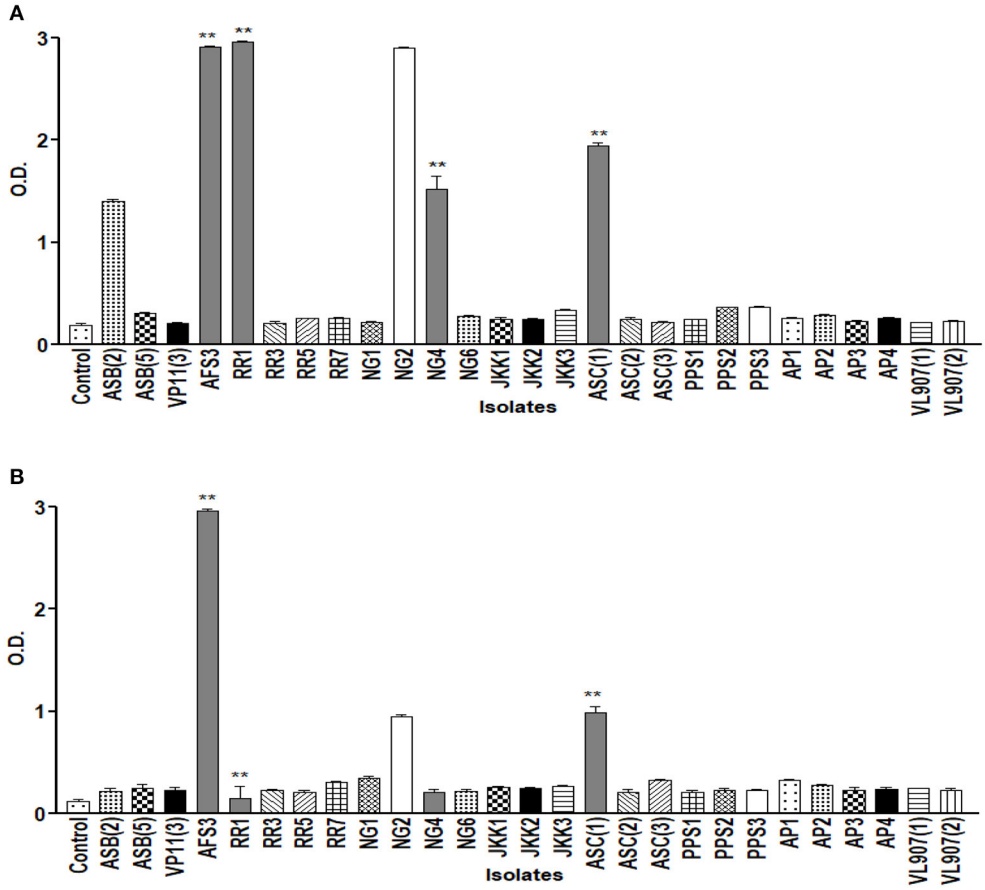

FIGURE 3
Graph showing growth curve at different pH **(A)** 4.5; **(B)** 9.5 of all 27 isolates. ** = p value < 0.01.

isolates exhibited this trait. The PGPR isolates were also tested for many indirect PGP attributes such as NH3 production, siderophore production, HCN production, and biofilm formation. Except for 5, all other PGPR isolates exhibited NH3 production whereas all of the isolates registered HCN production (Figure 4). Twenty-one isolates showed siderophore production whereas only 16 isolates showed biofilm/EPS secretion. The direct and indirect PGP traits of the PGPR isolates are shown in Table 2.

3.4. Plant growth-promoting activities of selected strains

To benefit plants, all 27 bacterial isolates were screened for plant promotion characteristics. Four best bacterial isolates were selected for the pot experiment, out of which three isolates namely ASC1, RR1, and AFS3 were the highest producers of siderophores, due to an orange halo around the colony. Similarly, the IAA production potential was found highest for these four isolates NG4, RR1, AFS3, and ASC1, respectively. In addition to the other PGPR traits, these 4 were also able to solubilize insoluble forms of phosphorus on the NBRIP plate as well. All four isolates also exhibited ACC deaminase activity. The PGPR isolates were also found positive for many indirect PGP attributes such as NH3 production, EPS production, and HCN production. The highest ammonia production was shown by the bacterial isolate NG4. Similarly, the greatest EPS and HCN production was recorded for isolate ASC1. Bacterial strains isolated from the rhizosphere of red kidney bean and hemp i.e., RR1 and ASC1, respectively, were able to tolerate drought for up to 35% PEG. Additionally, isolates AFS3 and ASC1 demonstrated remarkable pH tolerance with a range of 4.5–9.5. Based on variable plant growth-promoting traits AFS3, ASC1, NG4, and RR1, were selected for application as individual test subjects.

3.5. Identification and phylogenetic analysis of isolates based on 16S rRNA gene sequences

A 1,500 bp region of the the16S rRNA gene was purified and sequenced. All 4 bacterial strains were assigned to two distinct phyla based on their 16S rRNA gene sequences, including γ-proteobacteria, and Firmicutes. Out of the four test isolates, two strains belonged to the genus *Bacillus*, these were RR1 (*Bacillus velezensis*), and NG4 (*Bacillus cereus*), one strain belonged to the genus *Pseudomonas*, which was ASC1 (*Pseudomonas baetica*) and one strain belonged to the genus *Staphylococcus*, AFS3 (*Staphylococcus*

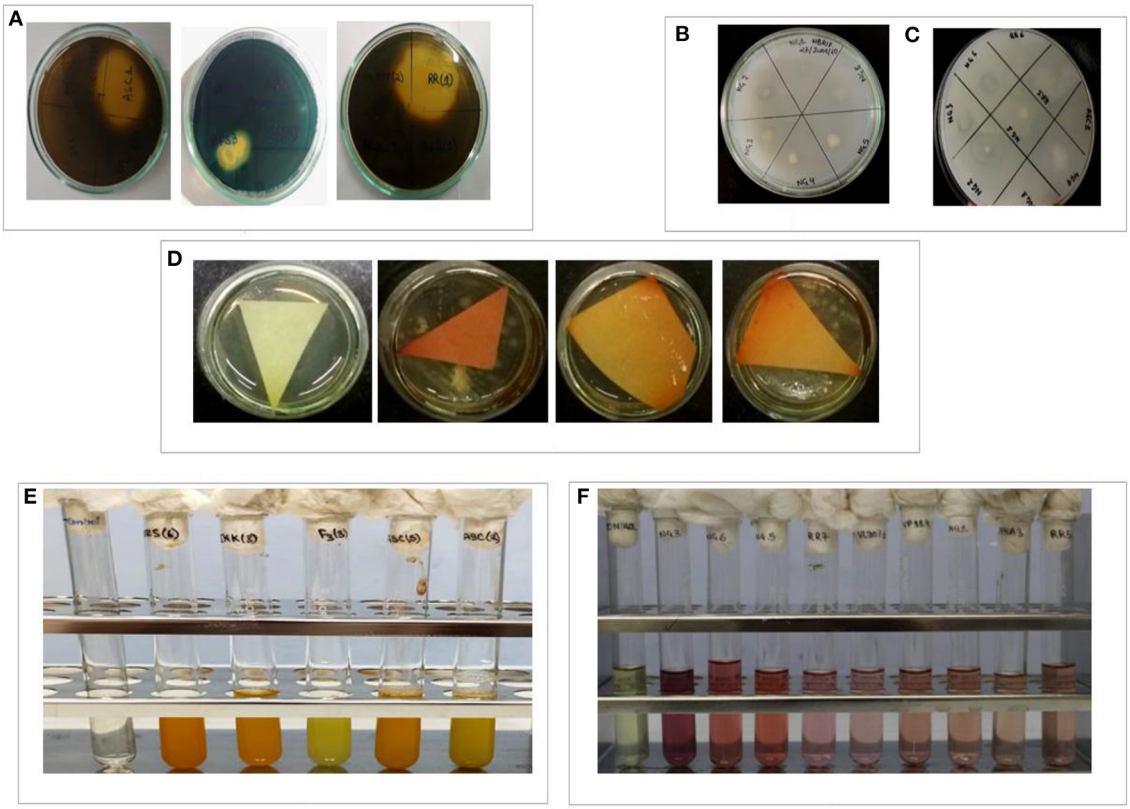

FIGURE 4
In-vitro depiction of different PGP parameters shown by isolated bacteria. **(A)** Siderophore production, **(B)** phosphate solubilization, **(C)** ACCd production, **(D)** HCN production, **(E)** NH3 production, and **(F)** IAA production.

pasteuri). A phylogenetic tree was built using 16S rDNA data to show the relationship between the tested isolates and related bacteria (Figure 5). Despite the two isolates belonging to the same genus as *Bacillus* sp., they exhibited sufficient variation in plant growth-promoting characteristics along with diverse seed vitality metrics. Owing to their stress adaption and nutrient solubilization potential, these PGPRs are highly effective at reducing stressful situations.

3.6. Effect of isolates RR1, ASC1, AFS3, and NG4 on growth promotion of wheat under drought stress

The final isolates AFS3, ASC1, NG4, and RR1 were chosen based on their PGP characteristics and capacity to endure high pH and higher concentrations of PEG in an NB medium. These isolates were analyzed for drought stress tolerance for seven consecutive days along with 3 days of the recovery phase. Plant root length, shoot length, and fresh and dry weights were calculated in PGPR inoculated and un-inoculated pots under normal and drought-stressed conditions (Table 3).

During normal circumstances, ASC1-primed wheat seedlings developed considerably longer shoots, and roots, over uninoculated control and AFS3, NG4, and RR1 seedlings, and their fresh and dry weight of shoot and root were also greater. Drought suffered, control plants greatly reduced in root length and shoot fresh and dry weights relative to PGPR primed plants. Under drought stress conditions, PGPR treatment was found to be beneficial in elevating the growth of wheat seedlings. PGPR-infected plants recovered from drought stress more effectively regarding the following growth parameters such as shoot length, root length, and shoot and root fresh and dry weights. However, the recovery of uninoculated plants under drought stress was minimal. A prominent upsurge in fresh and dry weight of root and shoot was observed with all bacterial treatments in comparison to the un-inoculated control. Thus, it was observed that isolate ASC1 emerged as the most effective PGPR for boosting plant growth under drought conditions and recovery.

4. Discussion

Wheat (*Triticum* sp.) is among India's major winter cereal crops. However, in hills, its production is impeded by various climatic constraints and its cultivation is mainly rain-fed. Also, wheat production is negatively affected by multiple abiotic stresses, predominantly, drought. Decreased wheat production and nutritive value in the hilly region are mostly caused by scattered agricultural

TABLE 2 Screening of isolated rhizobacteria for various direct and indirect plant growth-promoting traits.

S. no.	Strain code	IAA production (μg/mL)*	ACC d Activity	Phosphate solubilization (μg/mL)*	Siderophore production (%)*	EPS production (μg/mL)*	Ammonia production (μmol/mL)*	HCN production (μmol/mL)*
1.	ASB (2)	0.45 ± 0.02	+	0.33 ± 0.01	23.62 ± 0.93	52.73 ± 0.46	2.28 ± 0.09	0.02 ± 0.002
2.	ASB (5)	0.38 ± 0.02	+	0.21 ± 0.02	18.55 ± 1.04	28.69 ± 0.38	4.65 ± 0.14	0.024 ± 0.00 2
3.	VP11 (3)	0.19 ± 0.01	–	0.36 ± 0.02	16.62 ± 0.89	33.21 ± 2.28	3.54 ± 0.12	0.07 ± 0.05
4.	AFS (3)	0.69 ± 0.05	+	0.59 ± 0.03	51.36 ± 0.82	147.8 ± 0.96	5.68 ± 0.02	0.08 ± 0.003
5.	RR (1)	0.76 ± 0.01	+	0.63 ± 0.04	42.18 ± 1.20	140.42 ± 0.61	5.78 ± 0.14	0.084 ± 0.002
6.	RR (3)	0.34 ± 0.01	+	0.27 ± 0.01	32.87 ± 0.88	33.36 ± 1.40	4.88 ± 0.09	0.063 ± 0.002
7.	RR (5)	0.33 ± 0.01	+	0.19 ± 0.03	25.36 ± 1.09	27.45 ± 1.14	3.70 ± 0.21	0.06 ± 0.001
8.	RR (7)	0.20 ± 0.04	+	0.4 ± 0.02	18.02 ± 0.89	37.76 ± 1.00	5.51 ± 0.10	0.05 ± 0.002
9.	NG1	0.39 ± 0.04	+	0.16 ± 0.02	13.82 ± 1	75.83 ± 1.48	5.30 ± 0.10	0.05 ± 0.002
10.	NG2	0.28 ± 0.02	+	0.43 ± 0.02	12.86 ± 0.10	69.88 ± 0.19	5.17 ± 0.04	0.06 ± 0.001
11.	NG4	0.9 ± 0.02	+	0.42 ± 0.01	11.62 ± 0.41	171.61 ± 0.60	5.88 ± 0.07	0.094 ± 0.001
12.	NG6	0.69 ± 0.05	+	0.37 ± 0.02	36.51 ± 0.95	75.82 ± 0.44	5.16 ± 0.02	0.07 ± 0.001
13.	JKK 1	0.12 ± 0.01	–	0.25 ± 0.02	13.63 ± 1.28	12.31 ± 0.58	2.13 ± 0.03	0.01 ± 0.002
14.	JKK 2	0.11 ± 0.02	–	0.21 ± 0.02	17.20 ± 1.02	14.90 ± 0.18	2.38 ± 0.07	0.01 ± 0.001
15.	JKK 3	0.07 ± 0.005	–	0.23 ± 0.02	13.90 ± 0.09	18.62 ± 0.42	2.01 ± 0.02	0.017 ± 0.001
16.	ASC (1)	0.54 ± 0.01	+	0.72 ± 0.03	56.24 ± 1	182.99 ± 0.05	5.66 ± 0.17	0.18 ± 0.003
17.	ASC (2)	0.27 ± 0.03	+	0.84 ± 0.01	22.53 ± 1.21	175.81 ± 0.62	5.03 ± 0.04	0.09 ± 0.002
18.	ASC (3)	0.27 ± 0.03	+	0.76 ± 0.03	13.41 ± 1.37	177.94 ± 0.89	5.41 ± 0.16	0.09 ± 0.002
19.	PPS (1)	0.16 ± 0.01	+	0.34 ± 0.01	9.04 ± 0.11	64.12 ± 0.21	3.17 ± 0.01	0.042 ± 0.001
20.	PPS (2)	0.36 ± 0.01	+	0.27 ± 0.02	12.92 ± 0.75	61.01 ± 0.74	3.04 ± 0.05	0.043 ± 0.002
21.	PPS (3)	0.38 ± 0.02	+	0.21 ± 0.02	14.67 ± 0.49	53.54 ± 1.55	2.85 ± 0.11	0.045 ± 0.002
22.	AP (1)	0.15 ± 0.01	+	0.18 ± 0.02	12.2 ± 0.87	27.09 ± 0.23	4.06 ± 0.06	0.075 ± 0.002
23.	AP (2)	0.16 ± 0.02	+	0.24 ± 0.01	9.08 ± 0.65	24.14 ± 1.35	4.78 ± 0.13	0.076 ± 0.003
24.	AP (3)	0.33 ± 0.02	–	0.16 ± 0.01	15.09 ± 0.24	19.74 ± 0.57	2.84 ± 0.08	0.072 ± 0.003
25.	AP (4)	0.35 ± 0.01	+	0.24 ± 0.01	12.97 ± 0.5	25.21 ± 0.76	4.56 ± 0.25	0.075 ± 0.002
26.	VL 907 (1)	0.09 ± 0.02	+	0.36 ± 0.02	27.43 ± 0.61	75.45 ± 1.60	5.80 ± 0.04	0.028 ± 0.02
27.	VL 907 (2)	0.25 ± 0.01	+	0.38 ± 0.01	24.47 ± 0.83	73.26 ± 1.23	5.27 ± 0.04	0.01 ± 0.002

*Mean value (all values are triplicate).
± Standard deviation (SD); + means the presence of activity; and – means the absence of activity.

lands, poor farming techniques, and insufficient nutrient availability. Hence, the present study aims at exploring rhizobacteria from Kumaon Himalayas and analyzing their drought stress resistance. A total of 27 PGPR were isolated from the rhizosphere of various plants growing in the Kumaon region. The study demonstrates the role of these isolates to alleviate salinity and drought stress in wheat plants. Variation in soil types and quality is a common attribute of the hilly region. Fluctuating elevation and temperature influence soil properties and health, which further affect soil biological mechanisms, plant growth, and yield. Therefore, to check the abiotic stress endurance of rhizospheric isolates were screened in drought, pH, and salinity conditions. Out of the 27 isolates, nine were able to grow in moderate drought and four isolates showed extreme drought tolerance by growing in 35% PEG-supplemented NB. Similarly, a total of seven isolates demonstrated extreme pH tolerance, in which five isolates were able to grow in acidic pH of 4.5 while only 2 isolates showed growth in basic pH of 9.5. Salt tolerance was not detected in any of the isolates. Further, to confirm the plant beneficial properties of these 27 isolates, an extensive PGP-traits analysis was performed including IAA production, phosphate solubilization, ACC deaminase production, NH3 production, siderophore production, HCN production, and biofilm formation. Multiple PGP characteristics, including IAA generation, phosphate solubilization, ACC deaminase, siderophore production, etc., were present in the PGPR isolates. Out of a total of 27 isolates, we chose the best four for further analysis based on the greatest drought and pH stress tolerance and the presence of the greatest number of PGP traits. IAA production is a direct PGP

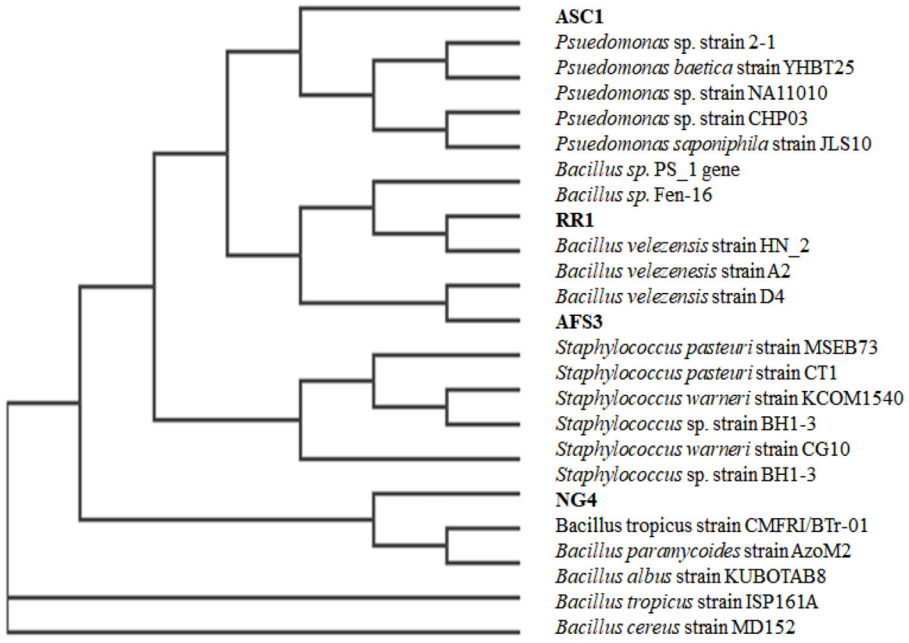

FIGURE 5
Phylogenetic tree constructed using the 16S rRNA gene sequences of the four selected isolates and some of their nearest phylogenetic taxa.

trait as this plant hormone help in plant growth, root development, tissue formation, cell elongation, etc. (Tsavkelova et al., 2007). In the present study, all four final isolates showed IAA production. Isolate NG 4 registered the highest IAA production (0.9 μg/ml of culture medium). The final four isolates were also capable of P solubilization. ASC1 was the highest P solubilizer among all, followed by RR1. Other similar studies of P solubilization have also been reported (Wang et al., 2020; López-Hernández et al., 2022). Additionally, all four selected isolates were noted to produce siderophores up to variable levels. A significant quantity of siderophore was observed in ASC1 and AFS3 ranging from 56.24 ± 1.0 to 51.36 ± 0.82. Production of siderophores, which aids in iron acquisition and accessibility for plants. This PGPR-triggered iron transport helps chlorophyll buildup and assists with iron retention in the edible sections of plants (López-Hernández et al., 2022). Wheat plants under drought stress were used to examine the ability of isolates AFS3, ASC1, RR1, and NG4 to promote plant growth. Under both non-stressed and drought-stressed conditions, all the isolates boosted the fresh and dry biomass of roots and shoots and significantly improved their length as well. The presence of various PGP characteristics in these four isolates, which could be the plausible explanation for their plant growth-promoting capacity, are well-known. Additionally, these isolates demonstrated adequate ACC deaminase activity, which could trigger stress-relieving effects. The 16S rRNA gene sequencing revealed RR1 as (*Bacillus velezensis*), NG4 as (*Bacillus cereus*), ASC1 as (*Pseudomonas baetica*), and AFS3 as (*Staphylococcus pasteuri*). The role of *Pseudomonas baetica* (González et al., 2021), *Staphylococcus pasteuri* (Bhattacharyya et al., 2020), *Bacillus velezensis* (López-Hernández et al., 2022), and *Bacillus cereus* (Akhtar et al., 2021) has been reported in earlier studies as PGPR with multifarious PGP activities and our observations are in line with these reports. The current study exhibited the diversity of indigenous PGPR from the Kumaon Himalayas. The PGPR isolates demonstrated multi-dimensional PGP traits along with a good tolerance range toward multiple abiotic stresses like drought and pH. The isolates are extensively capable of bio-inoculum formulations. The isolates ASC1 performed best among the four treatments with the highest growth augmentation of wheat saplings. Treating plants with PGPR is advisable in mitigating many abiotic stresses in wheat plants. To the best of our knowledge, this is the first report of exploring the diversity and characterization of PGPR from the Kumaon Himalayas and their drought evaluation. Still, advanced research on the communication of these bacterial treatments with other soil microflora and extensive field trials are required to authenticate their efficacy under real field circumstances and in curtailing the repercussions of drought stress. These indigenous rhizospheric isolates could be used as formulated as prospective biofertilizers for agroecosystem sustainability.

5. Conclusion

The present study illustrates the importance of rhizobacteria under *in-vitro* conditions with drought tolerance potential. These rhizospheric bacteria become crucial under agricultural droughts by not only protecting the plants from drought but also maintaining their productivity. It can be established from the above discussion that rhizobacteria have immense potential to enhance plant growth by imparting tolerance against drought stress. The present study also advocates the use of rhizobacteria as bio-inoculants for substituting chemical fertilizers to enhance the growth and productivity of plants under severe abiotic stress conditions like drought. Such bacteria can be introduced into the root system to augment their stress-tolerant potential without compromising their productivity and thus safeguarding the ecosystem. Hence, it is concluded that the isolated bacteria can be efficiently used in drought-suffered plants, and in

TABLE 3 Effects of bacterial treatments on root and shoot length and fresh, and dry weight of root and shoot of wheat under non-stress conditions, drought conditions, and during drought recovery under pot trial conditions.

Stress	Treatment	Shoot length (in cm)	Root length (in cm)	Shoot fresh weight (mg)	Root fresh weight (mg)	Shoot dry weight (mg)	Root dry weight (mg)
Non-stressed	Uninoculated	19.72 ± 0.85^{d}	9.89 ± 0.03^{d}	125.4 ± 0.54^{d}	48.42 ± 1.09^{c}	14.75 ± 0.43^{d}	9.48 ± 0.10^{d}
	AFS3	23.40 ± 0.46^{ab}	10.27 ± 0.04^{bc}	145.87 ± 2.88^{b}	64.54 ± 2.46^{a}	16.78 ± 0.25^{abc}	11.72 ± 0.11^{b}
	ASC1	23.43 ± 0.46^{a}	11.23 ± 0.04^{a}	156.79 ± 1.85^{a}	67.04 ± 2.40^{a}	18.16 ± 0.85^{a}	12.35 ± 0.02^{a}
	NG4	20.17 ± 0.68^{cd}	9.86 ± 0.01^{d}	125.46 ± 2.17^{d}	58.93 ± 2.16^{b}	16.00 ± 0.60^{bcd}	10.29 ± 0.02^{c}
	RR1	21.75 ± 0.20^{bc}	10.37 ± 0.01^{ab}	136.83 ± 1.40^{c}	62.78 ± 0.39^{ab}	16.86 ± 0.44^{ab}	11.7 ± 0.03^{b}
7 days drought	Uninoculated	17.25 ± 0.02^{e}	6.95 ± 0.01^{e}	105.66 ± 1.39^{c}	41.23 ± 0.04^{e}	12.43 ± 0.22^{d}	6.68 ± 0.12^{d}
	AFS3	20.27 ± 0.11^{ab}	8.37 ± 0.04^{bc}	116.18 ± 1.06^{a}	60.81 ± 0.64^{ab}	14.27 ± 0.17^{b}	8.13 ± 0.06^{b}
	ASC1	20.43 ± 0.22^{a}	10.83 ± 0.07^{a}	119.88 ± 1.62^{a}	59.45 ± 0.58^{abc}	16.64 ± 0.32^{a}	8.16 ± 0.02^{b}
	NG4	18.34 ± 0.02^{cd}	8.23 ± 0.04^{cd}	108.88 ± 0.66^{bc}	51.13 ± 0.02^{d}	14.09 ± 0.10^{b}	7.15 ± 0.03^{bc}
	RR1	19.14 ± 0.05^{bc}	9.22 ± 0.03^{ab}	109.18 ± 4.15^{b}	58.38 ± 0.06^{bc}	12.98 ± 0.03^{c}	9.22 ± 0.02^{ab}
Drought recovery	Uninoculated	17.30 ± 0.07^{e}	7.04 ± 0.03^{d}	106.33 ± 1.22^{c}	42.07 ± 0.04^{e}	13.14 ± 0.01^{d}	7.17 ± 0.02^{e}
	AFS3	21.24 ± 0.02^{bc}	11.14 ± 0.06^{abc}	118.98 ± 0.03^{a}	61.14 ± 0.02^{abc}	15.13 ± 0.02^{b}	9.15 ± 0.03^{bc}
	ASC1	22.18 ± 0.01^{a}	11.27 ± 0.01^{a}	117.01 ± 0.11^{a}	62.21 ± 0.07^{ab}	17.09 ± 0.06^{a}	9.78 ± 0.20^{bc}
	NG4	20.07 ± 0.04^{bcd}	9.16 ± 0.01^{bc}	110.27 ± 1.62^{b}	54.09 ± 0.05^{d}	15.21 ± 0.01^{b}	8.17 ± 0.02^{d}
	RR1	21.35 ± 0.03^{ab}	12.16 ± 0.02^{b}	110.30 ± 0.01^{b}	60.13 ± 0.02^{bc}	14.08 ± 0.06^{c}	10.1 ± 0.07^{ab}

Data represented as mean ± SD of triplicates; the significantly different mean values are indicated by different letters.

the future, further research can be done to assess the potential of these PGPR in a consortium to combat drought and facilitate growth promotion.

Data availability statement

The original contributions presented in the study are publicly available, with the following accession numbers NG4-Bacillus cereus-MT642947.1, RR1-Bacillus velezensis-MG727659.1, ASC1-Pseudomonas baetica- MG571730.1, and AFS3-Staphylococcus pasteuri-KP261074.1.

Author contributions

DS conceptualized, designed, performed the experiment, and wrote the first manuscript. VP assisted with the manuscript's writing and editing. MS supervised the research, supported data analysis, and reviewed and edited the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Siccibacter colletis as a member of the plant growth-promoting rhizobacteria consortium to improve faba-bean growth and alleviate phosphorus deficiency stress

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The rhizosphere is a hot spot and a source of beneficial microorganisms known as plant growth-promoting rhizobacteria (PGPR). From the alfalfa (*Medicago sativa*) rhizosphere, 115 bacteria were isolated, and from the screening for PGP traits, 26 interesting isolates were selected as PGP rhizobacteria for the next tests. The objective of this study was to use a consortium of PGPR to enhance the growth of faba-bean under phosphate (P) deficiency by taking advantage of their ability to release phosphorus from rock phosphate (RP). Several examined strains were found to have a relatively high activity on P solubilization, auxin, siderophore, ammoniac production, antifungal activity, and the ability to tolerate hypersalinity and water stress. 16S rRNA gene sequencing of the collection revealed six different genera, including *Bacillus* (46.15%), *Siccibacter* (23.07%), and *Acinetobacter* (15.38%) which were identified as the most abundant. Three of the interesting strains (*Siccibacter colletis*, *Enterobacter huaxiensis*, and *Pantoea* sp.) showed high plant growth promotion traits and no antagonism with *Rhizobium laguerreae*. These three bacteria were retained to establish a rhizobia-including consortium. The inoculation of faba-bean plants with the consortium improved growth parameters as root and shoot dried biomasses and some physiological criteria (chlorophyll content and P uptake under low P availability conditions), and the increase reached 40%. Our study could be the first report of faba-bean growth promotion by a multi-strain PGPR-rhizobia consortium involving *S. colletis, E. huaxiensis*, and *Pantoea* sp. Thus, this consortium could be recommended for faba-bean inoculation, particularly under P-limiting conditions.

KEYWORDS

rhizosphere, plant growth-promoting rhizobacteria, 16S rRNA, inoculation, phosphate deficient

1. Introduction

Soil mineral nutrients are essential for crop production. The limitations of soil nutrient availability as well as other abiotic and biotic stresses impact plant growth and its performance (Choudhary et al., 2016). In recent times, to deal with these constraints, sustainable biological strategies were implemented to increase the availability of nutrients and the ability of plants to tolerate stress (Umesha et al., 2018). For this purpose, microbial communities draw more attention and are involved due to their beneficial interactions with plants, such as the rhizobacteria that colonize the rhizosphere and are characterized by plant growth-promoting traits (Marschner et al., 2004; Msimbira and Smith, 2020). The majority of recognized PGPR for the enhancement of crop production were isolated from the rhizosphere (a hotspot of multi-beneficial microorganisms colonization) and have a generally positive impact on plants (Chamkhi et al., 2022). Indeed, the PGPR are a group of bacteria that can be found in the rhizosphere in interaction with the plant root systems and that can improve plant performance by multiple direct mechanisms such as biological nitrogen (N) fixation, phosphorus (P) solubilization, and phytohormone production such as cytokinins (CKs) and indole-3-acetic acid (IAA) (Goswami et al., 2016; Chamkhi et al., 2022). There are also PGPR-stimulating substances such as bacterial volatile compounds like 1-hexanol, pentadecane, and indole that are capable of promoting plant growth and its regulation (Blom et al., 2011; Kanchiswamy et al., 2015). Moreover, PGPR can induce the production of hydrogen cyanide (HCN), ammonia, and bioactive metabolites (biosurfactant, siderophore, and phenazine) (Patil et al., 2017). In addition to their indirect actions related to enzyme production, such as synthesizing stress-alleviating enzymes, such as the 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Chandra et al., 2018), antibiotic production, and induced systematic resistance, the PGPR can modulate plant stress markers under abiotic stress (Goswami et al., 2016). Moreover, they can induce numerous metabolic changes in host plants, including the accumulation of secondary metabolites (Chamkhi et al., 2021).

Low soil P availability has a negative impact on legume performance, mainly when the growth is dependent on biological N fixation, which is a high energy-demanding process (Oukaltouma et al., 2021). Furthermore, under conditions of P deficiency in the soil, P solubilization is one of the most common modes of action implicated in increasing the amount of PGPR available in the soil that could be easily absorbed by the plants. The increase in P solubilization involves some physiological mechanisms, such as the acidification of the rhizosphere and the excretion of phosphatases into the rhizosphere (Vance et al., 2003).

Indeed, rhizospheric bacterial communities contain highly diverse populations of P-solubilizing bacteria such as *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Burkholderia*, *Serratia*, *Enterobacter*, *Paenibacillus*, *Pantoea*, *Methylobacterium*, *Azotobacter*, *Ochrobactrum*, *Rhizobium*, and *Acetobacter* (Kour et al., 2021).

Similarly, it was shown that PGPR, individually or in combination (consortium), increased the nutrient contents (N, P, K, Fe, Zn, and Mn) and improved the absorption of macronutrients and micronutrients in the host plant (Rana et al., 2012; Behera et al., 2021). Indeed, Kumawat et al. (2021) reported that, in comparison to the uninoculated control treatment, the inoculation of spring mung bean plants with a bacterial dual combination enhanced the shoot contents of N (170.54%), P (79.01%), K (20.98%), Mn (100%), Fe (49.65%), Zn (65.96%), and Cu (89.52%) under salt stress conditions.

While soils of many legume-growing areas are affected by drought, low P availability further reduces the performance of these crops. To alleviate stress effects, inoculation with efficient rhizobia and synergistic PGPR could be a promising approach. Thus, this research aimedto isolate beneficial multi-trait rhizobacteria from legume rhizosphere in order to evaluate their potential effectiveness in promoting growth, and all bacterial isolates were tested for their *in vitro* putative PGP traits (direct and indirect PGP traits), including P solubilization, biological N-fixation, tolerance to osmotic stress, auxin production, siderophore production, ammonia production, antifungal activity, and phytase activity. Through this original research article, we screened the most promising PGPR that colonize the rhizosphere of *Medicago sativa*, testing their beneficial biological activities and the molecular identification of these bacteria to determine their species. The promising rhizobacteria were selected for the conception of a consortium to test their ability under stressful conditions. This study highlights the impact of PGPR as a biologically sustainable fertilizer to enhance plants under P deficiency by direct application of phosphate rock (PR) as a source of phosphorus.

2. Material and methods

2.1. Prospected legume areas and rhizobacteria isolation

Soil samples were collected from *M. sativa* rhizosphere from two different agricultural regions, namely, Daraa-Tafilalet and Chaouia of Morocco. Two zones, Imiter (WT3E5) and Tizagazouine (WT2E4), were sampled from the Daraa-Tafilalet region. From the Chaouia area, two zones, Ouled Said (CH3E5) and Sidi El Aydi (CH2E3), were sampled. Soil samples were conserved at −20°C until they were used.

Rhizospheric soil samples (4 g) were suspended in 16 ml of physiological sterile water, diluted serially, and homogenized for 30 min. Petri dishes containing TY medium [tryptone (5 g), yeast extract (3 g), and $CaCl_2$ H_2O (0.8 g)] were spiked with 0.1 ml of each soil dilution and incubated at 28°C for 48 h (Becerra-Castro et al., 2012).

2.2. Screening and characterization of rhizobacteria isolates for their PGP traits

2.2.1. Biological nitrogen fixation ability

The isolates were tested for their biological nitrogen fixation ability on a selected medium of biological nitrogen fixation (NFB) (KH_2PO_4 1.20 g, K_2HPO_4 0.80 g, glucose 5.0 g, $MgSO_4{\cdot}7H_2O$ 0.20 g, NaCl 0.20 g, $CaCl_2{\cdot}2H_2O$ 0.02 g, $FeSO_4{\cdot}7H_2O$ 0.002 g, distilled water 1 L, and 2.0 ml of metal solution (0.40 g $CaCl_2{\cdot}2H_2O$, 0.30 g H_3BO_3, 0.04 g $CuSO_4{\cdot}5H_2O$, 0.10 g KI, 0.20 g $FeSO_4{\cdot}7H_2O$, 0.40 g $MnSO_4{\cdot}7H_2O$, 0.20 g $NaMoO_4{\cdot}2H_2O$, and 10.0 ml

concentrated HCl in 1.0 L of distilled water). Adjusted fresh culture (DO = 0.1) of the isolates were inoculating plates containing *NFb* medium with or without the addition of NH_4Cl (positive control). Plates were incubated at 28°C for 7 days (Zhou et al., 2013).

2.2.2. Phosphate solubilization ability on solid medium

The obtained isolates were tested for their phosphate solubilization potential in a selective Pikovskaya medium (PVK) [glucose 10 g, $Ca_3(PO_4)_2$ 5 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.2 g, $MgSO_4{\cdot}7H_2O$ 0.1 g; KCl 0.2 g, yeast extract 0.5 g, $MnSO_4{\cdot}H_2O$ 0.002 g, $FeSO_4{\cdot}7H_2O$ 0.002 g; and Bromocresol Purple 0.1 g in 1.0 L of distilled water] supplemented with (0.5 g/L) of rock phosphate (RP) (Pikovskaya, 1948). A sample of 10 µl of each bacterial suspension (DO = 0.1) was dispensed into the center of Petri dishes containing the medium and incubated for 7 days at 28°C. The presence of a clearing zone around bacterial colonies (halo zone) is an indicator of positive phosphate solubilization (Xie et al., 2009).

2.2.3. Osmotic potential tolerance

The isolates obtained were screened for their osmotolerance levels by assessing their ability to grow in a medium supplemented with 30% (−1.69 MPa), 40% (−2.25 MPa), and 0% (0 MPa) as the control of polyethylene glycol (PEG) (8000). Appropriate uninoculated controls were maintained. The tubes were inoculated with 200 µl inoculum of each isolate (DO = 0.1) and incubated at 28°C for 2 days on an orbital shaker. Later, the O.D. of the broth was measured at 600 nm (Ilyas et al., 2020). The selected isolates were purified and stored at −80°C in 40% glycerol.

2.3. Assessment of isolated rhizobacteria for PGP traits

2.3.1. Quantification of phosphate solubilization

The P solubilization activity was determined according to Murphy and Riley (1962). In 250-ml Erlenmeyer flasks, 100 ml of liquid PVK medium were inoculated with 100 µl of fresh pre-cultures of each isolate (DO = 0.1). The incubation was done for 7 days at 28°C in the Shaker-Incubator at 158 rpm. A volume of 2 ml of the culture was centrifuged, and the pH of the culture rest was determined. Soluble P concentrations were measured spectrophotometrically by using the molybdenum blue method at 665 nm (Murphy and Riley, 1962).

2.3.2. Auxin quantification

The Salkowski colorimetric method was used to determine the auxin production of the isolates. Liquid pre-cultures (DO = 0.1) were inoculated into liquid YEM medium (Vincent, 1970) supplemented with tryptophan (YEM-Try) (5 g Mannitol, $3H_2O$, 0.12 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 1 g yeast extract, 0.46 g KH_2PO_4, 1 L distilled water, and 0.5 mg tryptophan) and then incubated at 28°C for 7 days. At 12,000 rpm for 10 min, 2 ml of each culture were centrifuged, and the supernatants were used to determine the auxin concentration following the method of Glickmann and Dessaux (1995).

2.3.3. Siderophore production

A sample of 100 µl of 24-h broth culture (DO = 0.1) was inoculated into 20 ml of iron-free succinic acid broth medium. The flask was incubated at 28°C for 7 days. According to Rachid and Ahmed, the concentration was calculated using the absorption maximum (400 nm) and the molar extinction coefficient (ε = 20,000/M/cm) (Rachid and Ahmed, 2005).

2.3.4. Ammoniac production

2.3.4.1. *Qualitative test*

The purified isolates (DO = 0.1) were grown in 10 ml of peptone broth in a test tube and placed on a rotary shaker at 120 rpm for 72 h at 28°C. After this incubation, 0.5 ml of Nessler's reagent was added to the culture test tube. The change in color from yellow to brown indicated that ammonia production was occurring (Mukherjee et al., 2017).

2.3.4.2. *Quantitative test*

The ammonia concentration was estimated for the best bacteria selected in the qualitative test. The supernatant of the isolates grown on peptone medium was used to quantify the ammonia produced by each bacterium using NH_4Cl as standard and measured at 420 nm (Zhao et al., 2019).

2.3.5. Halotolerance test

Bacterial isolates were screened for their salinity tolerance levels using TY media supplemented with NaCl at different concentrations (1%, 5%, 10%, and 15%). The plates were inoculated with 20 µl volumes of inoculum (DO = 0.1), and the cultures were incubated for 7 days at 28°C (Albdaiwi et al., 2019).

2.3.6. Antifungal activity

The isolates were tested against *Fusarium oxysporum* on potato dextrose agar (PDA) to test their antifungal activity. Hence, fungal disks were placed in the middle of agar plates, which is 2 cm away from the isolated spots. The antagonistic activity was observed after incubation at 28°C for 7 days. The percentage of inhibition was measured using the following formula: I (%) = (C – T/C) × 100, where I is the percentage of growth reduction, C is the diameter of the control hyphal growth (without bacterial spot), and T is the diameter of the treated hyphal growth (with bacterial spot) (Petatán-Sagahón et al., 2011).

2.3.7. Phytase activity

Bacterial isolates were assessed for their phytase activity by plate assay using phytase-specific medium agar in Petri plates after incubation at 28°C for 14 days. Bacterial isolates able to hydrolyze calcium phytate can grow on a specific medium (Singh et al., 2013).

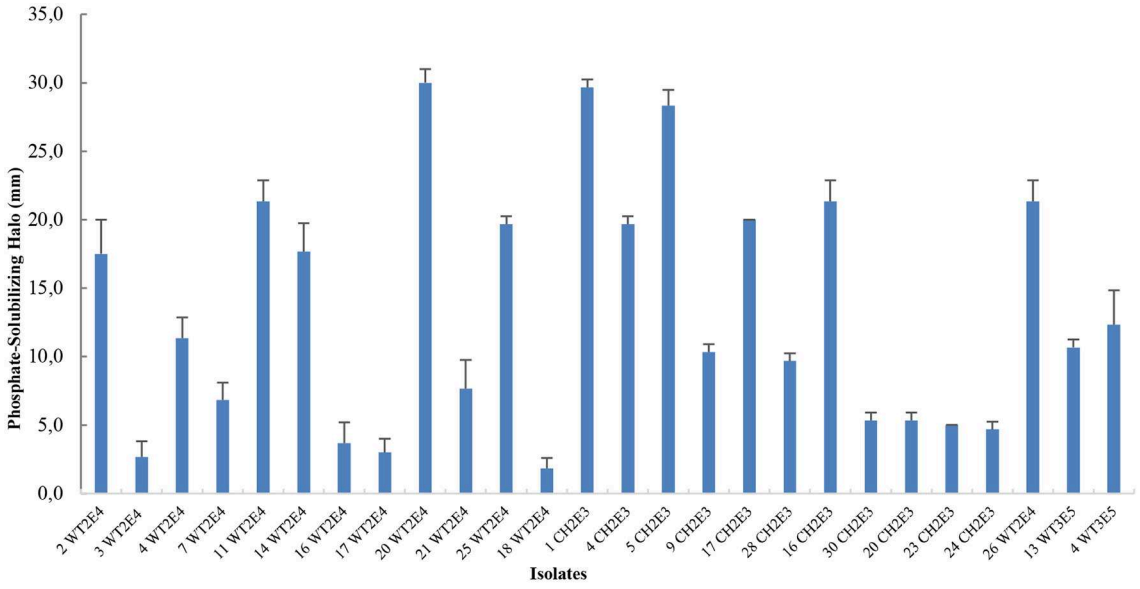

FIGURE 1
Qualitative test of P solubilization on *Pikovskaya (PVK) medium*.

2.4. Diversity analysis of selected PGPR

2.4.1. DNA extraction and PCR amplification

The genomic DNA was extracted (Rodríguez et al., 2014), and the quantity of extracted DNA was checked by using a NanoDrop spectrophotometer. PCR amplification of partial nucleotide sequences of the 16S rRNA gene was performed using the universal primers 7f (5′-AGA GTT TGA TYM TGG CTC AG-3′)/1510r (5′-ACG GYT ACC TTG TTA CGA CTT-3′). The PCR procedure used was as follows: initial denaturation for 3 min at 96°C, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 64°C for 20 s, and extension at 72°C for 45 s. Purified PCR products were then sequenced in both senses by using 1510r and 7 f based on the Sanger sequencing platform.

2.4.2. Phylogenetic analysis

The sequences of sense 1510r were converted into inverse complements by using the bioedit software and then aligned with the sequences of sense 7f by using the MEGA11 software and the ClustalW tool. The almost complete 16S rRNA gene sequence (2,400 bp) was obtained by assembling the sequences that were then compared to the 16S rRNA gene sequences obtained from the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed from the ClustalX results and the maximum likehood test with the Kimura 2-parameter model by using the MEGA11 program. The bootstrap method was used as the phylogeny test. Reference sequences were added to optimize the comparison (Tamura et al., 2011).

2.5. Bacterial inoculation, consortium preparation, and trial assessment

2.5.1. Rhizobacteria selection for consortium establishment

The isolates showing highly interesting PGPR traits were selected to establish a rhizobacterial consortium with rhizobia for faba-bean inoculation under P deficiency. The isolates 20WT2E4, 21WT2E4, and 25WT2E4 were selected for their highest PGPR traits and then tested for their eventual antagonism before consortium establishment with rhizobia *R. laguerreae* from the collection of Cadi Ayyad University, Team of Biotechnology and Symbiosis Agrophysiology, Faculty of Sciences and Techniques, Marrakech, Morocco. Primary screening of three rhizobacterial strains (20WT2E4, 21WT2E4, and 25WT2E4) and *R. laguerreae* was carried out for the selection for the consortium and evaluated by the Cross-Streak method. Each of the four isolates was streaked on a YEM medium in a straight line and incubated at 28°C for 6 days. The plates were then seeded with four other similar isolates by a single streak at a 90° angle to the previous streaked isolates and incubated at 28°C for 24 h. The absence of antagonistic interactions among the tested isolates was confirmed by the absence of an inhibition (clear) zone around the crosses between streaked lines of colonies (Velho-Pereira and Kamat, 2011).

For secondary screening based on the disk-diffusion method, strain inocula of 0.1 ml (final concentration of 10^6 CFU ml) was swabbed on the TY agar plates, and the isolates were directly spot inoculated onto sterilized Whatman paper disks of 7 mm diameter, which were deposited on the agar surface, and 15 µl of each strain was added on the top of the disk. The disks were then incubated at 28°C for 24 h. The antagonistic interaction was detected by the presence of the inhibition zone (Tendencia, 2004).

TABLE 1 Biological atmospheric N fixation potential according to different selected isolates.

	Isolates	Nitrogen fixation ability
1	2 WT2E4	+ + +
2	3 WT2E4	+ + +
3	4 WT2E4	+ + +
4	7 WT2E4	++
5	11 WT2E4	+
6	14 WT2E4	++
7	16 WT2E4	++
8	17 WT2E4	+
9	20 WT2E4	+
10	21 WT2E4	+
11	25 WT2E4	+ + +
12	18 WT2E4	+ + +
13	1 CH2E3	+ + +
14	4 CH2E3	++
15	5 CH2E3	++
16	9 CH2E3	+ + +
17	17 CH2E3	++
18	28 CH2E3	+ + +
19	16 CH2E3	+ + +
20	30 CH2E3	++
21	20 CH2E3	+
22	23 CH2E3	+ + +
23	24 CH2E3	++
24	26 WT2E4	++
25	13 WT3E5	+ + +
26	4 WT3E5	++

+ + +, high N fixation potential; ++, moderate N fixation potential; and +, low N fixation potential.

2.5.2. Inoculation of faba-bean plants with a selected rhizobacterial consortium

2.5.2.1. *Bacterial inoculum production*

Each bacterial strain was grown in a liquid YEM medium shaken at 180 rpm for 24 h at 28°C. The bacterial inoculum concentration used was approximately 10^8 UFC ml for each strain. The mixed consortium inoculum was prepared by mixing equal amounts of the three strain solutions (20WT2E4, 21WT2E4, and 25WT2E4) and *R. laguerreae* (the consortium) compared with the inoculation only with *R. laguerreae*.

2.5.2.2. *Plantlets growing and inoculation*

Seeds of the faba-bean (*Vicia faba*), Alfia variety, were surface sterilized using acidified 0.2% mercury chloride for 3 min, rinsed five times thoroughly with sterile distilled water, and allowed to germinate on sterile agar plates. After 4 days, seedlings were transferred into plastic pots (10 cm × 12 cm × 12 cm) containing 1 kg of a mixture of sterilized agricultural soil with a low available P level (3 ppm). Rock phosphate was added to the substrate as the only P source at a rate of 1.5 g per 1 kg. Two plantlets per pot were considered, and each one was inoculated with 5 ml of inoculum consisting of rhizobia only (*R. laguerreae*) or the bacterial consortium [*R. laguerreae* + rhizobacteria (20WT2E4 + 21WT2E4 + 25WT2E4)]. The pots were designed in triplicate, with two seeds per pot and one inoculated pot without seed as a control for each inoculated treatment. Four treatments were tested: the individual strain [Rhizobia (*R. laguerreae*) and the consortium of the three rhizobacteria and rhizobia] and the controls with non-inoculated plants [a negative control (T–), pots without Hoagland nutrient solution, and a positive control (T+), pots were watered when required with Hoagland nutrient solution]. The pots were transferred to a growth chamber (phytotron) under controlled conditions (25°C, 70% humidity, 16:8 h photoperiod, and illumination intensity of 240 mmol m^{-2} s^{-1}). A second reminder inoculation was conducted after 4 days of the first one.

2.6. Assessment of inoculation effect on agrophysiological parameters

2.6.1. Pre-harvest studies (late vegetative parameters)

Stomatal conductance was measured (mmol m^{-2} s) by the SC-1 leaf porometer of treated plants (T+, T–, *R. laguerreae*, and consortium) to estimate the rate of gas exchange and transpiration through the leaf stomata was determined by the degree of stomatal opening. The Chlorophyll Content Index (CCI) was assessed on the central part of well-extended young leaves in plants 30 days after inoculation using the chlorophylometer CL-01 (Hansatech Instruments Ltd., UK) (Shrestha et al., 2012).

2.6.2. Dry weight determinations

Root and shoot parts of the plants were dried in an oven at 70°C for 48 h to determine their dry weights.

2.6.3. Determination of P and minerals contents in plants

2.6.3.1. *Dosage of total P in the plant*

Samples of 0.5 g of dry matter from different parts of the faba-bean plant were incinerated at 600°C for 6 h. The ashes were recovered and dissolved in 3 ml of HCl (10 N), and after filtration, the filtrate was adjusted to 100 ml with distilled water. A volume of 1 ml of the filtrate was added to 4 ml of distilled water and 5 ml of the ascorbic acid reagent and then incubated in a water bath at 95°C for 10 min. The OD was measured with a spectrophotometer at 825 nm (Wieczorek et al., 2022).

2.6.3.2. *Dosage of other minerals in the plant*

Mineral elements (K and Ca) in the different parts of the plants were determined on the same filtrates used for the P assay with a spectrophotometer (model AFP100, Biotech Management Engineering Co. Ltd., UK) (Liu et al., 2021).

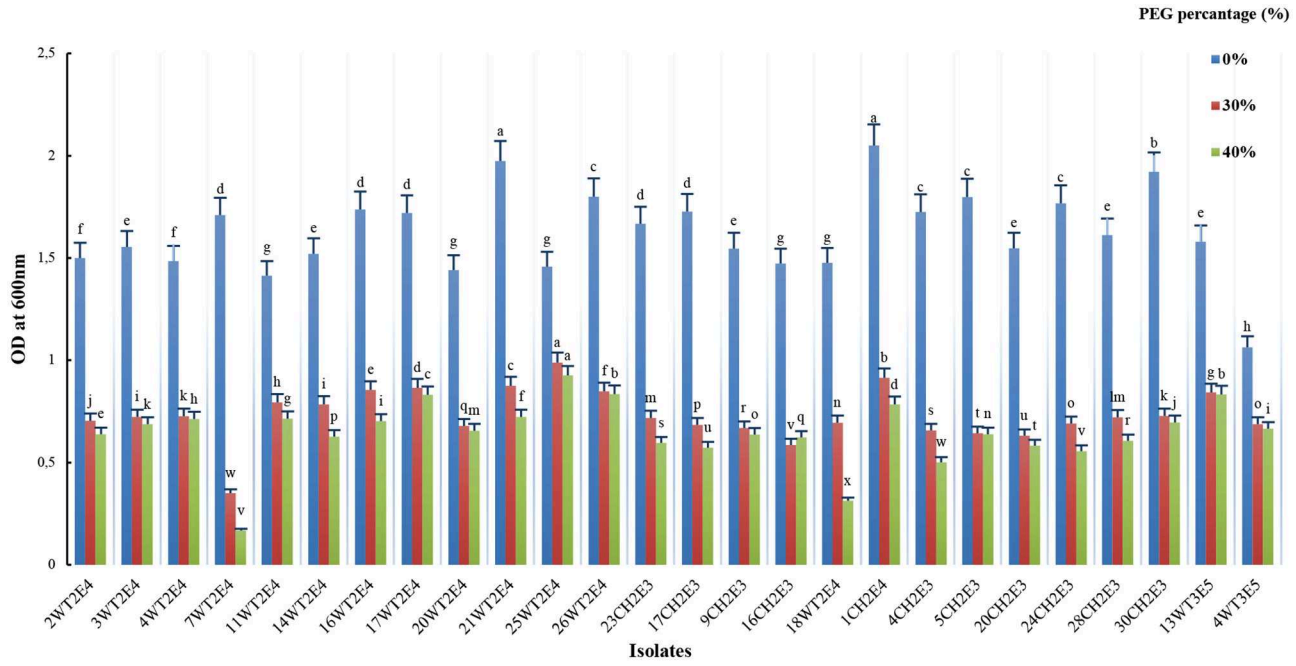

FIGURE 2
Effect of osmotic stress on the growth of the tested isolates. Growth obtained by the isolates on different concentrations of (0%, 30%, and 40%) PEG 8000. Different letters indicate that values are statistically different at a *P*-value of ≤ 0.0.

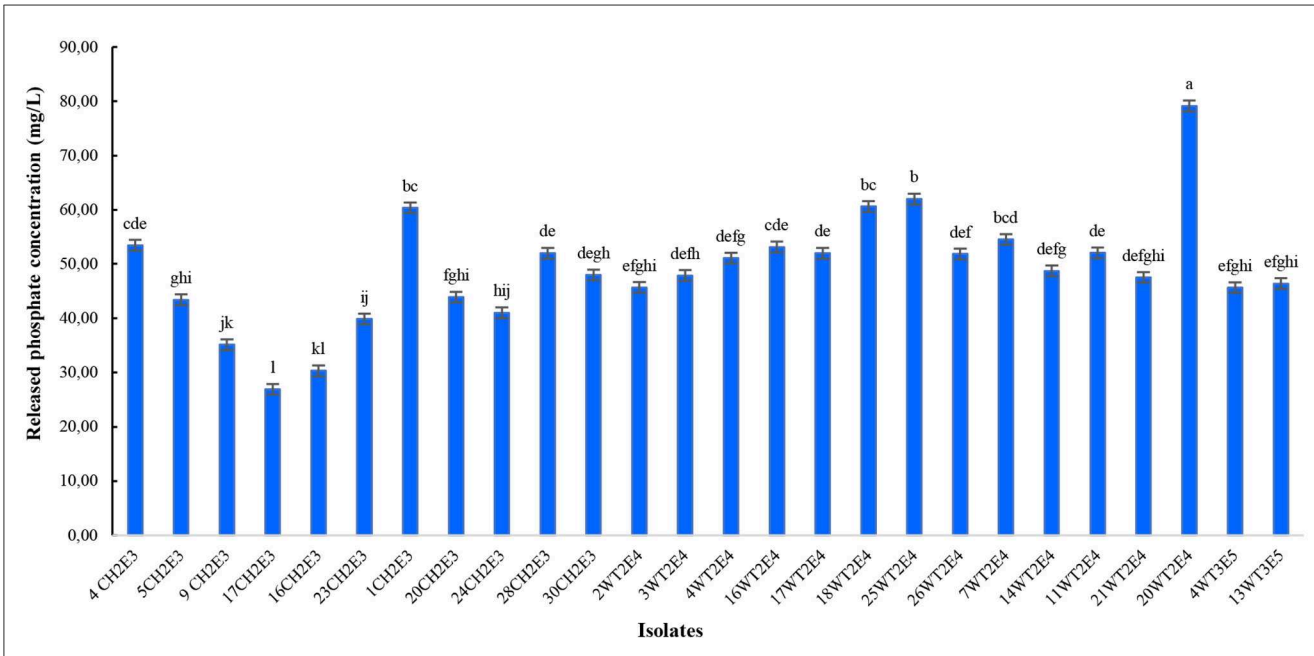

FIGURE 3
Quantification of soluble P content released by different selected isolates. Different letters indicate that values are statistically different at a *P*-value of ≤0.05.

2.6.4. Roots morphological parameters

After harvesting the plants, the roots were first washed with distilled water, and then, their architecture was immediately analyzed using the LA2400 scanner and WinRHIZO software (Regent Instruments Inc., Canada). Total root length, average root diameter, root volume, and root surface area were measured as the main root parameters (Gaudin et al., 2011).

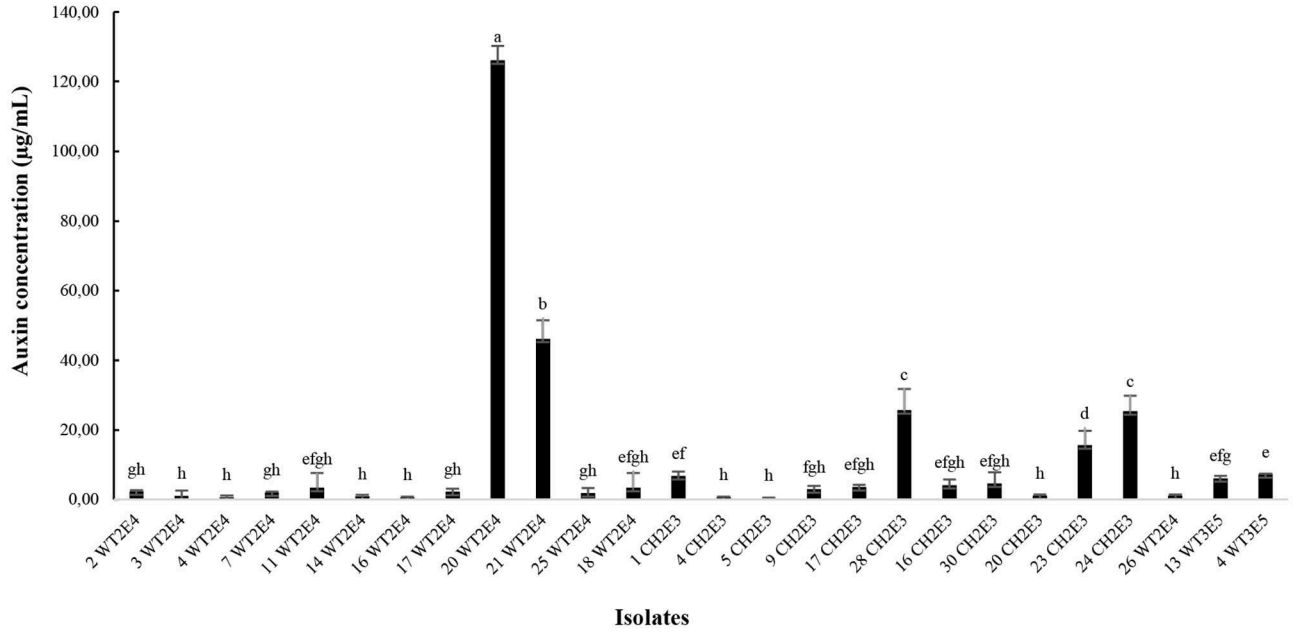

FIGURE 4
Auxin production by different selected isolates. Different letters indicate that values are statistically different at a *P*-value of ≤0.05.

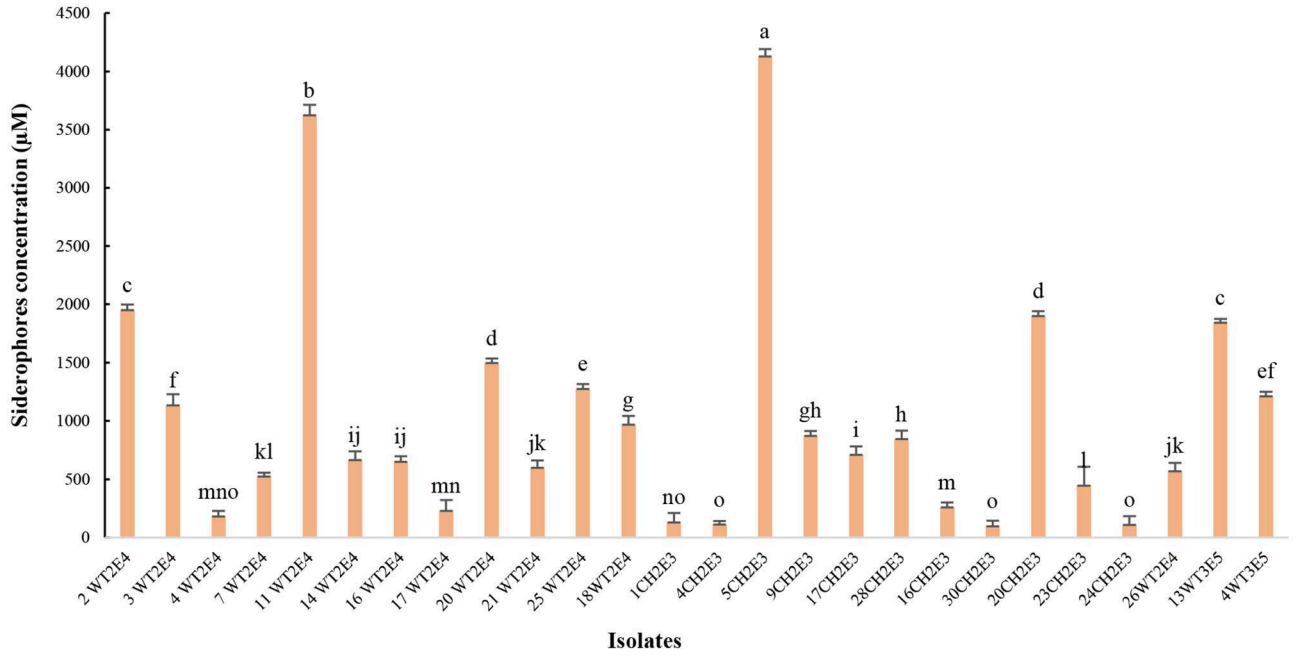

FIGURE 5
Siderophores concentrations (µM) produced by different selected isolates. Different letters indicate that values are statistically different at a *P*-value of ≤0.05.

2.6.5. Soil available P and acid phosphatase activity analysis

Available P content in the rhizosphere soil was measured using the Olsen (1954) method. Available P was determined after its extraction from the soil with 0.5 M $NaHCO_3$, adjusted to pH 8.5. The acid phosphatase (APase) activity of the soils was determined according to Tabatabai and Bremner (1969) using the para-nitrophenolate colorimetric assay method and buffer acetate (pH < 6.5). The reaction was stopped by adding 1 ml of NaOH (0.5 N). The APase activity was determined by measuring the formation of para-nitrophenolate at a wavelength of 410 nm.

10.3389/fsufs.2023.1134809

TABLE 2 Ammoniac production activity corresponding to different tested isolates.

	Isolates	Ammoniac production
1	2 WT2E4	+
2	3 WT2E4	++
3	4 WT2E4	++
4	7 WT2E4	++
5	11 WT2E4	+
6	14 WT2E4	++
7	16 WT2E4	+
8	17 WT2E4	++
9	20 WT2E4	+++
10	21 WT2E4	+
11	25 WT2E4	+
12	18 WT2E4	++
13	1 CH2E3	+
14	4 CH2E3	+++
15	5 CH2E3	++
16	9 CH2E3	++
17	17 CH2E3	++
18	28 CH2E3	++
19	16 CH2E3	++
20	30 CH2E3	+++
21	20 CH2E3	+++
22	23 CH2E3	+++
23	24 CH2E3	+++
24	26 WT2E4	+
25	13 WT3E5	+
26	4 WT3E5	+

+++, high; ++, moderate; +, low ammonia production.

2.7. Statistical analysis

The experimental data for different assessments were statistically analyzed using an analysis of variance (ANOVA) by the SPSS version 20 software (IBM-SPSS Inc., Chicago, IL). The collected data were analyzed by the HSD Tukey's test with a 5% probability threshold. The graphs were performed using the GraphPad Prism 8 software.

3. Results

3.1. Rhizobacterial isolation and screening of PGP rhizobacteria

In total, 115 bacteria were isolated from the collected legume rhizospheric soils, 34 were obtained from the CH2E3 area of *Sidi El Ayedi*, 24 from the CH3E5 area of *Ouled Said*, 26 from the WT3E5 of *Imiter*, and 31 from the WT2E4 area of *Tizagazouine*. The isolate that demonstrated a halo around the colonies as an indication of phosphate solubilization depends on the biological N fixation activity, the tolerance level to osmotic stress, and the qualitative phosphate solubilization in *Pikovskaya medium (PVK)* and *NBRIP medium*. A collection of 26 isolates were selected and conserved for subsequent tests.

3.1.1. Phosphate solubilization ability on Pikovskaya medium

Halo production is an indication of natural phosphate solubilization, as demonstrated. All selected bacteria can produce phosphate solubilizing halo (Figure 1). The test was done in *Pikovskaya medium* (PVK) agar supplemented with natural rock phosphate. As a result, 11 isolates showed a high ability for P solubilization. The isolates 20WT2E4, 1CH2E3, and 5CH2E3 revealed a strong ability for P solubilization with the presence of a clear halo around the colonies measuring 30 ± 1.0 mm, 27.7 ± 0.6 mm, and 28.3 ± 1.2 mm, respectively.

3.1.2. Biological nitrogen fixation

The results of the biological N fixation screening showed that 42.30% of the tested collection presented high N fixation potential (+++), 38.46% fixed N moderately (++), and 19.23% had weak potential (+) for N fixation (Table 1).

3.1.3. Effect of osmotic stress

The effect of different levels of osmotic stress on the growth of the selected isolates was determined (Figure 2). All selected isolates were able to grow at higher osmotic stress induced by 30% (−1.5 MPa) and 40% (−1.99 MPa) of PEG 8000, although growth was drastically reduced. The highest growth of 30% PEG 8000 was obtained by the isolates 25WT2E4, 1CH2E4, 21WT2E4, 17WT2E4, and 16WT2E4, respectively. However, the highest growth of 40% PEG 8000 was recorded in the isolates 25WT2E4, 26WT2E4, 13WT2E4, 17WT2E4, and 1CH2E4, respectively. Moreover, it was also noticed that the isolates 25WT2E4, 17WT2E4, and 1CH2E4 were ranked, respectively, as being tolerant to high osmotic stress. In addition, the highest reduction in growth as compared to control conditions was observed for the isolate 7WT2E4, in which the growth declined remarkably with the increase in PEG concentration.

3.2. Evaluation of isolates for PGP traits

3.2.1. Quantification of released phosphate in the NBRIP medium

All isolated rhizobacteria solubilize the P in the *NBRIP medium*. The isolates 20WT2E4, 25WT2E4, 18WT2E4, and 1CH2E3 were the most P solubilizing bacteria, with concentrations of released P of 79.15 ± 0.2a mg/L, 61.95 ± 0.3b mg/L, 60.60 ± 0.2bc mg/L, and 60.37 ± 0.2bc mg/L, respectively. The weakest concentration of released P was approximately 26.90 mg/L, corresponding to isolate

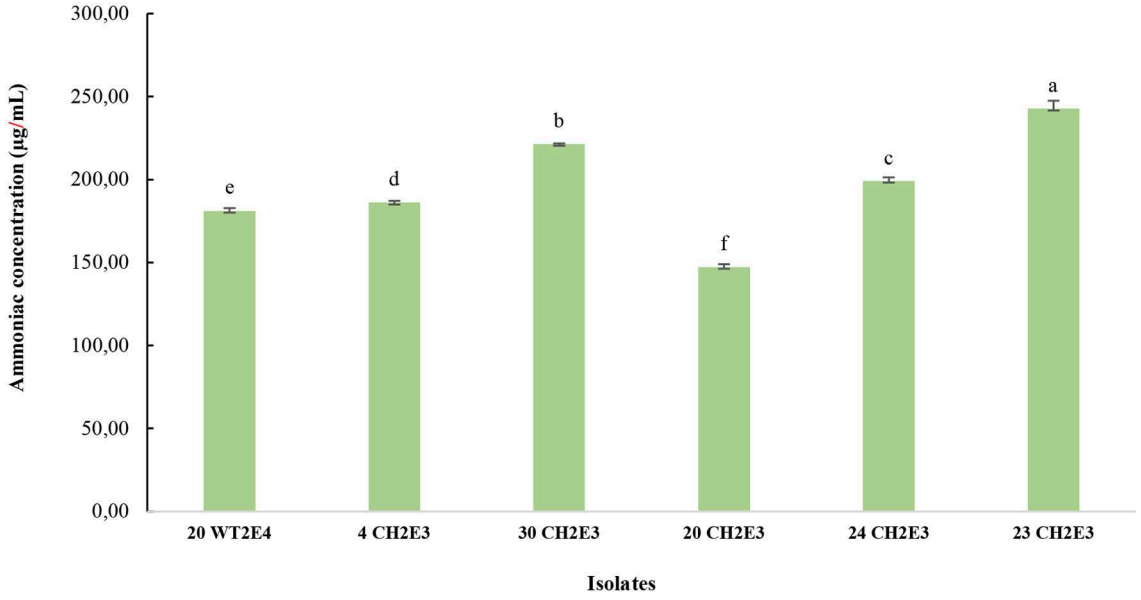

FIGURE 6
Ammoniac production by the selected isolates for the quantitative test. Different letters indicate that values are statistically different at a *P*-value of ≤0.05.

17 CH2E3 (Figure 3). However, other isolates presented moderate P solubilization activities.

3.2.2. Auxin production

The quantification of auxin biosynthesis by the rhizobacteria showed that the concentrations varied between the isolates. It was noticed that the isolate 20WT2E4 was more performing in auxin production and revealed the highest concentration of 126.14 ± 4.1a µg/ml followed by the isolate 21WT2E4 with 46.22 ± 5.3b µg/ml and then the isolates 28CH2E3 and 24CH2E3 with auxin concentrations of 25.64 ± 6.1c µg/ml and 25.42 ± 4.4d µg/ml, respectively (Figure 4). In general, the remaining isolates were low producers of auxin, with concentrations that did not exceed 10 µg/ml.

3.2.3. Siderophore production

The results presented in Figure 5 show a significant variation in siderophore production, on a standard succinate medium, between the tested isolates. Indeed, the isolates 5CH2E3 and 11WT2E4 produced the highest siderophore concentrations, with more than 3,500 µM. The isolates obtained with 2WT2E4, 20WT2E4, 20CH2E3, and 13WT3E5 showed relatively high concentrations of siderophore between 1,700 and 2,000 µM. Seven isolates produced very low siderophore concentrations that did not exceed 400 µM (Figure 5).

3.2.4. Ammoniac production

3.2.4.1. *Qualitative test*

As indicated by the results in Table 2, the ammonia production test showed that 23.07% of the isolates produced was highly (+ + +) ammoniac as indicated by the intensity of the brown color. Moreover, it was revealed that 42.30% of the isolates produced was moderately (++) ammoniac and 34.61% produced was weakly (+) ammoniac. The results of this qualitative test allowed us to select isolates with higher ammonia revelation for the quantitative test.

3.2.4.2. *Quantitative test*

From the qualitative test, six isolates (20WT2E4, 4CH2E3, 30CH2E3, 20CH2E3, 23CH2E3, and 24CH2E3) were selected as the most producer of ammonia. As detailed in Figure 6, the quantitative test demonstrated that the isolate 23CH2E3 registered the highest concentration of ammonia (242.72 ± 4.9a µg/L), followed by the isolates 30CH2E3 (221.41 ± 0.3b µg/L), 24CH2E3 (199.17 ± 2.2c µg/L), 4CH2E3 (186.08 ± 1.19d µg/L), 20WT2E4 (181.17 ± 1.8e µg/L), and 20CH2E3 (199.17 ± 2.2f µg/L).

3.2.5. Assessment of isolates for their halotolerant levels

The selected collection demonstrated an interesting halotolerant ability, as detailed in Table 3. All 26 tested isolates were able to tolerate a salinity of up to 5% NaCl. The majority of them, 21 isolates, were able to tolerate salinity levels up to 10% NaCl, and only 6 isolates tolerated the concentration of 15% NaCl (Table 3).

3.2.6. Antifungal activity against *Fusarium oxysporum*

The bacterial strain 21WT2E4 showed the highest antifungal activity against *F. oxysporum*, restricting mycelial growth in a dual culture plate assay with an inhibition percentage of up to 70% (Figure 7). The isolates 13WT3E5, 20CH2E3, and 4WT2E4 also

TABLE 3 Results of the halotolerance test corresponding to the selected isolates at four salinity levels (1%, 5%, 10%, and 15% NaCl).

	Isolates	1%	5%	10%	15%
1	2 WT2E4	+++	+++	+	–
2	3 WT2E4	+++	+++	+	–
3	4 WT2E4	+++	+++	+	–
4	7 WT2E4	+++	+++	++	+
5	11 WT2E4	+++	+++	+	–
6	14 WT2E4	+++	+++	+	–
7	16 WT2E4	+++	+++	–	–
8	17 WT2E4	+++	+++	+	+
9	20 WT2E4	+++	+++	++	–
10	21 WT2E4	+++	+++	+	–
11	25 WT2E4	+++	+++	+	–
12	26 WT2E4	+++	+++	+	–
13	18 WT2E4	+++	+++	–	–
14	23 CH2E3	+++	+++	++	+
15	17 CH2E3	+++	+++	–	–
16	9 CH2E3	+++	+++	++	–
17	16 CH2E3	+++	+++	++	+
18	1 CH2E3	+++	+++	++	+
19	4 CH2E3	+++	+++	–	–
20	5 CH2E3	+++	+++	–	–
21	20 CH2E3	+++	+++	+	–
22	24 CH2E3	+++	+++	++	+
23	28 CH2E3	+++	+++	++	–
24	30 CH2E3	+++	+++	–	–
25	13 WT3E5	+++	+++	+	–
26	4 WT3E5	+++	+++	+	–

+++, high; ++, moderate; +, low; –, no salinity tolerance.

presented interesting inhibition percentages of *F. oxysporum* at 46.42%, 44.04%, and 40.47%, respectively. Nine other isolates tested did not present any antagonistic activity against *F. oxysporum*.

3.2.7. Phytase activity

Out of 26 isolates, only isolate 21WT2E4 grew on *phytase-specific medium agar*.

3.3. Phylogenetic analysis

The 16S rRNA gene sequences obtained were analyzed using bioinformatic tools (Figure 8). Bacterial isolates belonged to 13 different bacterial species, namely, *Siccibacter colletis*, *Siccibacter turicensis*, *Enterobacter* sp., *Enterobacter huaxiensis*, *Pantoea* sp., *Pseudomonas punonensis*, *Acinetobacter dijkshoorniae*, *Acinetobacter calcoaceticus*, *Acinetobacter lactucae*, *Bacillus qingshengii*, *Bacillus* sp., *Bacillus huizhouensis*, and *Brevibacterium frigoritolerans*.

Among the 6 genera identified, *Bacillus* is the predominant genus with 46.15%, followed by the genus *Siccibacter* with 23.07%, *Acinetobacter* with 15.38%, *Enterobacter* with 7.69%, and then *Pantoea* and *Pseudomonas* with the same percentage of 3.84%.

3.4. Effect of inoculation test

Different parameters of the faba-bean plant growth were determined to assess the impact of the inoculation on the individual strains and the consortium.

3.4.1. Effect of inoculation on stomatal conductance and chlorophyll content index as physiological parameters affecting biomass accumulation

The bacterial inoculation impacted positively the stomatal conductance of the plant compared with non-inoculated plants. Notably, the consortium ameliorates the plant stomatal conductance up to 207 mmol/m^2 s, followed by the plant inoculated with rhizobia only up to 160 mmol/m^2 s compared with the positive control (129 mmol/m^2 s) and the negative control (98 mmol/m^2 s) (Supplementary Figure S1).

On the contrary, the results of the Chlorophyll Content Index (CCI) estimation showed a positive effect of the inoculation compared to non-inoculated plants. The index of chlorophyll content increased significantly in the faba leaves inoculated with the consortium. As recorded, the index of chlorophyll was about 54.8, followed by the plant inoculated with *R. laguerreae* (36.15) compared to the non-inoculated plants, the positive control (26.03), and the negative control (20.43) (Supplementary Figure S2).

3.4.2. Determination of minerals in plants

As summarized in Figure 9, inoculation impacts the content of some minerals (K and Ca) and P in the plants. The measurement of P in the faba-bean shows that the consortium enhances the P in the inoculated plants (0.224 ± 0.04 B mg/g) compared to the rhizobia (0.074 ± 0.043 C mg/g) and the negative control (0.074 ± 0.02 C mg/g). In addition, the measurement that the inoculation impacts the content of K and Ca. While the inoculation with bacterial inoculum influences the content of minerals, the plant inoculated with *R. laguerreae* had k = 60.49 ± 3.5b mg/gDM and Ca = 12.51 ± 0.05b mg/gDM and the plant inoculated with consortium had k = 31.69 ± 2.1b mg/gDM and Ca=5.59 ± 0.2c mg/gDM.

3.4.3. Dry weight determination

The inoculation of the faba-bean plants with rhizobia alone induced a slight increase in root biomass and shoot biomass compared to the non-inoculated negative control (T–) and positive control (T+) (Figure 10A). Meanwhile, an important increase in this parameter was noticed when the plants were

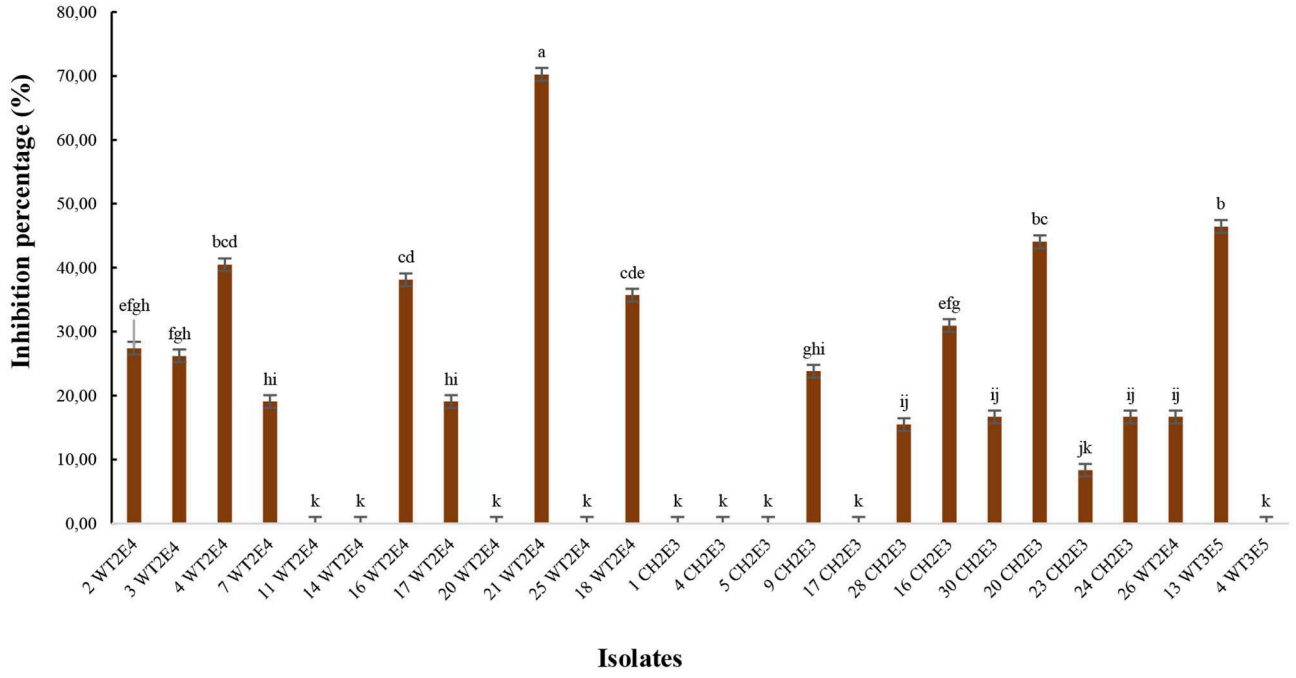

FIGURE 7
Antifungal effect of bacterial isolates against *F. oxysporum*. Different letters indicate that values are statistically different at a *P*-value of ≤0.05.

inoculated with the consortium compared to the other treatments (*R. laguerreae*, positive and negative controls). As shown in Figure 10B, the consortium significantly improved shoot biomass, and it induced an increase of 1.83 times more than the positive control and an increase of 1.29 times more than *R. laguerreae*.

In contrast, we noticed a slight increase of root biomass in plants inoculated with the consortium (0.77 g) compared to those inoculated with *R. laguerreae* (0.64 g) and with the positive control (0.65 g), respectively.

3.4.4. Roots morphology parameters

The observation and analysis of root morphological traits of the faba-bean plants submitted to different treatments were done using *WinRHIZO* (LA2400 scanner). The data collected showed the difference between the inoculated and the non-inoculated control plants. Based on the root architecture observation, the inoculation of plants improved root growth, especially in the plants inoculated with the consortium. The analysis of morphological traits revealed that the plants' inoculation with consortium shows a significant promoter effect on the total length of roots (Supplementary Figure S3). Similarly, inoculation of plants with these rhizobia including consortium significantly improved the surface area (Supplementary Figure S3A) and the length of the roots (Supplementary Figure S3B), compared to controls "T (+) 440 cm^2" and "T (−) 360 cm^2" for surface area and "1.33 mm" and "1.45 mm" compared to controls "T (+) 1.05 mm" and "T (−) 0.80 mm" for diameter, respectively.

3.4.5. Available P and acid phosphatase analysis

The inoculation of the faba-bean plants with the consortium or rhizobia induced an increase of released available P in the rhizosphere compared to the non-inoculated negative control (T−) (no nutrient solution added). We noticed that there was no significant difference between the rhizosphere of plants inoculated with rhizobia alone and those of plants inoculated with the consortium.

The analysis of acid phosphatase (APase) activity showed that the rhizospheric soil of plants inoculated with rhizobia alone showed an increase in APase activity compared with non-inoculated plants and exceeded 50 nmol/g/min (Figure 11). The soil of plants inoculated with rhizobia-including consortium showed the highest APase activity that exceeded 85 nmol/g/min, indicating the synergistic effect between PGPRs and rhizobia under low availability of P.

4. Discussion

The use of beneficial plant growth-promoting rhizobacteria for plant inoculation to enhance crop production, even under stress conditions, is an important practice for agriculture sustainability.

In the present study, rhizospheric soils were collected from the alfalfa plant grown on low P fertilized for the isolation of PGP rhizobacteria. In addition, *in vitro* assays and physiological characterizations were carried out to evaluate PGP traits. Based on the screening tests of PGP traits, including P solubilization, N fixation, and osmotolerance ability it allowed the obtention of a collection of 26 PGPR selected for further studies and molecular identification. The physiological traits were

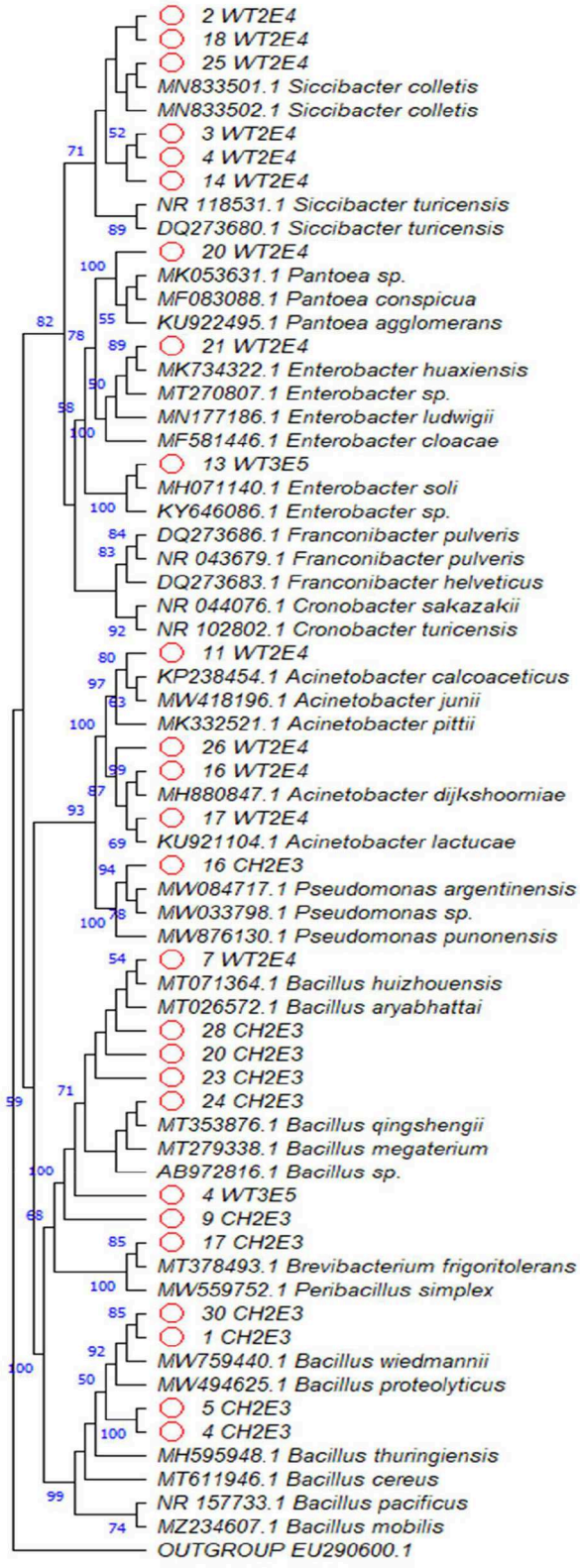

FIGURE 8
Phylogenetic tree based on the 16S rRNA gene sequences (consensus alignment) corresponding to selected PGPR from alfalfa rhizosphere. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model with 1,000 bootstrap replications. The percentage of trees in which the associated taxa clustered together are shown next to the branches. Evolutionary analyses were conducted in MEGA11.

chosen to establish a bacterial consortium based on specific biological activities to create complementarity among its members and act in a synergistic way for promoting plant growth under stress.

The most important biological activity focused on the rhizobacteria, as a PGP trait, is the inorganic P solubilization due to the importance of this nutrient for biological N fixation, plant growth, and productivity (Etesami and Maheshwari, 2018; Elhaissoufi et al., 2021). In this study, all the selected rhizobacteria solubilized the complex rock phosphate (RP) with important levels of released, available P that presented variation between isolates. Different studies identified several bacterial isolates that can promote plant growth, improve rhizosphere area, and solubilize different sources of immobilized P (Chen et al., 2006; Granada et al., 2018). In addition, as a complementary activity of P solubilization, there is auxin production. This phytohormone is responsible for the different stages of plant development (Jahn et al., 2021). In addition to their effect on the architecture and the development of the roots, the auxin also enhances nutrient absorption, especially P (Talboys et al., 2014). Our study demonstrated that the selected rhizobacteria produced the auxin-indole-3-acetic acid (IAA) with varying concentrations between them, and this result was reported for 80% of the tested bacteria by Gilbert et al. (2018). Nutrient availability in the soil is a crucial condition as is the availability of iron. For this objective, the PGPR isolates were tested for their ability to siderophores production. As chelates of insoluble iron, siderophores can have alternative functions such as non-iron metal transport, toxic metal sequestration, protection from oxidative stress, and antibiotic activity (Kramer et al., 2020). In our study, it was noted that the majority of the selected isolates from the alfalfa rhizosphere in arid areas showed siderophores production. This behavior may be related to the adaptation against stressing conditions prevailing in these poor soils and their pedological characteristics. Indeed, Nicolitch et al. (2016) confirmed the hypothesis of a variable selection of specific rhizosphere bacterial communities according to the soil conditions and the plant's nutritional requirements.

In contrast, among the isolated PGPR collection, 23.07% of the isolates produced highly ammoniac. In addition, from the 26 tested isolates, 21 isolates were able to tolerate salinity levels up to 10% NaCl and only 6 isolates tolerated it up to 15%. This can be an interesting PGP trait that impacts the growth of plants under extreme conditions of saline and/or dry areas. For this issue, the PGPR collection was also tested for its ability to resist water deficit, and our isolates grew in a medium containing 40% of PEG 8000. Our PGPR isolates were also tested for their antifungal activity and some of them showed antagonistic activity against *F. oxysporum*, isolated from field-infected faba-bean plants, and caused high inhibition that reached 70% of the mycelial growth.

Based on the interesting PGP traits and the test of antagonistic activity between the retained isolates and the rhizobia strain, a bacterial consortium was established. The three interesting rhizobacteria were selected as candidates for the PGPR rhizobia consortium and the inoculation test on the faba-bean plants.

The results of molecular studies, based on the 16S rRNA gene, demonstrated a diverse taxonomic structure, while 6 genera were identified, noting that *Bacillus* was the predominant genus (46.15%), followed by the *Siccibacter* (23.07%) and *Acinetobacter*

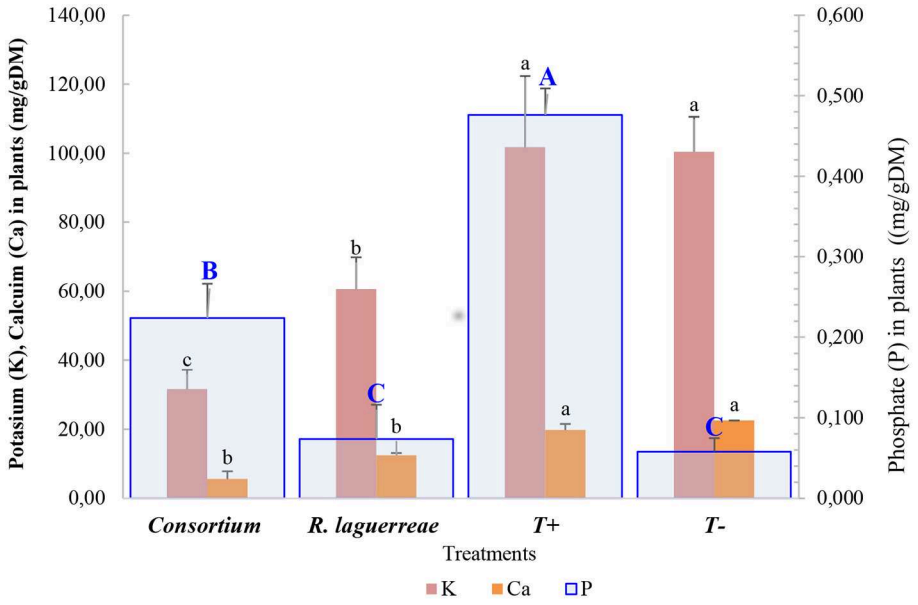

FIGURE 9
Minerals (K and Ca) and P content in the faba-bean plant in response to different treatments. For each element, different letters indicate that values are statistically different at a *P*-value of ≤0.05.

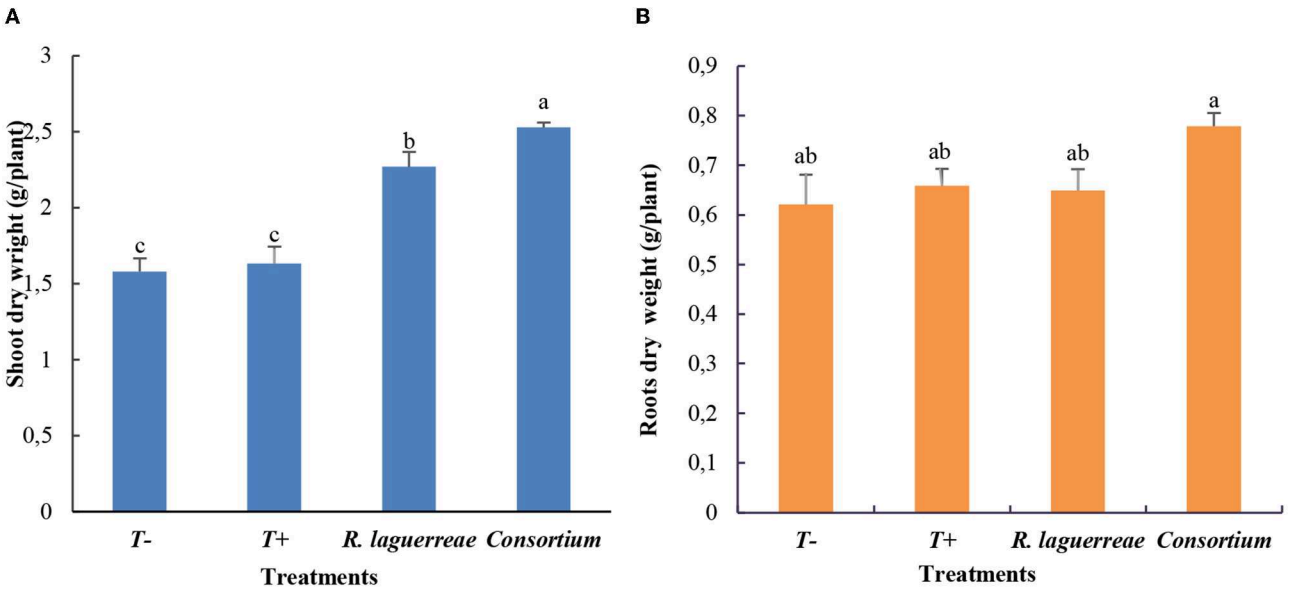

FIGURE 10
Root **(A)** and shoot **(B)** biomasses of faba-bean in response to different treatments. Each value represents the mean ± of three independent replicates; different letters indicate that values by Tukey's test are statistically different at a *P*-value of ≤0.05.

(15.38%) genera. The *Siccibacter* genus is a new phylum with some well-defined characteristics as described by Stephan et al. (2014). As revealed by our study, one of the retained PGPR members of the consortium (25WT2E4) is *Siccibacter colletis*, a species characterized by important PGPR traits such as P solubilization, N fixation, and its halotolerant. The first description of *Siccibacter colletis* sp. as a novel species was reported in 2015 and has been isolated from poppy seeds and tea leaves (Jackson et al., 2015). Up to the present time, the study of Salazar-Ramírez et al. (2021) reported the isolation of *Siccibacter colletis* from the candelilla rhizosphere (*Euphorbia antisyphilitica*). This is the first study to isolate *Siccibacter colletis* from the alfalfa rhizosphere and assess their PGP traits such as siderophores production, P solubilization, indole-3-acetic acid (IAA) production, and tolerance to water and salt stresses, all of which make them useful for enhancing crop production under stress. Thus, this species was included in our

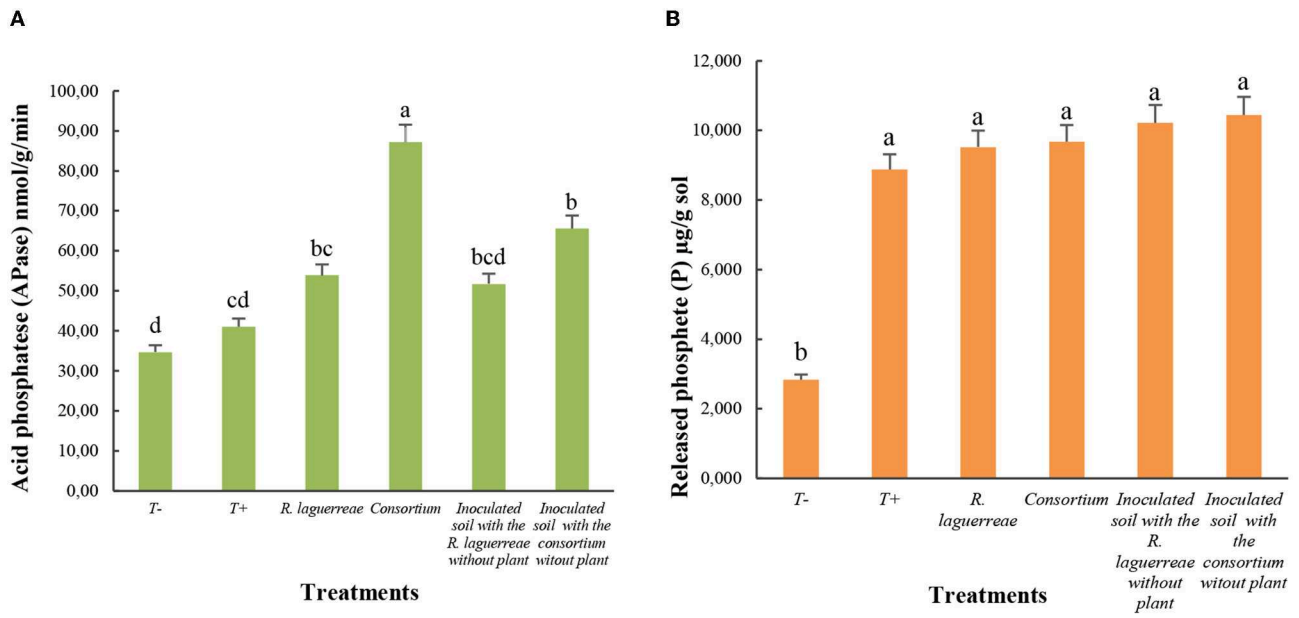

FIGURE 11
Available P **(A)** and acid phosphatase activity **(B)** and analysis in rhizospheric soil of plants inoculated with rhizobia alone or with the consortium. Each value represents the mean ± of three independent replicates; different letters indicate that the values by Tukey's test are statistically different at a *P*-value of ≤0.05.

established rhizobacterial consortium for inoculation. The second candidate, 20WT2E4, was identified as *Pantoea* sp. and the third one, 21WT2E4, was identified as *Enterobacter huaxiensis*. Recently, Ka-Ot and Joshi (Ka-Ot and Joshi, 2022) reported that the strain *E. huaxiensis* was able to be resistant to acidic conditions as well as Fe, Cd, and Cr and was able to remove 89%, 90%, and 82.45% of Fe, Cd, and Cr, respectively. Therefore, it could be used for the inoculation of plants in heavy metal-contaminated soils. However, the synergistic effect of more than one PGPR strain gathered as a consortium could be very interesting as a biofertilizer compared to as an individual strain. Eventually, the positive interaction between *Enterobacter* sp. Z1 and *Klebsiella* sp. Z2 exhibited great capacities for heterotrophic nitrification-aerobic denitrification (HNAD) and intracellular P accumulation. Strikingly, the co-cultured strains enhanced the removal efficiency of total N and P, with removal efficiencies of ammonia, nitrate, nitrite, and soluble P of 99.64%, 99.85%, 96.94%, and 66.7%, respectively (Zhang et al., 2019). In addition, the cooperation of the two strains belonging to the *Pantoea* and *Enterobacter* genera showed their potential for improving plant tolerance to stress and promoting maximum plant growth (PGP). It was found that they possess the *nifH* and *acdS* genes associated with N-fixation, ethylene production, and nitrogenase activity. The application of these strains to two sugarcane varieties increased several sugarcane physiological parameters, i.e., plant height, shoot weight, root weight, leaf area, chlorophyll content, and photosynthesis, in plants grown under greenhouse conditions (Singh et al., 2021). These findings support our choice to retain the three species, namely, *Siccibacter colletis*, *Pantoea* sp., and *Enterobacter huaxiensis*, for consortium establishment with *R. laguerreae* for the inoculation of the faba-bean plant under P deficiency. Such an inoculation increased the root parameters (weight, volume, diameter, length, and surface) and the shoot growth and other physiological parameters of the faba-bean inoculated plants under this nutrient constraint. Furthermore, the inoculation with this consortium increased the availability of inorganic P in the rhizosphere of inoculated plants. This P solubilization could be due to the production of organic acids, which are ubiquitous among rhizosphere P-solubilizing rhizobacteria in P-deficient soils (Elhaissoufi et al., 2021). Based on our knowledge, our study is the first report on faba-bean growth promotion by *Siccibacter colletis*, *Pantoea* sp., and *Enterobacter huaxiensis* as synergistic PGPRs with rhizobia in a consortium, highlighting their PGPR traits particularly under the P-limiting conditions. Our results also point to the suggested role that our selected PGPR-rhizobia consortium could play, besides the nutritional effects (N and P), in helping inoculated plants cope with other abiotic stresses such as salt stress and water deficit, which are the most prevailing stresses affecting legume crops in the Mediterranean basin and Africa.

5. Conclusion

The isolation of rhizobacteria from the *M. sativa* rhizosphere and their characterization allowed us to select a collection of 26 isolates with interesting PGP traits. Based on 16S rRNA gene sequencing, we identified eight different genera. In addition, the molecular characterization revealed for the first time the identification and isolation, from the *M. sativa* rhizosphere, of the *S. colletis* species and the identification of its interesting PGPR traits, and thus, it could be used for legume inoculation as a member of the rhizobia-including consortium. The results of the inoculation of faba-bean with the rhizobacterial consortium (*S. colletis*, *E. huaxiensis*, *Pantoea* sp., and *R. laguerreae*) under P

deficiency significantly improved the plant growth compared to non-inoculated controls or plants inoculated only with rhizobia, confirming thus the synergy between the PGPRs strains and the rhizobia for their beneficial effects for faba-bean inoculation under P deficiency conditions.

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

IC planned and designed the research, investigation, methodology, resources, software, visualization, and writing—original draft. JZ performed the isolates' molecular identification. AI performed soil analysis and conducted data analysis. SC performed plant analysis and conducted data analysis. RS conducted data analysis and prepared figures. JG performed project administration, conceptualization, and review and editing. YZ and LK performed funding acquisition, investigation, and project administration. AB conducted investigation and project administration. CG performed conceptualization, project administration, funding acquisition, supervision, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1134809/full#supplementary-material

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Inoculation with rhizobacterial consortia alleviates combined water and phosphorus deficit stress in intercropped faba bean and wheat

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Our study aimed to assess the role of inoculation of faba bean/wheat intercrops with selected rhizobacterial consortia (composed of one rhizobium and two P solubilizing bacteria "PSB") to alleviate the effects of combined water deficit and P limitation on faba bean/wheat intercropping vs. monocropping under greenhouse conditions. One *Vicia faba* L (Aguadulce) and one *Triticum durum* L. variety (Karim) were grown as a sole crop or were intercropped in pots containing a sterilized substrate (sand:peat 4:1 v/v) with either rock phosphate (RP) (unavailable P) or KH_2PO_4 in the nutrient solution (available P). Plant inoculation was performed using the rhizobacterial consortia C1 (*Rhizobium laguerreae*, *Kocuria* sp., and *Pseudomonas* sp.) and C2 (*R. laguerreae*, *Rahnella* sp., and *Kocuria* sp.). Two weeks after inoculation, the plants were subjected to water deficit with 40% substrate water holding capacity (WHC) vs. 80% WHC for the well-watered plants. The trial was assessed at the flowering stage, and the results showed that inoculation with both consortia (C1 and C2) improved faba bean biomass in terms of shoot, root, and nodules dry weight compared to inoculation with rhizobia alone. C2 improved these parameters by 19.03, 78.99, and 72.73%, respectively. The relative leaf water content decreased under combined stress, especially in response to C1 conferring significant improvement of this parameter in wheat intercrops. In faba bean under P limitation, inoculation with C2 increased stomatal conductance (gs), phosphatase, and phytase activity by 35.73, 166.94, and 26.16%, respectively, compared to plants inoculated with rhizobia alone. Furthermore, C2 also improved membrane stability under P deficit by 44.33 vs. 16.16% for C1 as compared to inoculation with rhizobia alone. In sole-cropped faba bean, inoculation with both consortia improved N accumulation compared to single inoculation with an increase of 70.75% under P limitation. Moreover, under combined stress, inoculation with C2 improved biomass and N content (112.98%) in intercropped wheat compared to the sole crop. Our findings revealed that consortium C2 might

offer an agronomic advantage under water and P deficit and could serve as a useful inoculum for enhancing faba bean and wheat production in monocropping and intercropping systems.

KEYWORDS

drought, phosphorus, cropping, PBS, rhizobia, *Triticum durum*, *Vicia faba*

1. Introduction

By the middle of the 21st century, the human population will pass the nine billion mark and thus put high pressure on food security (Gerland et al., 2014). Nitrogen (N) and phosphorus (P) are essential nutrients in crop production systems. They control plant growth, development, and yield due to their important role in many physiological processes such as signal transduction, respiration, photosynthesis, and energy transduction (Turuko and Mohammed, 2014; Meena et al., 2017). For legumes, P is an essential macronutrient for efficient nodulation and biological N fixation (BNF). Indeed, almost 20% of the P taken up by plants is allocated to nodules to ensure optimal N fixation (Mandri et al., 2012). It was also reported that part of the P applied as fertilizer is either assimilated by soil microbes or complexed by cations such as aluminum (Al), iron (Fe), calcium (Ca), or magnesium (Mg), and only 15–30% of the fertilizer is used by the plants, which reduces the use efficiency of P fertilizers (Sharma et al., 2013). Unfortunately, Morocco has suffered from water limitation in the last decade (Benabdelouahab et al., 2019), and many soil types in dry areas present low available P, which have caused double stress in crops combining water deficit and P limitation.

Furthermore, Morocco is known for its large reservoirs of rock phosphate (RP), which represents an important source of mineral phosphate fertilizers. A main research goal is the optimization of P solubilization to make it more available to plants (Hamdali et al., 2008; Chang and Yang, 2009; Park et al., 2011). The application of RP seems to be an affordable and environment-friendly solution to replace chemical P fertilizers. Additionally, the application of organic fertilizers often did not show the same positive effects as mineral fertilizers (Abbasi and Manzoor, 2018). The utilization of phosphate-solubilizing bacteria (PSB) in combination with RP could increase the level of plant-available P in soils (Wahid et al., 2016).

One solution to enhance plant growth under stressful conditions is to use beneficial plant growth-promoting rhizobacteria (PGPR) capable of mobilizing different forms of P in soils (Shilev, 2020), particularly when plants are co-inoculated with synergistic consortia of strains (Kumar et al., 2017). Furthermore, the cropping system could also affect fertilizer efficiency. Intercropping is defined as growing two or more crops on the same piece of land at the same time (Nasar et al., 2020) to maximize the use of nutrient resources and enhance plant production with rational nutrient inputs (Bargaz et al., 2017). The positive effect of intercropping is generally related to below-ground complementarity or facilitation phenomena between two crop species (Li et al., 2014). Complementarity leads to lowering plant species competition, which may be related to the use of different pools of nutrients unavailable to the associated crop, or to different root architectures that can explore different soil horizons (Bechtaoui et al., 2019; Chamkhi et al., 2022). In addition to the complementarity, the facilitation effect is manifested when a plant makes an unavailable resource available to the other intercrop plant. In this context, the legume facilitation effect toward cereals can be regarded as the partial supply of symbiotically fixed N and available P by the production of protons, organic acids, or phosphatases (Hinsinger, 2001; Li et al., 2008; Betencourt et al., 2012).

The effects of the most prevailing abiotic stresses on legume crops in Morocco, such as drought and P limitation (Bargaz et al., 2012; Mouradi et al., 2015, 2018; Kabbadj et al., 2017), and on intercropping legume/cereal systems (Bargaz et al., 2017; Mouradi et al., 2018) were studied separately. However, only a few studies have considered inoculation with PGPR consortia including rhizobia to enhance legume/cereal growth and production under stressful conditions. This could be an environment-friendly alternative to enhance plant nutrition under abiotic stress. Our research work was based on the hypothesis that inoculation of faba bean/wheat with rhizobia-containing PSB consortia enhances the tolerance of these crops to drought and P limitation and intercropping will improve wheat performance under these conditions. Our research approach included the assessment of the impact of inoculation with rhizobacterial consortia containing multifunctional species, notably rhizobia (for BNF) and two rhizobacteria (for P solubilization and other PGP traits), expecting that this biotechnological measure will allow for plant–microbe and plant–plant beneficial interactions to alleviate combined stress on inter- and sole crops.

2. Material and methods

2.1. Biological material

A randomized block design was carried out using *Vicia faba L.* variety Aguadulce (Ag) characterized as tolerant to water deficit (Kabbadj et al., 2017), and *Triticum durum* variety Karim (K). These varieties are commonly grown by farmers in the Haouz area (Hadria et al., 2007; Oukaltouma et al., 2021).

The bacterial inocula consisted of *Rhizobium laguerreae* and other rhizobacteria (*Kocuria* sp., *Pseudomonas* sp., and *Rahnella* sp.) presenting high P solubilization capacity and at least one PGP trait. These rhizobacteria were isolated from the nodules of faba bean collected from two different sites of Marrakech-Al

Haouz region *Rahnella* sp. from Sidi Ghiat (latitude 31°28′20.0″N, longitude 7°46′31.9″W) and *Rhizobium laguerreae.*, *Kocuria* sp., and *Pseudomonas* sp. from Souihla (latitude 31°40′30.1″N, longitude 8°12′17.8″W). These strains were characterized as tolerant to a wide range of pH and temperature and high salinity levels and were endowed with a high capacity to solubilize tricalcium phosphate. They were also characterized for not being antagonistic to each other, which allowed for the composition of two consortia, C1 (*R.* sp., *Kocuria* sp., *Pseudomonas* sp.) and C2 (*R. laguerreae*, *Rahnella* sp., and *Kocuria* sp.), for plant inoculation under greenhouse conditions.

2.2. Bacterial inoculum production and seeds germination

The inoculum was produced separately in a liquid yeast extract mannitol (YEM) for each bacterial species after incubation for 3 days at 28°C under agitation. The bacterial cultures were then centrifuged at 13,000 rpm for 10 min, and the inoculum was prepared by equally mixing the three bacteria for each consortium.

Faba bean and wheat seeds were first surface-disinfected by immersion in 6% sodium hypochlorite for 10 min before they were rinsed five times with sterile distilled water. Faba bean seeds were germinated in sterilized sand for 5 days. The inoculation was performed by soaking seedling roots in the inoculum solution for 20 min and transplanting them into pots (diameter: 16.5 cm; height: 20 cm) presenting in their bottom two draining holes and containing 2.2 kg of substrate consisting of a mixture of sterilized sand and peat at a ratio of 4:1. The sand was sterilized for 3 h at 180°C for three cycles, and peat was autoclaved 1 h at 121°C and 2 bar of pressure three times. The substrate was supplemented with 800 mg/kg of ground rock phosphate as the only source of mineral P. To ensure proper inoculation of faba bean plants, each one received another 5 ml of inoculum at transplantation. Sterilized wheat seeds were soaked in inoculum solution for 20 min and were sown on the same substrate, and 3 ml of inoculum solution was added per seed. A second inoculation was applied by drenching the seedlings 1 week after transplantation. For the sole crop, two plants of faba bean and six plants of wheat were grown per pot. For intercropping, one faba bean plant and three wheat seedlings were grown in the same pot.

The experiment was performed in a greenhouse with a day/night temperature of 25/20°C, an approximate relative humidity of 60–70%, and a 16-h photoperiod with a light intensity of 11.3 Klux.

At 2 weeks after sowing, water deficit was applied by maintaining the pot substrate at 40% WHC for the stressed plants vs. 80% WHC for non-water-stressed (Kabbadj et al., 2017; Oukaltouma et al., 2021). For P limitation, RP was used as the only mineral P source at a rate of 800 mg/Kg substrate. For positive control, plants were irrigated with Hoagland nutrient solution containing 125 μmol/l of KH_2PO_4 and 46 mg/l of NKO_3, and for the negative control (Rh + RP), the substrate was supplemented with RP and plants were inoculated only with rhizobia instead of using KNO_3. For the P limitation treatment (RP, 80% WHC), the substrate containing RP was maintained at 80% WHC and for the stress combining P limitation and water deficit (RP, 40% WHC), the substrate was maintained at 40% WHC. The plants were irrigated once a week with an N-free Hoagland nutrient solution. The plants were stressed for 40 days during which physiological parameters were assessed *in situ*. Afterward, plants were harvested for growth and biochemical assessment. Five replicated pots per treatment were considered.

2.3. Dry biomass measurement

At the flowering stage of faba bean and the appearance of wheat spikes, which corresponded to 60 days after sowing, plants were harvested. Shoots were separated from the roots including rhizosphere soil and nodules were carefully detached from the roots. The three plant parts were washed and dried at 70°C for 72 h, and the dry weights (DW) were determined by weighing the plant tissues for each treatment.

2.4. Stomatal conductance

Stomatal conductance (gs) was measured on the second fully expanded and healthy leaf. The measures were taken at noon under 28 ± 2°C and 60 ± 4% of relative humidity with a porometer (SC1 Model, Decagon Devices, version 2012).

2.5. Leaf relative water content

According to Ghoulam et al. (2002), relative water content (RWC) was determined in well-developed leaves (flag leaves) from three plants per treatment and plant species. Fresh foliar disks of faba bean and wheat were sampled and weighed to determine their fresh weight (FW) and then immersed in distilled water for 6 h to reach full turgidity. Turgid weight (TW) was determined after wiping the surface of the leaf disks. Then, the samples were dried for 24 h at 70°C and their dry weights (DW) were determined. RWC was defined as follows:

$$RWC\,(\%) = \frac{FW - DW}{TW - DW} * 100$$

2.6. Leaf water potential

Leaf water potential (LWP) was measured at noon on leaves of the same level using a pressure chamber (PMS Instrument Co, Model 600, USA). This measurement was repeated three times per treatment.

2.7. Leaf area

At the flowering stage of the faba bean, the development of wheat spike leaf area (cm^2) was determined on three plants per treatment and three leaves per plant using the "Mesurim version 3.4.4.0" software.

2.8. Proline content

The determination of the plant proline content was carried out following the method of Bates et al. (1973), based on the interaction of proline with ninhydrin, which forms a colored complex. Samples of 100 mg of fresh material (faba bean or wheat leaves) were ground in 2 ml of 40% methanol and centrifuged at 5,000 rpm for 20 min. To 1 ml of the supernatant, 1 ml of a mixture of glacial acetic acid and 6 M orthophosphoric acid (3:2 v/v) and 25 mg of ninhydrin were added. Subsequently, the tubes were incubated in a water bath for 1 h at 100°C to allow the formation of the colored complex that was extracted by adding 3 ml of toluene. The solution was stirred for 5 min. The optical density was measured at 520 nm. Proline contents were determined using a standard curve established with known concentrations of proline.

2.9. Chlorophyll "a" fluorescence

Measurement of chlorophyll "a" fluorescence was performed by using a portable fluorometer (plant efficiency analyzer, Hansatech Instruments Ltd.). Before the *in situ* measurement, leaves were covered with black leaf clips to mimic at least 15 min under dark conditions. The maximum quantum yield (Fv/Fm) was used as the chlorophyll "a" fluorescence-derived parameter. The differential curves were obtained by subtracting the curve of samples of the control plants from the curve of samples of plants that received different treatments.

2.10. Electrolyte leakage

To determine the electrolyte leakage (EL), the method described by Ghoulam et al. (2002) was used. Samples of five disks of 1 cm diameter from faba bean leaves and 50 mg from wheat leaves were rinsed three times with distilled water to remove minerals from the surfaces of the disks. Afterward, the samples were collected in tubes containing 10 ml of deionized water and incubated for 24 h under shaking at 25°C. Subsequently, the initial electrical conductivity (L0) of each sample was determined at 25°C with a conductometer. After autoclaving the samples for 20 min at 120°C followed by cooling for 30 min at 25°C under agitation, the total electrical conductivity (Lt) of the samples was determined. The electrolyte leakage was determined by the formula:

$$Electolyte\ leakage\ (\%) = \frac{L0}{Lt} * 100$$

2.11. Acid phosphatase activity in nodules

Nodule APase activity was determined according to the method described by Araújo et al. (2008). Fresh nodules (100 mg) were homogenized in 500 µL of sodium acetate buffer (0.1 M, pH 5.5) containing 2.2% of polyvinylpyrrolidinone (PVP) and 5 µL of beta-mercaptoethanol. After 15 min of centrifugation at 12,000 ×g, 100 µL of the obtained supernatant was added to 200 µL of p-nitro-phenyl phosphate (pNPP) and the mixture was incubated for 30 min at 38°C. The reaction was stopped by adding 1 mL of 1 N NaOH and the OD was recorded at 410 nm. The p-nitrophenol (enzyme substrate) concentration was determined by reference to the standard curve.

2.12. Phytase activity in nodules

Phytase activity was determined by mixing 200 µL of 10 mM phytic acid with 100 µL of nodule enzymatic extracts according to Eeckhout and Paepe (1994). The reaction was maintained at 37°C and stopped after 90 min by adding 1 mL of 10% TCA (trichloroacetic acid). For each sample, a control was prepared by immediately adding 1 mL of 10% TCA to the reaction medium containing phytic acid at t_0. The reaction media were centrifuged at 12,000 ×*g* for 5 min. The concentration of Pi in the extract was determined by colorimetry using sodium molybdate and hydrazine sulfate. The phytase activity was defined as the difference between the Pi in the extract and its corresponding blank sample and expressed in µmol Pi min^{-1} g^{-1} FM.

2.13. Determination of shoot nutrient contents

Dried wheat and faba bean plants (80°C for 3 days) were finely ground for total N, P, and K contents analyses. The wheat and faba bean plant powders were digested using nitric acid and analyzed for P and K contents using inductively coupled plasma optical emission spectrometry (Agilent 5110 ICP-OES, USA). The total N content was determined by the Kjeldahl method (KjelMaster K-375, Netherlands).

2.14. Statistical analysis

The statistical analysis was carried out using IBM® SPSS® Statistics V. 20 software. A multivariate analysis of variance was used followed by the Tukey *post-hoc* test to determine the significant difference between the means of the treatments at the $p < 0.05$ significance level. All tested parameters and their correlation with treatments were subjected to principal component analyses (PCAs) using the same software.

3. Results

3.1. Plant growth and nodulation

Inoculation with either consortium (C1 or C2) and intercropping significantly (Supplementary Table S1) affected shoot dry weight (SDW), root dry weight (RDW), and nodule dry weight (NDW) under combined stress. The application of both consortia improved faba bean shoot dry weights for both cropping systems. In particular, plants inoculated with C2 and

supplied with RP showed the highest increase rate of 53.59 vs. 30.56% C1 compared to inoculation with rhizobia alone (Table 1). Plants inoculated with C2 under the same condition showed the highest SDW (2.89 g plant^{-1}) for the sole crop. Intercropping reduced legume SDW no matter which inoculant was applied (Rh, C1, or C2) with the highest reduction rate of 40.48%, when plants were inoculated with C2 under combined stress (Table 1). In wheat under combined stress, inoculation with both consortia and intercropping significantly affected SDW and RDW ($p < 0.001$) (Supplementary Table S2). Under P limitation, the highest value for SDW (2.48 g plant^{-1}) was recorded in plants inoculated with C2 with an increase of 51.22% relative to the corresponding sole crop treatment under the same conditions (Table 1). We observed that under combined stress and sole crop, the application of C2 improved wheat SDW by 16.83% compared to plants inoculated with C1.

C2 significantly (Supplementary Table S1) improved faba bean RDW under P deficiency compared to the application of rhizobia alone, with an increase of 78.99% (Table 1). Combined stress reduced RDW in both cropping systems, particularly in plants inoculated with C1 compared to P limitation. P deficiency reduced RDW in wheat and combined stress reduced this parameter for all treatments compared to the controls, while intercropping increased RDW in wheat compared to the sole crop for both consortia under both stresses. This improvement was noticeable under the P limitation with an increase of 176.74% for plants inoculated with C2 compared to their corresponding plants grown as a sole crop (Table 1).

Inoculation with C2 significantly improved faba bean NDW by 36% in the intercropping treatments and by 72.73% in the sole crop (Supplementary Table S1), compared to P limitation and inoculation with rhizobia alone (Table 1). Moreover, intercropping and inoculation with C2 improved NDW by 80% compared to sole crops under combined stress.

3.2. Stomatal conductance

Among all treatments subjected to water deficit and P limitation, inoculation with both consortia and cropping system significantly ($p < 0.001$) (Supplementary Table S1) affected faba bean stomatal conductance. Figure 1A shows that under P limitation, faba bean "gs" was improved by inoculation with both consortia compared to inoculation with rhizobia alone, either for sole crop or intercropping. Particularly, faba bean inoculated with C2 presented a "gs" increase of 35.73% compared to plants under P limitation inoculated with rhizobia alone. Furthermore, plants inoculated with C2 presented the highest value of conductance of 139.02 mmol H_2O m^{-2} s^{-1}. Combined stress significantly reduced "gs" in plants inoculated with both consortia and those inoculated with C2 presented the highest "gs" value of 86.73 mmol H_2O m^{-2} s^{-1}. Intercropping reduced the "gs" of faba bean compared to sole crop in all treatments except for plants inoculated with C2 and the controls.

Statistical analyses revealed that combined stress in wheat and inoculation with both consortia, as well as intercropping, significantly affected "gs" ($p < 0.001$) (Supplementary Table S2). Intercropping improved "gs" under P limitation but did not affect it

TABLE 1 Faba bean and wheat dry biomasses (SDW, shoot dry weight; RDW, root dry weight; NDW, nodule dry weight) grown as a sole crop or intercropped (FSC, Faba bean sole crop; FIC, faba bean intercropped; WSC, wheat sole crop; WIC, wheat intercropped) under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) inoculated with two rhizobacterial consortia C1 and C2.

		SDW (g plant^{-1})				RDW (g plant^{-1})				NDW (g plant^{-1})	
		FSC	FIC	WSC	WIC	FSC	FIC	WSC	WIC	FSC	FIC
80% WHC	Control	6.56 ± 0.13^{a}	5.4 ± 0.15^{bc}	2.79 ± 0.07^{ab}	3.28 ± 0.16^{a}	3.22 ± 0.18^{a}	2.09 ± 0.09^{bc}	1.90 ± 0.23^{bc}	2.77 ± 0.09^{a}	*	*
	RH+RP	4.73 ± 0.30^{bc}	3.37 ± 0.19^{d}	*	*	1.38 ± 0.25^{de}	1.38 ± 0.09^{de}	*	*	0.11 ± 0.02^{bcd}	0.14 ± 0.01^{abc}
	C1+RP	5.03 ± 0.17^{bc}	4.40 ± 0.19^{c}	1.6 ± 0.03^{cd}	2.22 ± 0.23^{bc}	1.69 ± 0.05^{cde}	1.80 ± 0.05^{cd}	0.98 ± 0.07^{def}	1.60 ± 0.05^{cd}	0.14 ± 0.001^{abc}	0.14 ± 0.01^{abc}
	C2+RP	5.63 ± 0.24^{ab}	5.21 ± 0.36^{c}	1.64 ± 0.12^{cd}	2.48 ± 0.15^{b}	2.47 ± 0.18^{b}	1.84 ± 0.07^{cd}	0.86 ± 0.03^{def}	2.38 ± 0.24^{ab}	0.19 ± 0.001^{a}	0.19 ± 0.02^{a}
40% WHC	C1+RP	2.34 ± 0.06^{ef}	2.24 ± 0.09^{ef}	1.01 ± 0.05^{ef}	1.74 ± 0.09^{cd}	1.41 ± 0.04^{de}	1.19 ± 0.04^{e}	0.46 ± 0.02^{f}	1.05 ± 0.08^{def}	0.07 ± 0.003^{d}	0.05 ± 0.01^{de}
	C2+RP	2.89 ± 0.06^{de}	1.72 ± 0.22^{f}	1.18 ± 0.015^{de}	1.75 ± 0.06^{cd}	1.51 ± 0.04^{cde}	1.46 ± 0.10^{de}	0.83 ± 0.08^{ef}	1.44 ± 0.30^{cde}	0.10 ± 0.03^{cd}	0.18 ± 0.01^{ab}

The positive controls were treated with 125 µmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls were supplemented with RP and inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates. Values with different letters are significantly different at 0.05 level of probability by Student-Newman- Keuls. *It means that there is no data available in this case.

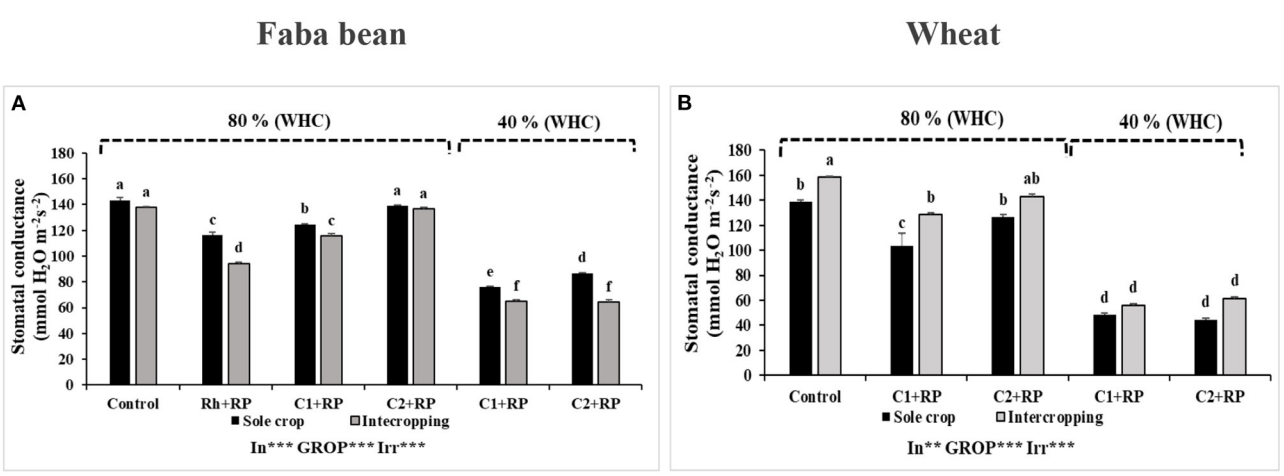

FIGURE 1
Stomatal conductance of faba bean **(A)** and wheat **(B)** grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 μmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls.
*Represent the significance level of treatments.

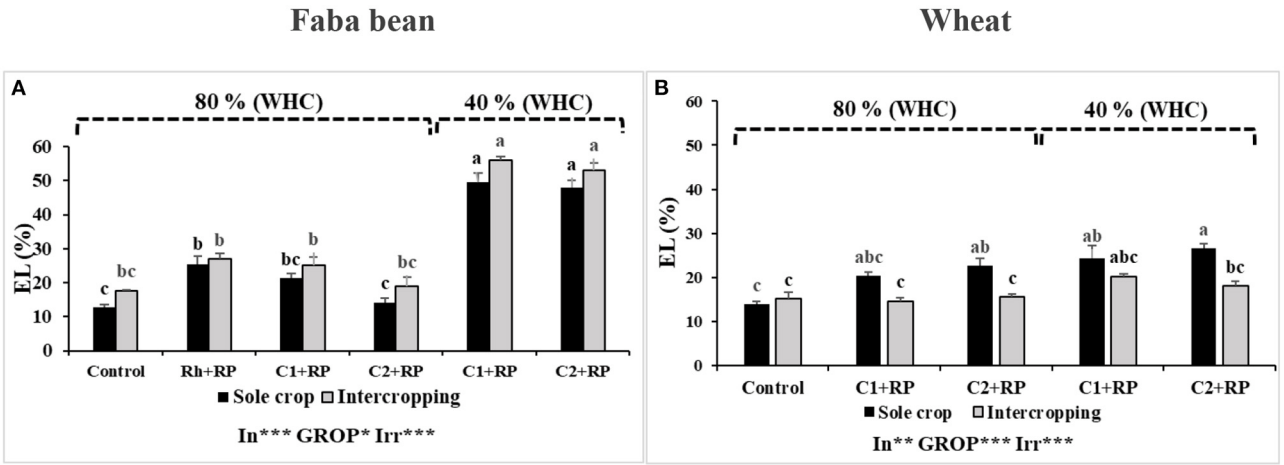

FIGURE 2
Electrolyte leakage in faba bean **(A)** and wheat **(B)** grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 μmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls.
*Represent the significance level of treatments.

under combined stress, no matter which inoculant was (C1 or C2) used (Figure 1B).

3.3. Electrolyte leakage

Measurement of electrolyte leakage (EL) revealed a significant effect ($p < 0.001$) in response to combined stress, inoculation with both consortia, and in the intercropping system ($p < 0.05$). For the faba bean (Figure 2A), EL was reduced when plants were inoculated with both consortia compared to inoculation with rhizobia alone. This reduction was more pronounced when plants were inoculated with C2 in the sole crop, with a reduction of 44.33% compared to 16.16% when plants were inoculated with C1, relative to inoculation with rhizobia alone under

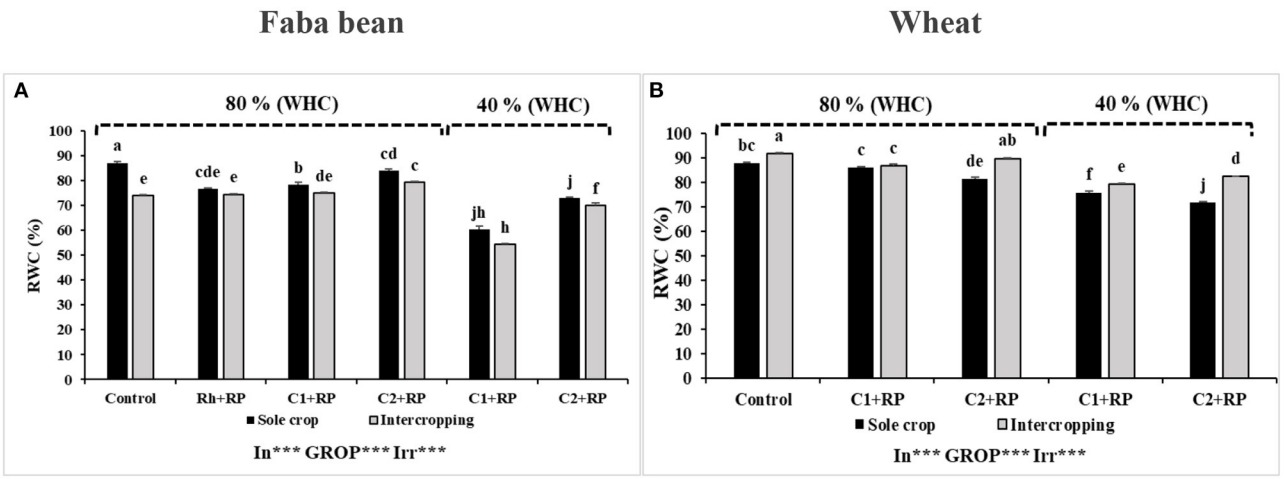

FIGURE 3
Relative water content in faba bean **(A)** and wheat **(B)** grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 μmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls.
*Represent the significance level of treatments.

P limitation. The combined stress highly increased the EL, with the highest value detected in plants inoculated with C1 (53.10%).

For water deficit and P limitation in wheat, inoculation with both consortia and intercropping significantly affected EL ($p < 0.05$). Figure 2B shows that P deficiency increased the EL values, compared to the controls, no matter which consortium was used (C1 or C2). The combined stress did not induce any additional increase in EL, compared to P deficiency alone. Intercropping reduced EL significantly, particularly when plants were inoculated with C2, as compared with the sole crop.

3.4. Relative water content

Combined stress, inoculation, and cropping system showed significant effects ($p < 0.001$) on RWC under P limitation. The combined stress reduced RWC in sole-cropped plants inoculated with C1 or C2, relative to their corresponding plants under P limitation. The decrease was more pronounced in plants inoculated with C1 (11.81 and 8.6%, respectively) (Figure 3A). In general, intercropping with wheat decreased RWC for faba bean with the lowest value recorded for plants inoculated with C1 under combined stress (54.26%).

Considering wheat, statistical analyses revealed significant effects ($p < 0.05$) of combined stress, inoculation, and cropping system on RWC. For sole-cropped wheat plants, the combined stress induced a decrease of RWC that was more evident in plants inoculated with C2 compared to the control (Figure 3B). The intercropping increased RWC for most treatments, and the highest increase was achieved with plants inoculated with C2 (15.06%) compared to sole-cropped plants under combined stress.

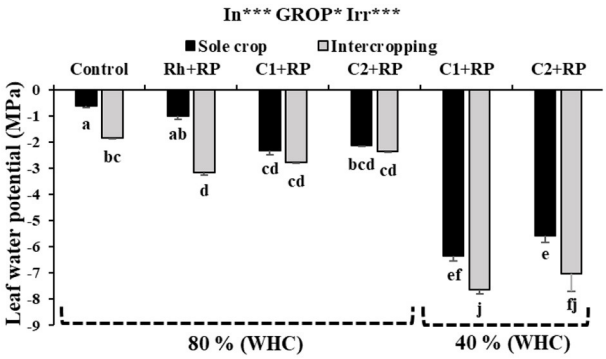

FIGURE 4
Leaf water potential in faba bean grown as a sole crop or intercropped with wheat under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 μmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls.
*Represent the significance level of treatments.

3.5. Leaf water potential

Under the combined stress and intercropping system, inoculation with both consortia significantly affected LWP ($p < 0.001$) in faba bean plants (Figure 4). P limitation induced a decrease of LWP in sole-cropped faba bean inoculated with C1 or C2, compared to those inoculated with rhizobia only. The decrease

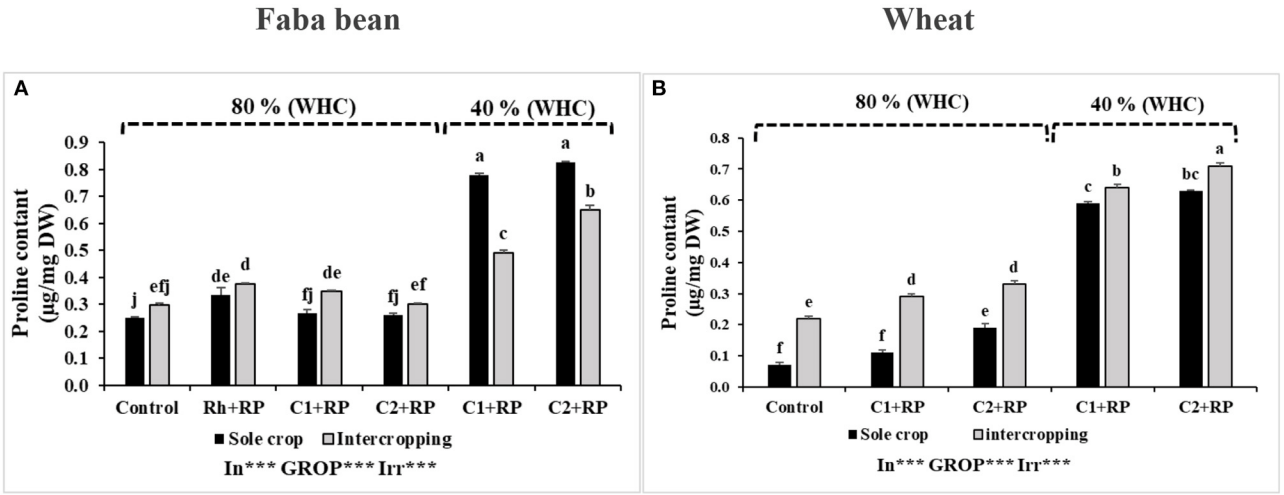

FIGURE 5
Proline content in faba bean **(A)** and wheat **(B)** grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 µmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls.
*Represent the significance level of treatments.

amounted to 133.33% compared to inoculation with rhizobia alone or when plants were inoculated with C1 (Figure 5). The combined stress together with intercropping decreased LWP, no matter which inoculum was used (C1 or C2). The plants inoculated with C1 showed the lowest value of −7.65 MPa.

3.6. Proline content

Statistical analysis detected significant effects ($p < 0.001$) of combined stress, inoculation, and intercropping on the plant proline content. The result shown in Figure 5A documents the accumulation of proline in faba bean plants. We noticed that proline was more enriched under combined stress than under P limitation alone, for both inocula (C1 or C2). Under combined stress, the highest proline value was observed in faba bean inoculated with C2 (0.83 µg/mg DW). Intercropping reduced proline accumulation under combined stress and the reduction become obvious when plants were inoculated with C1, as compared to C2, with reduced rates of 37.18 and 21.69%, respectively, compared to the sole crop.

Statistical analyses discovered significant effects of the combined stress on proline content ($p < 0.001$) in wheat, as well as in inoculation and cropping systems. Under P limitation, inoculation with C2 induced an increase of proline in both cropping systems, compared to inoculation with C1 and the positive control, with the highest value (0.33 µg/mg DW) observed in intercropped plants inoculated with C2 (Figure 5B). Under combined stress, we noticed a high accumulation of proline in plants inoculated with both consortia. Moreover, intercropped wheat plants showed an additional increase compared to their corresponding sole-cropped plants, and the highest value was recorded in plants inoculated with C2 (0.71 µg/mg DW).

3.7. Leaf area

Combined stress and inoculation significantly ($p < 0.01$) affected the leaf area of faba bean plants. The highest value was recorded for the positive control in the sole crop treatments (25.82 cm^2). However, under P limitation, it was significantly reduced, compared to the controls, and when plants were inoculated with rhizobia alone or C1, sole-cropped plants inoculated with C2 did not show any significant reduction (Table 2). Combined stress highly reduced the leaf area compared to the control and P limitation treatments with the highest value recorded for plants inoculated with C2 (14.80 cm^2). Intercropping did not significantly affect this parameter, no matter which stress or consortium was applied.

Combined stress and inoculation with both consortia significantly affected ($p < 0.05$) the leaf area in wheat (Table 2), except for the control, which showed an increase in leaf area. Intercropping did not show a significant variation in this parameter for all remaining treatments.

3.8. Chlorophyll "a" fluorescence

The combined effect of P limitation, water deficit, and inoculation with both consortia was studied through the analysis of the dark recovery kinetics curves of the Chl "a" fluorescent transient (OJIP-transient). All treatment curves showed a normal

TABLE 2 Leaf area of faba bean and wheat grown as a sole crop or intercropped (FSC, Faba bean sole crop; FIC, faba bean intercropped; WSC, wheat sole crop; WIC, wheat intercropped) under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs.

		Leaf area (cm^2)			
		FSC	FIC	WSC	WIC
80% WHC	Control	25.82 ± 1.51^{a}	21.14 ± 2.72^{abc}	20.29 ± 1.84^{bc}	22.12 ± 0.13^{a}
	RH + RP	19.37 ± 0.25^{bc}	16.46 ± 1.42^{bcde}	*	*
	C1 + RP	19.19 ± 0.44^{bc}	17.34 ± 0.55^{bcd}	15.54 ± 0.62^{bcd}	17.34 ± 0.55^{abc}
	C2 + RP	22.07 ± 1.23^{ab}	19.99 ± 0.93^{abc}	17.19 ± 1.29^{abc}	19.99 ± 0.93^{bc}
40% WHC	C1 + RP	12.42 ± 1.16^{de}	10.29 ± 0.29^{e}	10.43 ± 0.51^{e}	10.29 ± 0.29^{de}
	C2 + RP	14.8 ± 0.68^{cde}	11.45 ± 1.58^{de}	10.73 ± 1.46^{e}	11.45 ± 1.58^{cde}

The positive controls were supplemented with 125 µmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates. Values with different letters are significantly different at 0.05 level of probability by Student-Newman- Keuls. *It means that there is no data available in this case.

distribution of OJIP transients, which refers to the reduction phase of the electron chain transporters. For the sole faba bean (Figure 6A), the results of Chl "a" fluorescence presented a difference in the shape of all transition states (O, J, I, and P). In general, we observed that all treatments under sole crop showed differences in all transition states compared to the control treatments. The J-step and P-step showed the highest amplitude by inoculation with C1 compared to inoculation with C2 and rhizobia alone. However, for the I-step, inoculation with C2 presented the highest amplitude compared to all other treatments. For intercropped plants, the highest amplitude of all transition states was detected in inoculation with C2 compared to the other treatments (Figure 6B). In sole-cropped wheat (Figure 6C), inoculation with C1 presented the highest amplitude compared to the other treatments, while intercropped plants inoculated with C2 presented the highest amplitude between all steps when compared to inoculation with rhizobia alone and C1. This difference was most pronounced in the I-step (Figure 6D).

3.9. Acid phosphatase and phytase activities in nodules

Statistical analyses revealed significant effects of combined stress and inoculation on APase and phytase activities in the nodules of faba bean roots ($p < 0.001$). The cropping system did not affect these two parameters significantly. For APase, under P limitation, the inoculation with consortium C2 improved APase activity compared to inoculation with consortium C1 and inoculation with rhizobia alone (Figure 7A). This improvement was significant under sole crop with the highest increase of 66.92% compared to inoculation with rhizobia alone. Under combined stress, inoculation with C2 improved APase activity with the highest value of (158.43 µmol pNP min^{-1} g FM^{-1}) which was observed for faba bean in the sole crop inoculated with C2. Under P limitation, intercropping did not affect this activity but reduced it under combined stress for plants inoculated with C2.

The results revealed that P limitation and inoculation with both consortia did not affect phytase activity for sole crops. Plants inoculated with C2 presented the highest phytase activity of 217.98 µmol Pi min^{-1} g FM^{-1} (Figure 7B). Combined stress reduced phytase activity in nodules of plants inoculated with C1 or C2 compared to those inoculated with rhizobia alone. This reduction was more pronounced in plants inoculated with C1.

3.10. Major nutrient contents in faba bean and wheat

3.10.1. N content

Combined stress, inoculation, and intercropping induced a significant effect on N accumulation in faba bean and wheat ($p < 0.001$). In faba bean, inoculation with both consortia highly improved N accumulation, compared to the inoculation with rhizobia alone (Figure 8A). This improvement was significant for sole-cropped plants under P limitation inoculated with C2, compared to inoculation with C1, with improvement rates of 70.75 and 28.24%, respectively. The combined stress of water deficit and P limitation reduced N accumulation in sole cropping and the reduction was more pronounced for plants inoculated with C1.

In wheat, intercropping improved N accumulation compared to the sole crop, either under P limitation or under combined stress, where intercropped plants inoculated with C2 showed the highest increase (112.98%) compared to the sole crop (Figure 8B).

3.10.2. P content

ANOVA testing revealed a significant effect of combined stress, inoculation, and intercropping on P accumulation in faba bean and wheat ($p < 0.001$). Inoculation with both consortia improved P accumulation compared to inoculation with rhizobia alone in faba bean under P limitation (Figure 9A). Under combined stress, plants showed a severe decrease in P content with both inocula (C1 or C2), compared to their corresponding plants under P limitation. Intercropping induced a decrease of P content in plants inoculated with rhizobia alone or with C1, but other treatments did not affect this parameter.

In wheat, combined stress with inoculation (C1 or C2) induced a decrease in P content, compared to the corresponding results under P limitation alone (Figure 9B). Intercropping increased P

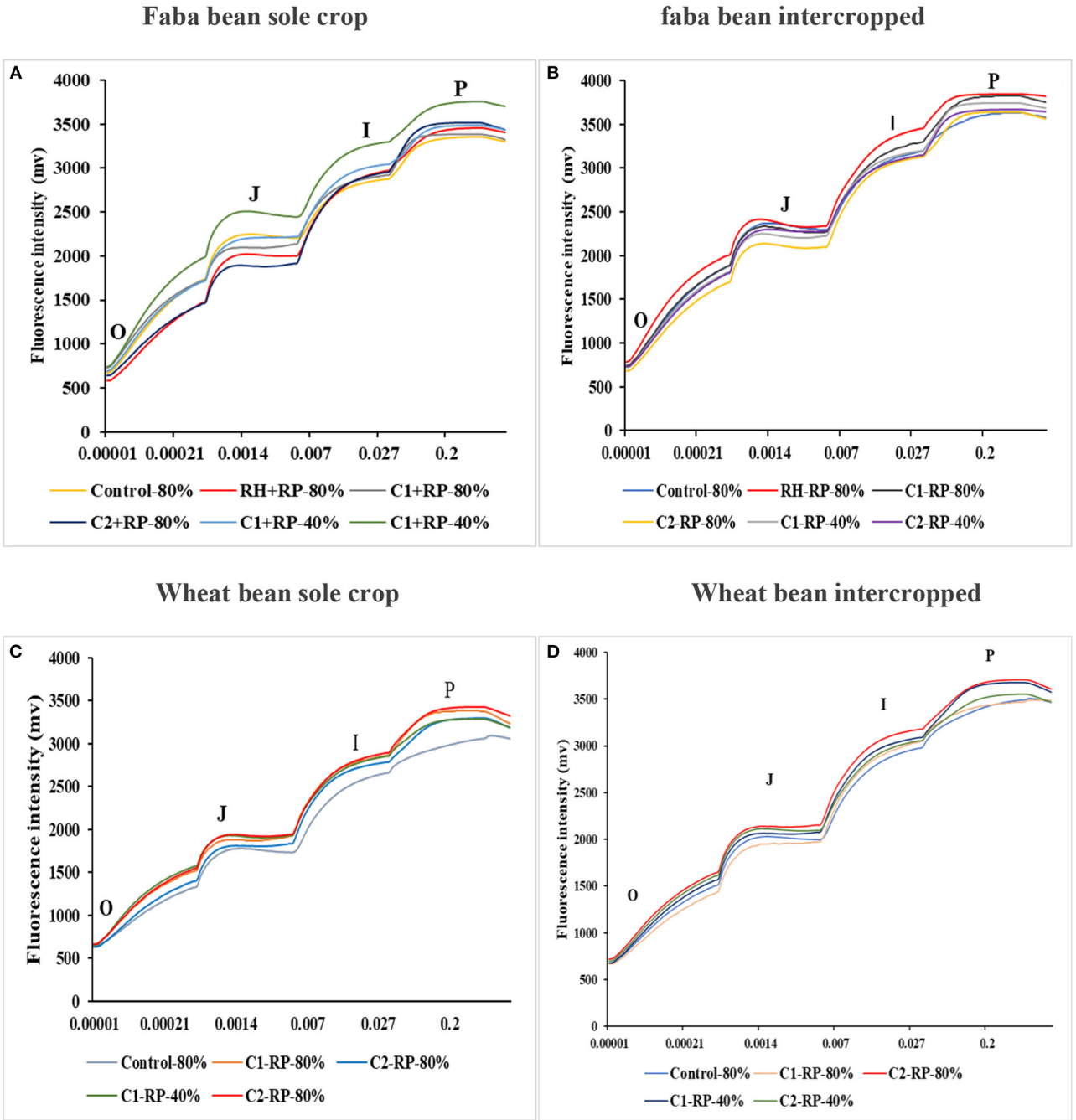

FIGURE 6
Faba bean sole crop chlorophyll "a" polyphasic fluorescence OJIP **(A)**, Faba bean intercropped chlorophyll "a" polyphasic fluorescence OJIP **(B)**, wheat sole crop chlorophyll "a" polyphasic fluorescence OJIP **(C)**, wheat intercropped chlorophyll "a" polyphasic fluorescence OJIP **(D)**, under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP), and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 μmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates. Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls. *Represent the significance level of treatments.

accumulation compared to sole crop in all considered treatments and the increase was more evident in plants under combined stress inoculated with C1 (122.32%) relative to sole-cropped plants.

3.10.3. K^+ content

Faba bean plants under P limitation inoculated with C2 showed improvement of K^+ content (245.19 mg plant^{-1}) in sole-cropped plants, but inoculation with C1 did not affect this parameter

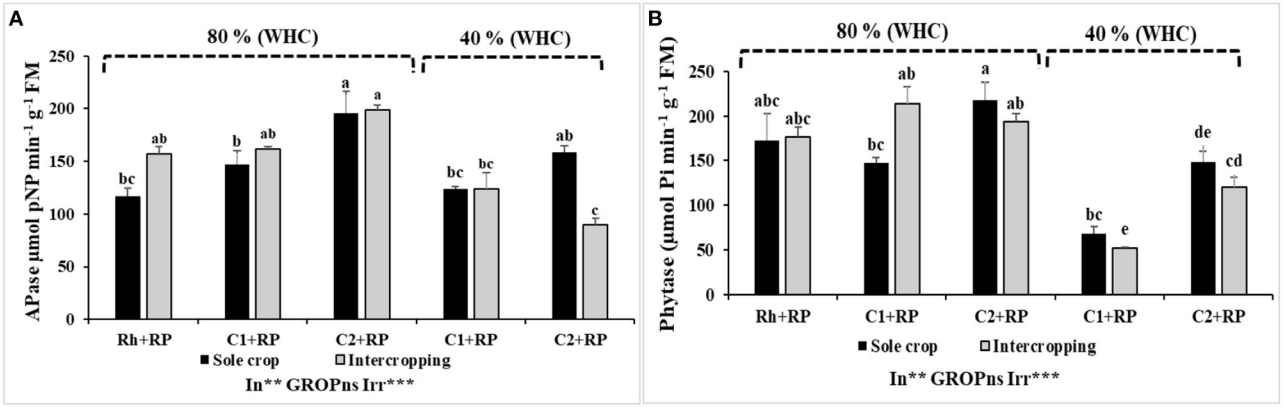

FIGURE 7
Faba bean nodules APase activity **(A)** and nodules phytase activity **(B)**. Plants were grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 µmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh+RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls. *Represent the significance level of treatments.

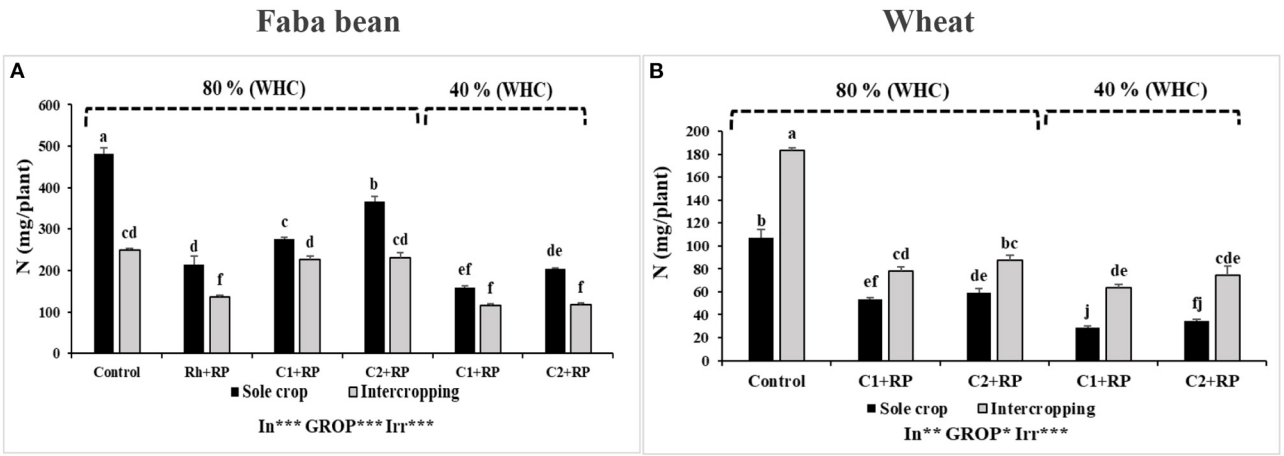

FIGURE 8
Faba bean **(A)** and wheat **(B)** N contents. Plants were grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 µmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls. *Represent the significance level of treatments.

when compared to inoculation with rhizobia alone (Figure 10A). Combined stress reduced the K^+ content in plants inoculated with C1 but showed no effect in plants inoculated with C2 compared to the corresponding treatment under the P limitation. In general, the intercropping practice reduced the K^+ content in faba bean, except for the variants inoculated with C1 under P limitation.

Intercropping induced a significant increase of K^+ accumulation in wheat, compared to the sole-cropped plants in all the considered treatments (Figure 10B). The highest increase rate of 99.65% was achieved in plants under the combined stress inoculated with C2 compared to sole-cropped.

3.10.4. Principal component analysis

The results of the pot experiments were analyzed using principal component analysis (PCA), which showed that the accounted proportion of variance for the first two axes was 65.15 and 20.64% (eigenvalues), respectively. For faba bean under P limitation inoculated with C1 and C2 (Figure 11A), PCA analyses showed that the treatments with the higher yield, nutrient accumulation, La, RWC, gs, APase, and Phy activities were correlated to intercropping faba bean and inoculation with C2, while RWC and Ph were correlated to sole plants inoculated with C1.

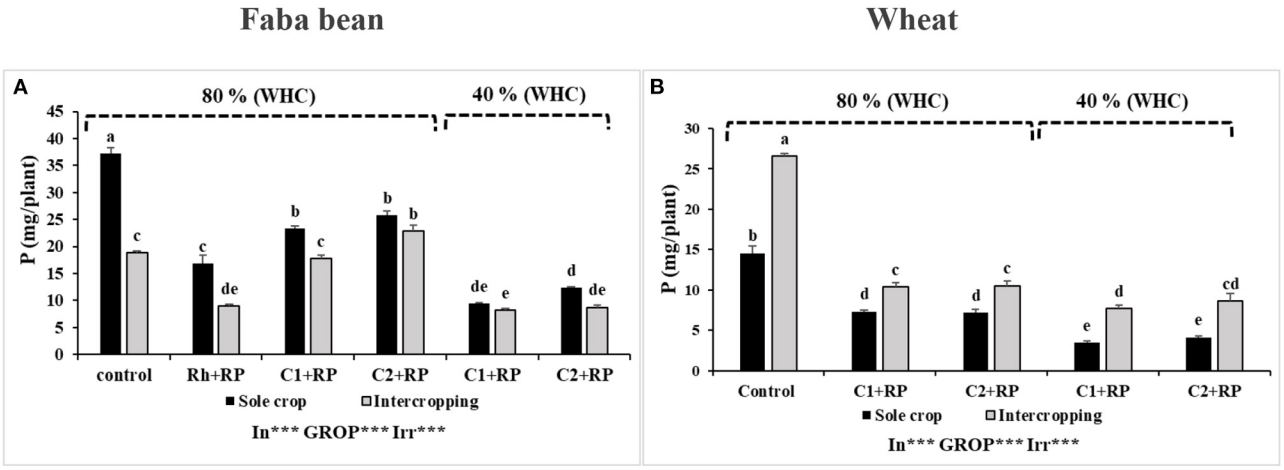

FIGURE 9
Faba bean **(A)** and wheat **(B)** P contents. Plants were grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 μmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls.
*Represent the significance level of treatments.

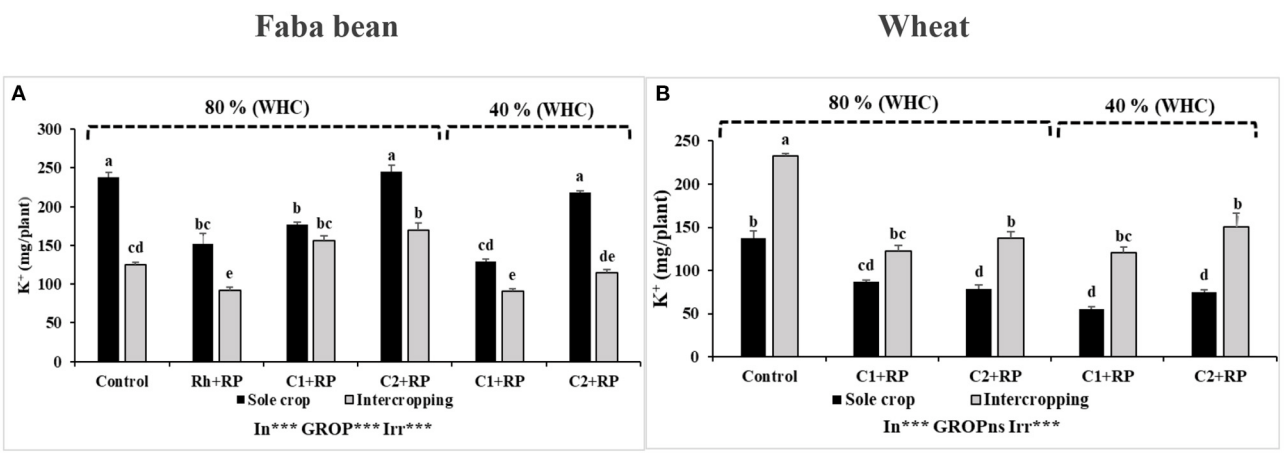

FIGURE 10
Faba bean **(A)** and wheat **(B)** K contents. Plants were grown as a sole crop or intercropped under water sufficiency and P limitation (80% WHC, RP) vs. combined water deficit and P limitation (40% WHC, RP) and inoculated with two rhizobacterial consortia (C1 and C2) containing rhizobia and two PGPRs. The positive controls were supplemented with 125 μmol/l of KH_2PO_4 and 46 mg/l of KNO_3 (control). The negative controls received RP and were inoculated with the rhizobia only (Rh + RP). Each value represents the mean ± standard error (SE) of three replicates (In, inoculation; GROP, type of cropping; Irr, irrigation). Values with different letters are significantly different at 0.05 level of probability by Student-Newman-Keuls.
*Represent the significance level of treatments.

For faba bean grown under water deficit and P limitation, PCA showed that the accounted proportion of variance for the first two axes was 68.71 and 25.78% (eigenvalues), respectively (Figure 11B). This analysis revealed that the treatments with the higher yield, nutrient content, APase, Phy, Pro, gs, and Ph were related to sole-cropped plants inoculated with C2. However, PSN and RWC were correlated to intercropped plants inoculated with C2.

For wheat plants under P limitation, PCA showed that the accounted proportion of variance for the first two axes was 82.77 and 11.50% (eigenvalues), respectively (Figure 11C). PCA showed that nutrient content, higher yield, RWC, Pro, gs, and La were highly expressed in intercropped plants inoculated with C2.

For wheat under combined stress, PCA showed that the accounted proportion of variance for the first two axes was 91.35 and 5.79% (eigenvalues), respectively (Figure 11D). The PCA analyses showed that yield, nutrient content, La, Pro, gs, and RWC were highly expressed in intercropped plants inoculated with C2.

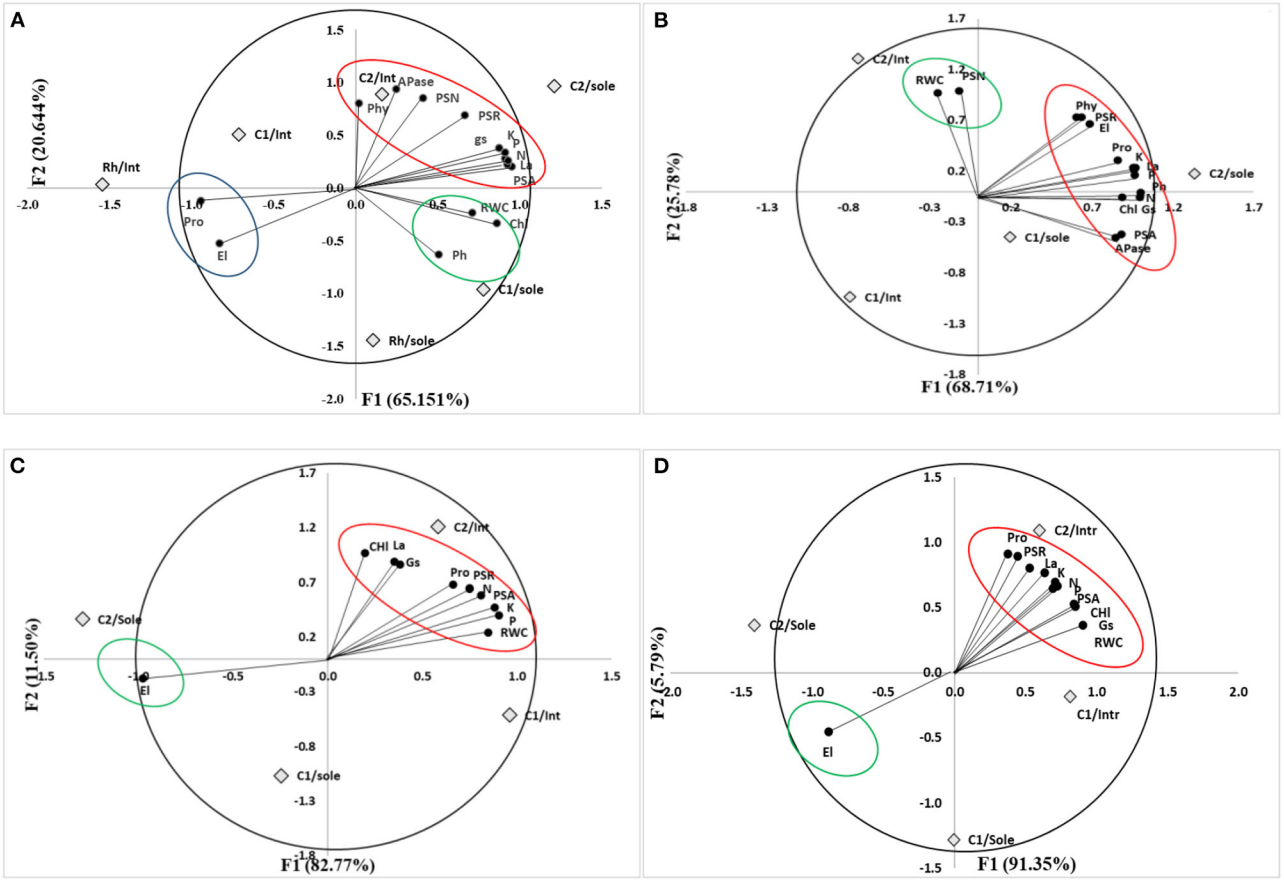

FIGURE 11
Principal component analyses (PCA) of faba bean inoculated with C1 and C2 under P limitation **(A)** and under water deficit and P limitation **(B)** and wheat inoculated with C1 and C2 under P limitation **(C)** and under water deficit and P limitation **(D)**. Faba bean sole-cropped (Sole): inoculated with rhizobia only; Rh. Faba bean intercropped (Int): Int, wheat sole-cropped; (Sole). wheat intercropped; (Int). PSA, shoot dry weight; PSR, root dry weight; PSN, nodule dry weight; gs, stomatal conductance; La, leaf area; RWC, relative water content; EL, electrolyte leakage; Ph, leaf water potential; Pro, proline content; aPase, phosphatase activity, Phyt, phytase activity; N, nitrogen content, K^+, potassium content; P, phosphorus content.

4. Discussion

The present study aimed to evaluate the effects of inoculation with rhizobacterial consortia and intercropping of faba bean/wheat on plant growth, performance, and physiology under combined stress of water deficit and low P availability. Our research addressed the hypothesis that inoculation with rhizobacterial consortia, including one *Rhizobium* sp. and two P solubilizing bacteria, could increase the nitrogen-fixing potential of faba bean—-rhizobia symbiosis and transfer this benefit to associated wheat plants in the intercropping system under stressful conditions.

The key findings of this study revealed that combined stress, P limitation, and water deficit reduced growth in both crop species and for both cropping systems (sole crop and intercropping). This decrease could be due to a reduction of some physiological properties determining plant growth and performance, e.g., cell water status, membrane stability, photosynthesis, and the activity of enzymes involved in plant nutrition. Our results showed that the combined stress induced a decrease in water potential, RWC, leaf area, and phytase and phosphatase activity, which are key enzymes in plant P nutrition. We observed increases in proline content and electrolyte leakage under stressful conditions in comparison to the untreated controls. Such changes would suggest that membrane stability is affected and that these structures would no longer function properly. Another study conducted by Abbasi and Manzoor (2018) showed that the RWC of wheat plants decreased under P deficiency and salt stress. For the same species, the combination of water and salt stress highly affected the genes that are responsible and related to growth and different trait indicators of nitrogen metabolism (nitrogen content, stable nitrogen isotope composition, glutamine synthetase, and nitrate reductase activities) and photosynthetic carbon metabolism (Yousfi et al., 2016).

Several other studies on wheat have shown that water and salt stress affect the activity of key enzymes involved in nitrogen metabolisms, such as nitrate reductase (NR) and glutamine synthetase (Munns, 2005; Munns and Tester, 2008). Bargaz et al. (2012) demonstrated the negative effect of P limitation on membrane stability. In addition, Farissi et al. (2013) reported that the highest electrolyte leakage levels were observed under severe water stress in alfalfa plants. Furthermore, Oukaltouma et al. (2021) reported that the highest level of malondialdehyde, a product of phospholipid peroxidation, was observed under combined stress

of water deficit and P limitation in faba bean, indicating a loss of membrane stability that was reflected in our study by high electrolyte leakage. Our results showed that the effectiveness of Photosystem II (PSII) decreased under the combined stress. These results corresponded to Mouradi et al. (2015), who observed a decrease in this parameter under water deficit in alfalfa plants. This decrease could be associated with a downregulated performance of PSII, linked to the degradation of chlorophyll and, therefore, photosynthetic inactivation (Blackburn, 2007).

Inoculation with the two different rhizobia-containing consortiums significantly improved most of the analyzed parameters compared to inoculation with *Rhizobium* alone. The improvement was particularly evident when plants were inoculated with consortium C2. This high performance of C2 could be related to the presence of *Rahnella* sp. in this consortium, compared to *Pseudomonas* sp. in consortium C1. Indeed, Magallon-Servín et al. (2020) proved that *Rahnella* sp. presented the highest P-solubilizing activity in solid media followed by *A. lannensis* with more effective production of indol acetic acid (IAA), siderophore, biofilm, and acid phosphatase, compared to *Pseudomonas* sp. In our study, inoculation with C2 and intercropping increased P and N accumulation under P limitation and under the combined stress of P limitation and water deficit compared to inoculation with rhizobia alone. This accumulation could be attributed to the effects of the rock phosphate-solubilizing activity of PSB and the fixation of atmospheric nitrogen by rhizobia. The solubilized P would be available for faba bean–rhizobia symbiosis to enhance nodulation and symbiotic nitrogen fixation, and consequently, N nutrition (Hinsinger, 2001; Maazaoui et al., 2016). Indeed, we noticed a higher nodulation density in faba bean roots inoculated with the consortium C2, which could be explained by the sufficiently available P supply related to the PSB, particularly *Rhahnella* sp. Similarly, Benjelloun et al. (2021) showed that the combined inoculation of chickpeas with *Mesorhizobium* sp. and PSB was equivalent to the effect of the combined application of N and P fertilizers on P-deficient soil.

The tested rhizobacteria present a potential for K solubilization ability (unpublished data) and contributed to the enhancement of K^+ nutrition in inoculated plants (Figure 10). Such adequate mineral nutrition, based on the major elements N, P, and K, could be the reason for the improvement of plant growth (shoot and root biomasses) noticed in plants inoculated with the consortia (with C2 being the more effective inoculum) compared to those inoculated with rhizobia alone (Table 1). This trend corroborates well with the first part of our hypothesis. Moreover, Iqbal et al. (2022) showed that the application of *Enterobacter* sp. and *Bacillus* sp. together with the fungus *Piriformospora indica* significantly increased plant growth, physiological parameters, nutrient uptake, and soil microbiological functions in canola. Furthermore, Govindasamy et al. (2020) proved that physiological stress responses, such as relative water content (RWC) and the cell membrane stability index, showed significant improvement in seedlings inoculated with rhizobacterial endophytes under drought conditions. The maintenance of plant water balance under combined water deficit and P limitation was recorded in plants inoculated with C2 and indicated by relatively higher water potential and water content. This could have contributed to plant growth improvement under these constraints. However, added K^+ supply could act as a mineral osmoregulation compound besides organic osmotica (e.g., proline), which accumulates under combined stress and alleviate osmotic stress imposed by water deficit. Such association of inorganic (K^+) and organic compounds (e.g., glycine betaine) against combined stress of P limitation and water deficit has been proven for faba bean (Oukaltouma et al., 2021). Proline is a compatible osmolyte involved in the protection of cell membranes and proteins against these disturbing stresses. However, our results did not support the osmo-protecting role of proline, since the plants accumulating proline exhibited high electrolyte leakage, which suggests membrane damage under combined stress (Figures 2, 5).

The intercropping of wheat and faba bean enhanced wheat growth either under P limitation or under combined stress. This enhancement appeared to be at the expense of associated faba bean plants since we observed faba bean growth reduction in intercropping. These results agree with a report by Khalid et al. (2021), who confirmed the improvement of cereal growth in the intercropping system with faba bean, even under water stress. The association was beneficial for the cereal crop, which was most probably due to the legume facilitation effect. It seems worthwhile to highlight that this legume–cereal association represents a successful model for intercropping since the two crops display a complementary root system allowing for the exploration of different soil horizons and hence avoiding competition for resources (Li et al., 2006; Chamkhi et al., 2022). Intercropping increased the RWC and membrane stability of wheat plants indicating an improvement of the plant water status that could be advantageous for metabolism and growth. Under combined stress, intercropping increased nodulation in faba bean inoculated with C2. This nodulation increase has been reported before in intercropped faba bean and wheat (Bargaz et al., 2017). Our results did not show any enhancement of nodular enzyme activities involved in P availability, e.g., acid phosphatase or phytase, in intercropped faba bean. However, previous studies reported that intercropping reduced APase activity in the faba bean rhizosphere, while it increased in the associated barley rhizosphere (Mouradi et al., 2018). This variation in enzyme activity could prove one of the facilitation actions through the release of enzymes by legume nodules into the rhizospheric soil of associated crops, in which we did not assess this activity. The intercropping system increased the major nutrient contents (N, P, and K) in wheat plants intercropped with faba bean compared to sole-cropped plants. These results confirm the importance of legumes and their microbiome for improving the performance of intercropped wheat, even under stressful conditions, which confirms the second part of our research hypothesis. The facilitation effect of legumes in nutrient mobilization and release into the rhizosphere and the high ability of the cereal root system in taking up these nutrients from the shared rhizospheric space substantiate the benefit of the legume–cereal model, despite the trade-off at the expense of the legume crop. The intercropping benefit involved in phosphorus solubilization by the secretion of legume organic acids, protons, and enzymes, such as phosphatase and phytase, improved P nutrition of wheat under stressful conditions and alleviated the impact of combined stress (Betencourt et al., 2012; Oukaltouma et al., 2021). The inoculation with consortia

containing PSB improved plant P nutrition based on rock phosphate solubilization to make it available for plant particularly under the intercropping system.

5. Conclusion

The present study demonstrated that P deficiency decreased plant growth and nodulation under both cropping systems, and this reduction was more pronounced under the combined stress of water deficit and P limitation in faba bean and wheat grown under greenhouse conditions. This reduction resulted from an adverse impact on physiological water parameters and plant mineral nutrition (N, P, and K). The inoculation with rhizobia–PSB-containing consortia alleviated the impact of P limitation alone and the combined stress compared to inoculation with rhizobia alone. Consortium C2 was more effective than C1, and it was retained for future confirmation under field conditions. Intercropping faba bean and wheat improved wheat growth at the expense of faba bean through enhancement of their water parameters and major nutrient acquisition. Our findings showed that combining inoculation with rhizobia–PSB consortia and intercropping is a promising agroecological practice to alleviate drought and P limitation by improving plant nutrition and soil fertility, mainly in low-input agrosystems.

Data availability statement

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

Author contributions

SC and KO: performed the greenhouse and laboratory experiments and wrote the original draft. IC, AI, and BB: contributed to microbiology work. AQ: supplied methodology and resources. LK: funding acquisition and project coordination. JG: project administration and review and editing. YZ: funding acquisition and project administration. AB: planning, investigation, review, and project management. CG: conceptualization, project administration, funding acquisition, supervision, review, and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1147939/full#supplementary-material

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Formulation of *Brevibacillus agri* and compost to improve growth and phytochemicals compound of *Piper caninum* herbal plant

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Most herbal plant farming operations still rely on conventional methods, negatively impacting human health and the environment. However, by using rhizobacteria to boost the yield and quality of herbal plants, farmers can make a more environmentally responsible and safe choice for consumers. Therefore, the present study aimed to determine the dosage of *Brevibacillus agri* added to the medicinal plant *Piper caninum* to boost its growth and phytochemical content. *Piper caninum* is a popularly used medicinal plant with antifungal and antibacterial properties and the ability to improve the quality of mouse sperm. The investigation was carried out in a greenhouse using a randomized group approach. The results indicated that the most effective formula for promoting growth and enhancing phytochemical composition was F1 (100 g of compost and 3 kg of soil plus 1% *Brevibacillus agri*), which contained 1% *B. agri*. Treating the *Piper caninum* plant with 1%, 2%, or 3% *B. agri* yielded positive results, likely due to the bacteria's nitrogen-fixing ability and favorable outcomes for the IAA test and protease enzyme. *Brevibacillus agri* was also found to colonize the roots of *Piper caninum* and produce the phytochemicals butanoic acid, propanediol, and cyclopropane. In conclusion, using rhizobacteria in sustainable agriculture was highly effective, providing an ecologically responsible and safe alternative to conventional farming methods.

KEYWORDS

compost, hormones, phytochemicals, plant herb, rhizobacteria

Introduction

Plants have been utilized as herbal medicines for centuries due to their high level of safety for consumption. In this modern era, there has been a significant global increase in using plants as herbs (Singh et al., 2021). These plants possess biologically active chemical components with medicinal properties in different parts,

such as leaves, roots, rhizomes, stems, bark, flowers, fruits, and seeds. Various phytochemicals establish herbal plants' quality, including alkaloids, saponins, phenolics, flavonoids, tannins, and antioxidants (Nasab and Sayyed, 2021; Ali et al., 2022). Phytochemicals are essential daily as biopesticides, cosmetics, and medications to prevent and treat various diseases (Parbuntari et al., 2018). According to Firenzuoli and Gori (2007) research, there has been an increasing demand for herbal medicines. This trend underscores the need to cultivate plants sustainably, employ environmentally friendly methods, and refrain from using harmful chemicals (Egamberdieva and da Silva, 2015). Therefore, it is crucial to ensure that the plants used for herbal medicines are safe for consumption without harmful substances.

Due to practical reasons, chemical fertilizers and insecticides are still primarily used in producing herbal plants (Agbodjato et al., 2015). However, overusing pesticides and fertilizers has a negative impact on crop quality (Joko et al., 2020), as seen by the presence of chemical pesticide residues (Zou et al., 2023). Various businesses emerged in organic agriculture to obtain sustainable, environmentally friendly, and safe products. Furthermore, the study of plant growth promotion rhizobacteria (PGPR) present in plant roots has recently garnered considerable attention (Bhat et al., 2022; Desai et al., 2022; Tan et al., 2022). PGPR is safe to consume because there are no pesticide residues, and it is environmentally friendly and sustainable (Hamid et al., 2022). The application in plant cultivation has a good impact on plants (Chandran et al., 2021; Sharma et al., 2021). It creates a variety of chemicals for plants, including biofertilizers, biopesticides, growth hormones, and enzymes (Habib et al., 2016; Khan et al., 2021; Gowtham et al., 2022), which are beneficial to the development and productivity of plants (Mohanty et al., 2021).

Trichoderma biostimulants can increase the content of antioxidants, phenolics, and alkaloids in olive plants Egamberdieva and da Silva, 2015). *Trichoderma album* and *Bacillus megaterium* (Zope et al., 2019; Enshasy et al., 2020; Sagar et al., 2022a) can increase antioxidants in onion plants (Younes et al., 2023). *Brevibacillus agri* can improve the content of antioxidants, anthocyanins, vitamin C, vitamin A, fiber, tannins, and total phenols in Bali red rice (Unggulan et al., 2021). *B. agri*, found in the root of Bali rice plants, contains IAA hormones and protease enzymes. Furthermore, it fixes nitrogen from the atmosphere and boosts secondary metabolic chemicals and antioxidants (Suriani et al., 2022).

Piper caninum contains antioxidants, alkaloids, phenols, flavonoids, and steroids (Suriani et al., 2019, 2020b), and antimicrobial essential oils (Salleh et al., 2011, 2015). The quality of mice's sperm can be improved by *P. caninum* leaf extract (Gede et al., 2022). According to Suriani et al. (2020b), the plants growing at 600 meters in tropical forests are still in a wild state and have not been widely cultivated by the community. Additionally, several studies have been conducted on using the *B. agri* rhizobacteria formula to enhance growth, phytochemicals, and antioxidants in *P. caninum* leaf. This study aimed to get a better quantity and quality of *P. caninum* herbal plants using *B. agri* rhizobacteria.

Materials and methods

Time and location of research

The current study was conducted between January 2022 and October 2022 at the biopesticide lab at Udayana University in Bali, Indonesia, and the greenhouse in Munduk Paku Village in Senganan Penebel, Tabanan, Bali, Indonesia (8°22′49.3 "S 115°09′43.2"E). The climate of this region is categorized as Type A by Schmidt and Ferguson, and it experiences ∼155.6 wet days per year, with an annual rainfall of between 2,000 and 2,800 mm. The region has 4 to 10 and 0 to 5 wet and dry months per year, respectively. Additionally, the average air temperature ranges from 25°C to 28°C (Suriani et al., 2022).

Research design

A randomized group design with four treatments and six replicates was used in the greenhouse, resulting in 24 experimental units, each comprising three clumps for 72 clusters. F0 is the control (untreated soil), F1 is treatment (100 g of compost and 3 kg of soil plus 1% B. agri), F2 is treatment (100 g of compost and 3 kg of soil plus 2% *B. agri*), and F3 is treatment (100 g of compost and 3 kg of soil plus 3% *B. agri*). Each polybag contains one ready-to-plant *P. caninum* plant. The P. caninum plants were obtained from the Bali villages of Munduk Paku and Senganan in the Penebel District and Tabanan Regency (Sudewi et al., 2020).

Composting

Compost is made from rice straw, chicken manure, and cow dung. Subsequently, a minor bit of water was added to bring the entire weight up to about 500 kg, with 1 liter of liquid biostater *B. agri*. The container was locked for 20 days before thoroughly stirring the liquid. It was then closed again for 40 days following the process described by Shilviana et al. (2021). The appropriate amounts of cow dung, chicken manure, and rice straw were combined and then slightly moistened with water to achieve a total weight of around 500 kg to create the fertilizer. Additionally, 1 liter of liquid biostater *B. agri* was added, and after locking the container for 20 days, the liquid was thoroughly agitated upon opening to release any available gas. The container was closed again for 40 days to complete the process; after that, the compost was ready to be used as a mixed media in research (Shilviana et al., 2021).

Test for indole acetic acid-producing ability

The isolates were first grown in a 5 ml test tube of tryptic soy broth, where they were kept in the dark at 28°C for 48 h. One cc isolate was first grown in a 5 mL test tube of tryptic soy broth and added with one cc of Salkowski's solution to the test tube; the hue changed. A pink tinge in the suspension indicates that the rhizobacteria can produce IAA. Spectrophotometry at 520 nm or higher was also used to quantify the data (Delgado-Ramírez et al., 2021).

Test for ability to fix nitrogen

Bacterial isolates were grown on a bromothymol blue malate medium that lacked nitrogen and contained 5 g of malic acid, 4 g of KOH, 0.5 g of K_2HPO_4, and 0.05 g of $FeSO_4$. Additionally, 0.01 g of $MnSO_4$ and $7H_2O$ and 0.01 g of $MgSO_4$ in $7H_2O$, 0.02% NaCl, 0.01% $CaCl_2$, and 0.002% Na_2 were added. The cultures were maintained at 28°C for 48 h, and rhizobacteria actively fixed nitrogen when the colonies turned yellow (Tang et al., 2019).

Proteolytic activity test

Bacterial isolates were cultivated on 2% SMA media to test for proteolytic activity (Skimmed Milk Agar). The media was prepared by mixing 2 g and 3 g of skimmed milk and Nutrient Agar, diluted with distilled water to a final volume of 100 mL. The mixture was sterilized using an autoclave at 121°C for 20 min. Meanwhile, the isolated bacteria were grown on 2% SMA media at 35–37°C for 24–48 h. A clear zone around the bacterial colony indicated protease enzyme activity (Kusuma et al., 2021).

Production of *B. agri*

Rhizobacteria with assessment number: OM510267 were found in Senganan village, Penebel sub-district, Tabanan Regency, Bali, Indonesia. It is the bacterium that produces the most potent IAA hormone compared to 20 others from Badung Regency and Tabanan Bali (Suriani et al., 2022). *B. agri* bacteria is grown in Nutrient Agar (NA) media containing Nystatin as much as 500 mg/L. To make 1 liter of biostimulant, 1 liter of Potato Dextrose Broth (PDB) media is prepared, media containing Nystatin as much as 500 mg/L, then five needles of Ose culture of *B. agri* are then incubated for three days between 28°C and 30°C.

Gas chromatography-mass spectrophotometry analysis of *B. agri*

The compound's analysis in the control and treatment cases was analyzed using GC-MS to determine the phytochemical compound. The isolate was incubated in potato dextrose broth medium for seven days, after which the culture was centrifuged at 10.000 rpm for 15 minutes to collect the supernatant. Subsequently, the supernatant from the biomass experiment was filtered through a 0.45 µm Millipore membrane (LTD, Yonezawa, Japan) for analysis (Maulina et al., 2022).

The supernatant was dissolved in a 1:1 v/v ratio of methanol and chloroform (5 mL total) to prepare the sample for GC-MS analysis. The sample was then subjected to GC-MS analysis using liquid nitrogen as the eluent. Meanwhile, the column had the following specifications: 4.6 x 200 mm, 1 mL/min flow rate, 250°C temperature, and UV detection at 254 nm. The compound was identified by comparing the isolated compound's molecular weight and fragmentation pattern with those in the GC-MS library. The bacteria were isolated by inoculating the compound in a Potato Dextrose Broth medium for seven days (Akubugwo et al., 2022).

N, P, K soil and leaf analysis

N analysis

About 0.5 g of specimens were weighed after being smoothed out and placed into a Kjeldahl flask. Next, 25 mL of sulfuric-salicylic acid solution was added, shaken, and allowed to sit overnight. The mixture was then heated at a low temperature until the bubbles disappeared after 4 g of $Na_2S_2O_2.5H_2O$ was added. The temperature was gradually increased until a maximum of 300°C (~2 h) was reached and then allowed to cool. The solution was transferred into a 500 mL measuring flask, diluted with distilled water, shaken, and adjusted to the line mark. Distillation was terminated when the distillation yield reached 100 mL. Subsequently, 25 mL was pipetted and added to a distillation flask with 150 mL of distilled water, 10 mL of 40% NaOH solution, a 20 mL of 1% boric acid solution. The solution was titrated with an H_2SO_4 0.05 N solution until the endpoint of the titration was reached (the green color changed to pink). Meanwhile, work was also carried out on the blank solution. Furthermore, nitrogen levels were determined using a UV-Vis spectrophotometer at a wavelength of 400 nm (Liu et al., 2022).

P analysis

A total of 0.5 g of soil is subjected to the ashing process with the addition of concentrated H_2SO_4 and HNO_3; after that, it is heated over a hot plate. Then 2.5 ml of concentrated H_2SO_4 was added, so it turned black such as ash, then added concentrated HNO3 until the smoke from the sample is gone black. The addition of HNO_3 was gradual until the sample did not emit black smoke after adding HNO_3. After the ashing process, the sample was added to 50 ml of distilled water, shaken, filtered, and kept in the Erlenmeyer flask, followed by adding 2.5 ml of vanadate molybdate, which will produce a yellow color. Furthermore, phosphorus levels were determined using a UV-Vis spectrophotometer at a wavelength of 400 nm (Elbasiouny et al., 2020).

K analysis

About 2.5 g of test-ready samples were weighed in a 250 mL flask, and 50 mL of 4% $(NH_4)_2C_2O_4$ and 125 mL of distilled water were used for the K analysis. The mixture was brought to a boil, boiled for 30 min, and then cooled. Once the mixture reached the mark on the flask, it was transferred to a 250 mL measuring flask and diluted with distilled water. The 15 mL solution was then filtered or left to stand until clear and was transferred into a 100 mL measuring flask to prepare the analysis solution. Furthermore, 2 mL of NaOH (20%), 5 mL of HCHO, and 1 mL of STPB for every 1% K_2O were added. After filling the flask with distilled water to the mark and stirring for 5 to 10 minutes, the solution was strained using Whatman filter paper No. 12, and about 50 mL of the filtrate was taken for further analysis (Alhaj Hamoud et al., 2019).

Scanning electron microscopy test of rhizobacteria on the roots of *P. caninum*

Scanning electron microscopy (SEM) was utilized to investigate the effects of rhizobacteria treatment on bacterial colonization of

plant roots. Control samples were created using the roots of *P. caninum*, while treatment samples were prepared by immersing the roots in a 2% solution of *B. agri* for 3 days. The samples were then dehydrated for 8 hours and ventilated for 1 week at 50 degrees until a constant weight was established. The root samples were examined using an FE-SEM MICROSCOPE SCAN ENERGY X-ray spectrometry, ZEISS Merlin, with a beam current of 0.2–30 kV to 400 nA and a lowest vacuum of few pA-300 nA. In this study, a 3kV acceleration was used for imaging, and 15 kV was used for EDX experiments, except on the skin where 10 keV was sufficient. The analysis was conducted in the Lab. UGM (Maulina et al., 2022).

Greenhouse trials

Preparation of planting medium

A specific concentration is mixed with compost after the soil medium (20 cm below the surface) has been boiled, prepared, and combined with the media under treatment. The material was ready for usage.

Planting

The seedlings used are previously prepared and treated using biostimulants. The healthy seedlings are uniform at ± 20 cm in height, free from pests and diseases. Planting is perpendicular to a depth of ± 5 cm (Suriani et al., 2020b).

Application

The application of *B. agri*, under the previously designed plan, was carried out at 2, 4, and 6 weeks after planting (MST).

Maintenance

Plant maintenance involves several important tasks, such as watering, weeding, fertilizing, and pruning. Embroidery, which consists of creating plant patterns, is typically conducted on plants growing uniformly without irregularities. To ensure consistent growth, these plants are prepared in advance. Watering is performed weekly in the morning to induce stress and promote plant resilience. Weeding is crucial to prevent the growth of unwanted plants competing for nutrients. This helps to maintain healthy plant growth and to avoid damage (Suriani et al., 2020b).

Harvest

Harvesting is carried out after the *P. caninum* plant within four months. The harvested leaves were washed, dried in a clean indoor wind, and served 8 hours before the oven.

Measured parameters

Parameters measured in the field include plant height, root length, leaf area, laboratorium analysis of chlorophyll content, N, P, K, Cu, Cd, Pb leaf, then analyzed phenolic content, flavonoids, and antioxidant activity.

Extract manufacturing

To prepare *P. caninum* leaves for chemical analysis, they are first chopped into 2 mm thick pieces and dried for 8 hours in a clean, dry room. Once the leaves are dry, they are ventilated at a temperature of 50°C for 10 hours until a constant weight and moisture content of 4% is obtained. Next, the leaves are macerated using ethanol, and the resulting solution is evaporated using a rotary evaporator (Lee et al., 2022). Finally, the leaves are tested for their phenolic, flavonoid, and antioxidant content.

Polyphenols

A 7% Na_2CO_3 reagent was prepared by dissolving 3.5 g of Na_2CO_3 in 50 mL aqua bidestillata. Total phenolic compounds were measured using the colorimetric approach with gallic acid (GAE) as the reference. Meanwhile, a standard solution of gallic acid was created by dissolving 10 mg of gallic acid in 10 mL of ethanol to make a solution with a concentration of 1,000 ppm. To obtain a concentration of 100 ppm, 2.5 mL of the stock solution was diluted with ethanol to a volume of 25 mL. Subsequently, 1, 2, 3, 4, and 5 mL of the solution were diluted with 10 mL of ethanol simultaneously to create concentrations of 10, 20, 30, and 50 ppm. For the gallic acid standard solution measurement, 0.4 mL of the Folin-Ciocalteau reagent was added to each concentration of 10, 20, 30, 40, and 50 ppm. The mixture was whipped for 4–8 min, and then 4.0 mL of the 7% Na2CO3 was added and stirred until smooth. Subsequently, up to 10 mL of aquabidestillata was added, and the mixture was allowed to stand for two hours at room temperature. A calibration curve was created by measuring the absorbance at a maximum wave of 744.8 nm and relating it to the gallic acid content (g/mL). To prepare the *P. caninum* extract solution, 10 mg of the extract was weighed and dissolved in 10 mL of ethanol. Up to 1 mL of the solution was pipetted into the mixture to measure total phenol levels.

Furthermore, 0.4 mL of the Folin-Ciocalteau reagent was added, and the mixture was agitated for 4–8 min before being mixed with 4.0 mL of the 7% Na_2CO_3 solution. Up to 10 mL of aquabidestillata was added, and the mixture was stirred and allowed to stand for two hours at room temperature. The maximum absorption was measured at a wave of 744.8 nm. This process was repeated three times, and the phenol levels were measured as mg of gallic acid equivalent per g of extract (Redondo-Gómez, 2022).

Flavonoids

A colorimetric method determined the total flavonoid levels with the steps and quercetin (QE). Standard quercetin solutions were created by measuring and dissolving 10 mg of standard quercetin in 10 ml of ethanol every hour to achieve a concentration of 1000 ppm. A typical quartzine solution of 1,000 ppm was diluted in 10 mL of p.a. ethanol for 100 ppm before being pumped into a range of 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm concentrations. To each standard solution, three milliliters of quercetin, 0.2 milliliters of 10% $AlCl_3$, 0.2 milliliters of potassium

acetate, and up to ten milliliters of aquadestilata were added. After incubating for another 30 minutes at room temperature, the sample was subjected to UV-Vis spectrophotometry at a wavelength of 431 nm to measure its absorbance. To determine the total flavonoid content of a 100 mg cadaver extract solution in 10 mL of ethanol, 0.2 mL of 10% $AlCl_3$, 0.2 mL of potassium acetate, and 10 mL of distilled water were added to the solution. The absorbance was then measured using UV-Vis spectrophotometry at 431 nm after allowing the mixture to sit in a dark room at room temperature for 30 min. Meanwhile, 3 replicates of the sample solution were prepared to obtain the flavonoid levels as quercetin equivalents (Perisoara et al., 2022).

Antioxidant

A standard gallic acid curve was created with different concentrations (0–2 mg/L). The sample was treated by weighing 0.05 g and diluting with 99.9% ethanol to a volume of 5 mL in a measuring flask, then centrifuging for 15 min at 3000 rpm. After adding the standard and supernatants, pipetting was used to introduce 0.5 mL of DPPH 0.1 mm (in 99.9% ethanol solvent) to the test tube. It was incubated at 25°C for 30 min to give DPPH enough time to react with the hydrogen atoms of the sample's antioxidants. Furthermore, its absorbance at 517 nm was measured. The antioxidant capacity was estimated using the formula y from the linear regression equation: $y = ax + b$ (Cappellari et al., 2020).

TABLE 1 IAA concentrations produced by rhizobacteria *B. agri*.

Repetition	IAA concentration (ppm)
1	681.77
2	656.15
3	602.96
4	612.81
5	624.63
6	629.98
7	617.87

TABLE 2 IAA Concentration, protease, nitrogen fixation.

Parameters	Qualitative
IAA	Positive
Protease	Positive
Nitrogen fixation	Positive

Heavy metal analysis Pb, Cd, Cu

Sample analysis was carried out for the control and treated leaves of *P. caninum*. *P. caninum* leaf samples weighing 0.5 g were placed in a Kjeldahl flask with 5 ml of concentrated HNO_3 and H_2SO_4. The sample was then wet-digested until a dark powdered solution was obtained, which appeared slightly yellow. The resulting solution was diluted with ion-free water using a 100 mL measuring flask and filtered until a filtrate was obtained. Furthermore, the filtrates were analyzed using AAS for metal grades and mineral standards (Aslanidis and Golia, 2022).

Data analysis

The data obtained were analyzed quantitatively using *analysis of variance* (ANOVA). This is continued with the *Duncans Multiple Range Test* (DMRT) tests at a level of 5% when the treatment causes differences in the observed variables (Suriani et al., 2020a; Hosseini et al., 2022).

Result and discussion

IAA hormone analysis, nitrogen test, and protease test

Table 1 shows that *B. agri* has produced qualitatively positive IAA hormones and quantitatively acquired the concentrations from 7 tests, ranging from 602.96–681.77 ppm. The results are highly favorable, demonstrating its capacity to bind nitrogen

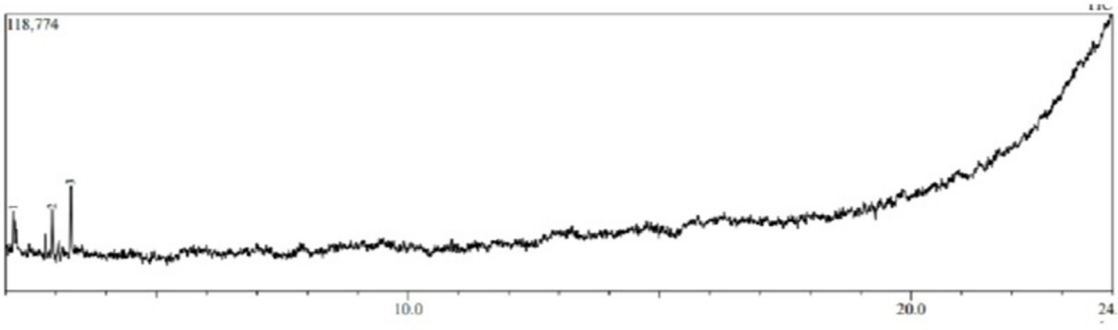

FIGURE 1
Chromatograms *B. agri*.

TABLE 3 Compounds of GC-MS analysis of *B. agri*.

Peak	Retention Time	Compound	Area (%)	Biology activities
1	2.159	Butanoic acid	28.94	Antibacterial, antimicrobial
2	2.926	Propanediol	39.25	Cosmetic ingredients, anti-aging, ingredients
3	3.297	Cyclopropane	31.81	Anti microbial and anti-fungal

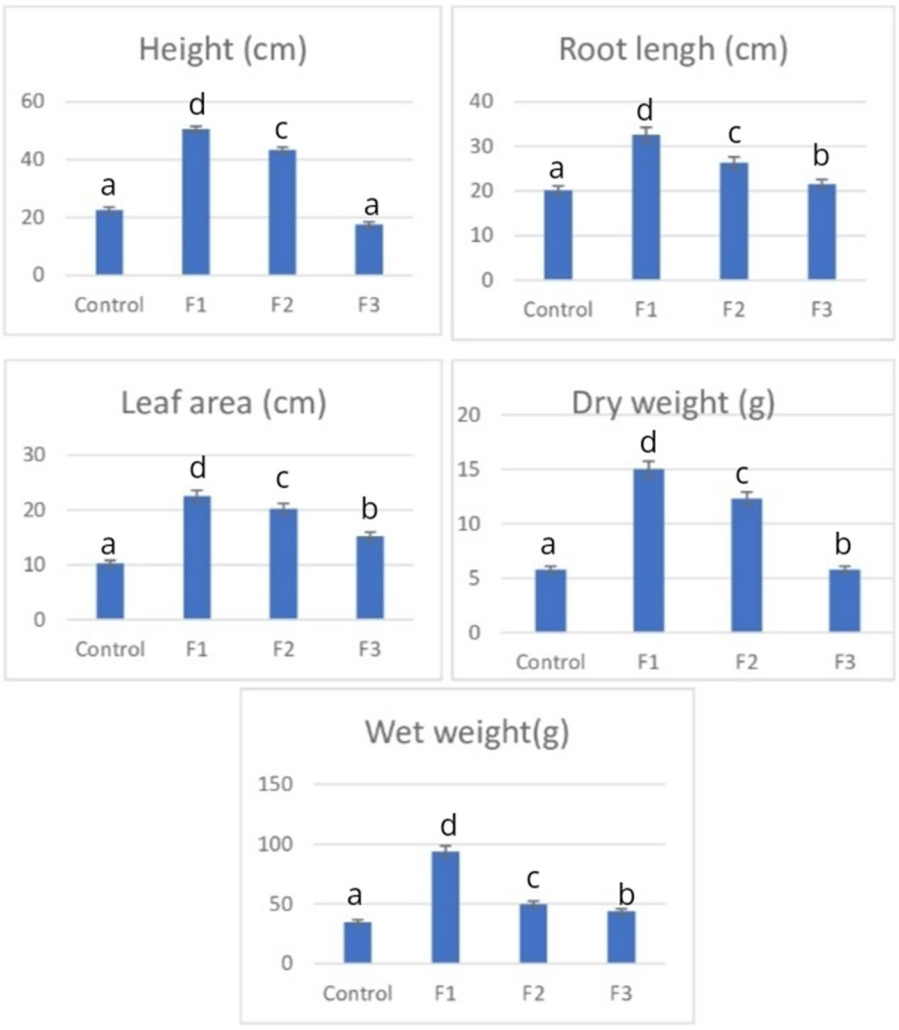

FIGURE 2
Everage groeth of *P. canium*. Letters a, b, c, d indicate significant differences between the values at $P > 0.05$.

from the air and break down proteins, as shown in Table 2. Balinese rice contains the IAA hormone, which enhances rapid development (Suriani et al., 2022). Rhizobacteria can produce IAA hormones to promote the growth of plant roots and shoots. For example, tomato plants' roots and shoots can grow faster when rhizobacteria like *Bacillus Substilis* and *Azospirillum brasilense* are present (Lobo et al., 2022). Plants benefit from *Arthrobacter pascens* ZZ21, located in the rhizosphere. These rhizobacteria can generate the phytohormone indole-3-acetic acid (IAA), promoting plant development and purging soil contaminated with fluoranthene. IAA synthesis increased by 4.5 times when tryptophan in the culture medium was supplied at 200 mg/L. (Li et al., 2018).

Meanwhile, rhizobacteria can also fix nitrogen, boost rice plants' willingness to take up N, and associate with the roots of rice plants (Sagar et al., 2020; Jabborova et al., 2022; Mir et al., 2022). In the Bali region, this bacteria can also raise the N content of maize crops and soil. Protease enzymes break down proteins into smaller components for plant nutrition, facilitating the synthesis of nutrients in bean plants

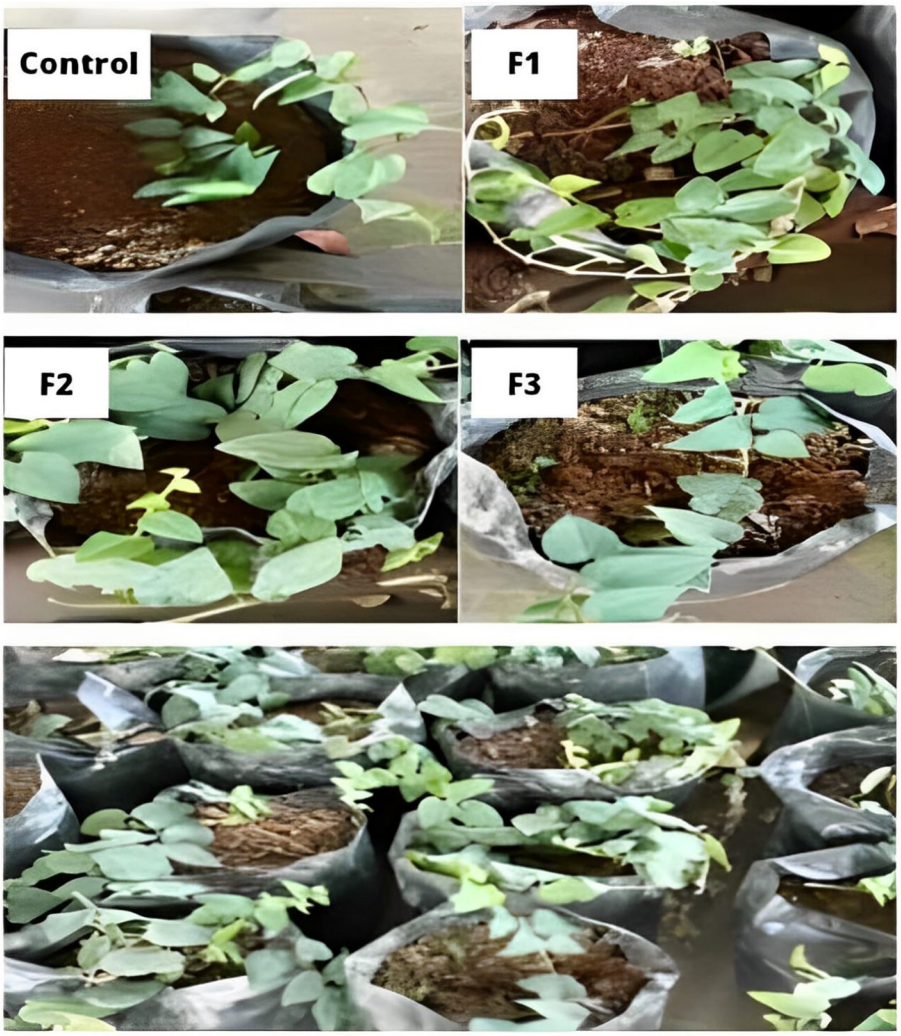

FIGURE 3
Growth of *P. canium* plant.

(Flores-Duarte et al., 2022). At the early seedling stage, PGPR increased leaf gas exchange rates, including photosynthesis, stomatal conductance, and transpiration in potato plants (Liu et al., 2022).

Gas chromatography-mass spectrophotometry analysis of *B. agri*

The results of the GC-MS analysis of *B. agri* (Figure 1) obtained 3 types of compounds, where propanediol has the most significant area at 39.25%, as shown in Table 3. Propanediol is a cosmetic ingredient produced by the bacterium *Propionibacterium freudenreichii* (Dank et al., 2021). Meanwhile, butanoic acid is antimicrobial (Suriani et al., 2022), and marine bacteria *Labrenzia sp*. 011, producing cyclopropane, can be antifungals (Moghaddam et al., 2018).

Effect of treatment growth of *P. caninum*

Data on *P. caninum* growth also indicated improvement following *B. agri* treatment, where 1% (F1) and 2% (F2) produced the best growth (Figures 2, 3). Rhizobacteria are the reason for the treatment's increased growth, significantly different from the control at all concentrations. IAA is a hormone that *B. agri* generates to promote plant development (Tables 1, 2). According to research, *B. agri* can improve Balinese rice growth at a concentration of 2% (Suriani et al., 2022). It creates phytochemicals with antifungal and antibacterial properties (Table 3). By directly promoting plant development through processes like nitrogen (N) fixation, phytohormone synthesis, and phosphate solubilization, PGPR can be employed as biofertilizers and biopesticides (Shah et al., 2021). It can increase vegetable growth and yield by creating hormones like IAA and phytochemicals inhibiting bacterial and fungal infections (Kumar et al., 2021). A plant's metabolism can be affected immediately by bacteria that support plant growth and use

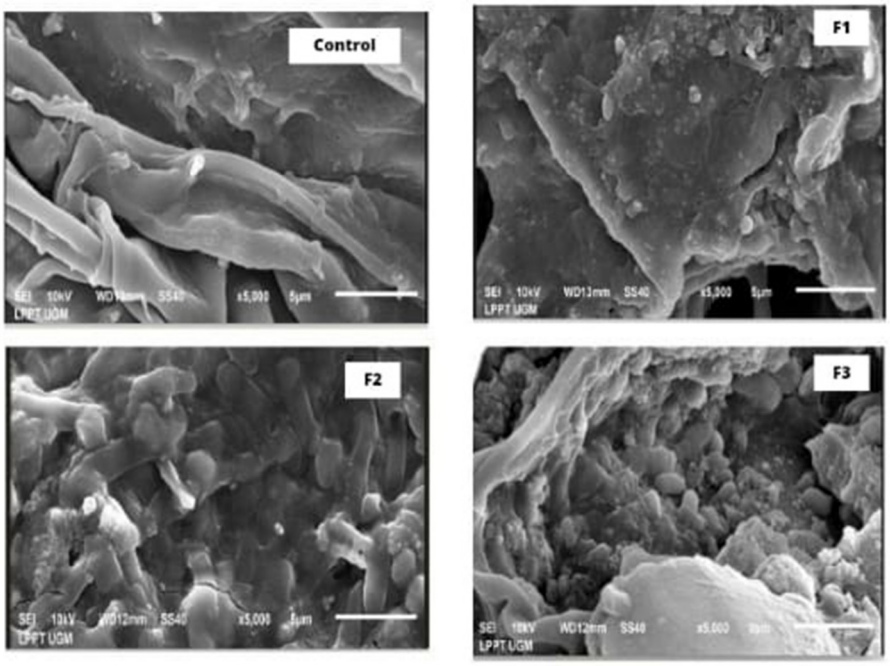

FIGURE 4
Colonization of rhizobacteria on the roote of *P. canium*.

their metabolism to solubilize phosphates, create hormones, and fix nitrogen.

Furthermore, PGPR enhances root growth and raises plant enzymatic activity. Several studies have shown numerous benefits of the application in maize and sugarcane crops (Kusale et al., 2021a; Saboor et al., 2021; Sagar et al., 2022b). PGPR can also stimulate other bacteria as part of a synergistic effect to improve their influence on plants, increasing growth or development (dos Santos et al., 2020). It increases the growth of medicinal plants because it can produce biofertilizers, dissolve phosphate and potassium, and fix nitrogen (Kumar et al., 2022).

Analysis of phytochemicals, chlorophyll, and heavy metal of *P. caninum*

Table 4 analyzes flavonoids, polyphenols, antioxidants, and chlorophyll. *P. caninum* plants treated with *B. agri* have been demonstrated to contain higher flavonoids, polyphenols, antioxidants, and chlorophyll than controls. Furthermore, 1–2% of *B. agri* treatment yields the best effects, whereas 3% results in lower levels of chlorophyll. Every treatment is significantly different from the control because of the influence of *B. agri*. The plant *P. caninum* has higher phytochemicals, antioxidants, and chlorophyll concentrations. According to studies (Ghorbanpour et al., 2016), PGPR boosts antioxidant activity in chickpeas (*Cicer arietinum* L.) and the activity of several antioxidant enzymes. *S. meliloti* increases the contents of phenolic compounds, flavonoids, and antioxidant capacity in the plant, which can be attributed to the ability of pounds, flavonoids, and antioxidants (Zapata-Sifuentes et al., 2022). *Hyssopus officinalis*, a member of the Lamiaceae family and one of the most significant medicinal plants producing essential oils can raise the chlorophyll content a, b, and total chlorophyll in plants by promoting rhizobacteria, *Azospirillum, Pseudomonas*, and *Bacillus* (Sharifi, 2017). Furthermore, the combination of *B. subtilis* and *B. amyloliquefaciens* had the most substantial significant impact, where the content of chlorophyll A increased by 30% and 27%, chlorophyll B by 20% and 16%, and total chlorophyll by 54% and 43%. Ascorbic acid also increased in tomato plants (Plants et al., 2022). *Thiobacillus thiooxidans, Frateuria aurantia*, and *Bacillus megaterium* can boost phenolic and antioxidant content (Eren, 2022). After being treated with PGPR *Glomus aggregatum, Trichoderma harzianum*, and *Bacillus coagulans, Glycyrrhiza glabra* L. (licorice) plants produced more phenols, ortho-dihydroxy phenols, tannins, flavonoids, and alkaloids (Egamberdieva and da Silva, 2015). Heavy metal data for Cu, Pb, and Cd were not detected, and there was no significant difference between the control and treatment. Therefore, the *P. caninum* plant is safe to consume and devoid of heavy metal contamination. There is no rich metal content of Cu, Pb, or Cd in the leaves of *P. caninum* due to the effects of *B. agri*. By changing the bioavailability of metal in the soil and enhancing metal translocation, PGPR may reduce phytotoxicity, supported by the analysis of soils showing no detected Cu (Table 5). PGPR can oxidize hydrocarbons and improve plant biodegradation activity (Vocciante et al., 2022). B. cereus inoculation increased the antioxidant enzyme activities in walnut seedlings and changed their photosynthetic characteristics (Ji and Huang, 2007).

ARAC 3221, ARAC 221, ARSI 2112, ARAI 3312, and ARAI 3221 were among the actinobacteria isolates that were successful in IAA is created by dissolving phosphate and producing chitinase

TABLE 4 Analysis of phytochemicals, antioxidant dan chlorophyll in *P. caninum* leave.

No	Parameters	Unit	Treatment			
			Control	F1	F2	F3
1	Poliphenols	mg GAE/100 g	2883.03 ± 0.7b	4024,40 ± 0.5d	3210.55 ± 0.9c	2327.38 ± 0.4a
2	Flavonoids	mg QE/100 g	607.54 ± 0.17b	873.38 ± 0.25d	748.27 ± 0.24c	567.72 ± 0.15a
3	Total chlorophyll	ppm	1343.39 ± 0.21a	2599.20 ± 0.25d	2277.88 ± 0.15c	1672.70 ± 0.13b
4	Chlorophyll a	ppm	817.14 ± 0.23a	1618.72 ± 0.15d	1435.13 ± 0.20c	1046.21 ± 0.18b
5	Chlorophyll b	ppm	526.63 ± 0.27a	981.21 ± 0.32d	843.37 ± 0.15c	626.96 ± 0.26b
6	IC 50%	ppm	861.75 ± 0,2a	383.05 ± 0.1d	520.20 ± 0.3c	616.34 ± 0.5b
8	metal Cu	ppm	No detected	No detected	No detected	No detected
9	Metal Pb	ppm	No detected	No detected	No detected	No detected
10	Metal Cd	ppm	No detected	No detected	No detected	No detected

Letters a, b, c, d indicate significant differences between the values at $P > 0.05$.

TABLE 5 Soil analysis.

No.	Parameters	Unit	Treatment			
			Control	F1	F2	F3
1.	Nitrogen (N)	%	0.29 861.75 ± 0.12a	0.43 ± 0.17c	0.36 ± 0.20b	0.672 ± 0.15d
2.	Phosphorus (P)	mg/kg	1.289.052 ± 0.22a	1.709.404 ± 0.19d	1.613.956 ± 0.32c	1.426.153 ± 0.34b
3.	Potassium (K)	mg/kg	560.704 ± 0.32a	1.080.292 ± 0.28d	607.665 ± 0.42b	867.972 ± 0.31c
4.	cadmium (Cd)	mg/kg	No detected	No detected	No detected	No detected
5.	Copper (Cu)	mg/kg	71.309 ± 0.23a	26.996 ± 0.18b	30.175 ± 0.41b	33.648 ± 0.32b
6.	Lead (Pb)	mg/kg	2.500 ± 43a	1.383 ± 51b	1.091 ± 21c	No detected

Letters a, b, c, d indicate significant differences between the values at $P > 0.05$.

enzymes. Only ARAI 3312 could not absorb nitrogen among the four isolates (Yanti et al., 2023). Most plants use indole-3-acetic acid (IAA) as their primary auxin. At the early stages of leaf development, in young leaves, and during seed germination, IAA is synthesized from tryptophan or indole. IAA also lowers intercellular concentrations and speeds up transpiration, stomatal conductance, and photosynthesis (Zou et al., 2023). Both nitrogen and phosphate, which are components of the nucleotides involved in the synthesis of amino acids and proteins, are macro elements that plants require for metabolism. Moreover, the primary component of plant chlorophyll is nitrogen (Etienne et al., 2018).

Analysis of N, P, K soil and *leaf of P. caninum*

Tables 5, 6 show notable differences in the NPK content of the soil and *P. caninum* leaves compared to the control group and the 1% treatment exhibited. There was a significant difference from the control in the case of P. caninum leaf NPK content. The 1% treatment yielded the highest N and K content, while the highest P was found in the 2%. B. agri plays a crucial role in increasing the NPK content in the soil, as it can fix nitrogen and dissolve phosphate and potassium. Therefore, the content in the leaf is also high because plants can easily absorb it after the nutrients are available. Rhizobacteria play a crucial role in providing nutrients, and PGPR can boost wheat plants' uptake of NPK minerals (Hafez et al., 2019). In the nitrogen fixation process, soil-dwelling microbes bind atmospheric nitrogen, making it available to plants as ammonia. Both non-symbiotic (free-living diazotrophs, *Azospirillum*) and symbiotic (*Azotobacter* spp., *Bacillus* spp., etc.) methods are possible with PGPR (Bhat et al., 2023). In the potato plant, PGPR and compost treatment enhanced P and k by 82.1% and 51.1% (Ekin, 2019). The concentration of N, P, and K in soil and maize crops can be increased by PGPR isolated from Bali (Maulina et al., 2022). P's solubilization and mineralization depend on the soil bacteria's actions. On the other side, phosphatase hydrolysis of phosphoric esters results in the mineralization of organic phosphorus (Vocciante et al., 2022). Experiments on cucumber and pepper plants show that *Paenibacillus* can enhance K solubility in the soil. Meanwhile, bacillus can also raise the willingness of K in the soil.

Colonization of rhizobacteria on the roots of *P. caninum*

The most favorable situation for plants is when rhizobacteria colonize their roots to gain the most benefits. According to a recent study, *B. agri* successfully colonized the roots of *P. caninum* after F1, F2, and F3 treatments but not the control plant, as shown in Figure 4. These three species can colonize the roots

TABLE 6 N, P, K analysis of *P. caninum* leaf.

No.	Parameters	Unit	Treatment			
			Control	F1	F2	F3
7.	Nitrogen (N)	%	3.26 ± 0.5a	3.43 ± 0.2d	3.29 ± 0.3b	3.27 ± 07a
8.	Phosphor us (P)	mg/kg	663.54 ± 0.21a	698.756 ± 0.32b	1.013.589 ± 0.43d	678.54 ± 0.91a
9.	Potassium (K)	mg/kg	19.879.219 ± 0.67a	20.857.707 ± 0.34c	19.541.517 ± 0.54b	18.876.236 ± 0.33a

Letters a, b, c, d indicate significant differences between the values at $P > 0.05$.

of maize plants, allowing for close interactions with the bacteria (Maulina et al., 2022). Free-living bacteria surrounding plant roots can exchange amino acids, proteins, enzymes, vitamins, and growth hormones in root exudates for nitrogen (Santoyo et al., 2021). Furthermore, PGPR alters the root system architecture by generating phytohormones and other signals that promote more lateral root branching and hair formation. It alters the plant's physiology, improves nutrition, and modifies the function of the root (Vacheron et al., 2013). To enhance their benefits to plants, PGPR colonizes roots, producing chemicals, fixing nitrogen, and dissolving phosphates (Ahemad and Kibret, 2014; Sayyed et al., 2015). Colonization of rooted rhizobacteria is closely related to the ability of rhizobacteria to produce hormones, enzymes (Sagar et al., 2022b), antibiotics (Vinay et al., 2016; Zakaria et al., 2016; Reshma et al., 2018), and biological fertilizers (Vejan et al., 2016; Kusale et al., 2021b). The rhizosphere should be created appropriately for plant growth, the bioavailability of N, P, K, and antagonistic characteristics should be increased, and PGPRs should be able to colonize host plant roots sufficiently. For PGPRs to be used as effective and successful bioinoculants, they must have specific properties. It must survive in soil, be compatible with the crop being inoculated, and interact with both abiotic and biotic soil microorganisms. The bioinoculants should be stabilized in soil systems, and any non-target effects should be prevented by taking the necessary precautions. These actions will ensure the longevity of the plant growth effect and the successful application of PGPRs as bioinoculants (Basu et al., 2021).

Conclusions

Treating 1%, 2%, and 3% B. agri on the *P. caninum* plant effectively improves growth and phytochemicals compound, with F1 (1% *B. agri*) as the best formula. *B. agri* is positive for the IAA test, protease enzyme, and can fix nitrogen. Furthermore, it can colonize the plant's roots and produce phytochemicals compounds butanoic acid, propanediol, and cyclopropane.

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, methodology, and writing original draft: NS and NR. Data analysis: DS, KP, and JA. Writing and review: IS. Editing: EH, Rusdianasari, KP, and JA. Fund acquisition: KP.

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Conflict of interest

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

Publisher's note

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Mitigation of drought or a combination of heat and drought stress effects on canola by Thuricin 17, a PGPR-produced compound

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Plant growth-promoting microorganisms(PGPMs) and the specific compounds they produce have the capacity to mitigate the adverse effects of stressors on plants. An example in this regard is Thuricin 17 (Th17), a signal molecule produced by *Bacillus thuringiensis* NEB17 (*Bt* NEB17), a plant growth-promoting rhizobacterium. In this study, we aimed to determine the efficacy of Th17 in mitigating drought and the combination of drought and heat stress in canola [*Brassica napus (L.)*]. Two of the best Th17 concentrations, 10^{-9} (Th1) M and 10^{-11} (Th2) M, were used either as seed treatment plus root drenching or foliar spray. Leaf area and biomass accumulation was increased by both application methods of Th1 under moderate and severe drought stress, whereas more promising results were seen from Th2-treated plants under the combination of stressors. Additionally, root length, root surface, and root volume were increased by 21%, 22%, and 23%, respectively, for plants grown from Th1 seed treatment plus root drenching compared to controls under severe drought conditions. Moreover, SOD, POD, and CAT contents were increased by spraying Th1 and Th2 under individual stresses and the combination of heat and drought, respectively. Accordingly, increases in physiological variables were observed for sprayed plants, which also had higher antioxidant contents. These results indicated that plant responses to the compound varied with concentration of Th17 and plant growth conditions. Specifically, when plants were grown under an individual stress condition, either drought or heat, the higher level of Th17 was more effective, whereas the lower dose demonstrated higher positive impacts under the combination of heat and drought. Regarding application method, both seed treatment plus root drenching and foliar spray had the ability to assist plants in alleviating stresses through growth stimulatory mechanisms. Therefore, Th17 has potential to become an environmentally friendly biostimulant, particularly under stressful environmental conditions.

KEYWORDS

microbial compounds, bacteriocins, canola, stressful conditions, biostimulants

Introduction

Crops are often simultaneously exposed to a variety of abiotic stresses in natural environments, which negatively impacts field crop development and productivity. Extreme temperatures and water deficit are two of the abiotic stressors posing the greatest threats to crop growth and yield, and consequently food security, under constantly changing climate conditions. Results of a meta-analysis from 120 studies regarding the combination of heat and drought demonstrates considerable negative influences on yield components and crop production (Cohen et al., 2021).

The climatic estimates for the year 2100 anticipate a 50% rise in the number of regions affected by drought, hence, a significant reduction in agricultural production seems almost certain. Heat waves and drought periods have both increased in frequency and severity, with more detrimental effects on agriculture than other climatic extremes (IPCC et al., 2022). Plants can tolerate, avoid, or escape abiotic stresses through evolved abiotic stress adaptation mechanisms or deliberate selection in agricultural breeding programs. However, the responses to the individual stresses could not be directly extrapolated to a combinations of stresses. Heat and drought responses are commonly regulated by several genes, and the underlying mechanisms are more complex than other stressors, such as biotic stress, which is largely defined by monogenic resistance. Moreover, other abiotic and/or biotic factors frequently have an additive effects on heat and drought responses, which causes studies in this field to be more complex (Deutsch et al., 2018; Lamaoui et al., 2018). Indications of drought stress, when soil water drops below a specific threshold, as detected through plants roots, results in ABA hormonal-signal transduction. Transduction of this hormonal signal within xylem sap causes stomatal closure, to reduce transpiration. A decrease in the stomatal conductance and carbon dioxide (CO_2) diffusion tremendously decreases carbohydrate production, leading to less crop production (Chatterjee and Solankey, 2015; Giordano et al., 2021). Heat waves, which are typically associated with drought, make this effect worse by hastening soil drying and worsening water vapor pressure deficit. Heat stress also hinders photosynthesis in plants, largely by interfering with metabolic processes including denaturation of proteins, enzymes, nucleic acids, and cell membranes (Nadeem et al., 2018; Janni et al., 2020). Additionally, the equilibrium of reactive oxygen species (ROS) production and scavenging could be disrupted by abiotic stressors, causing accumulation of these extremely reactive and toxic materials. Accordingly, plants have evolved defense mechanisms through activation of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) to scavenge ROS in cells (Zhou et al., 2019; Zhanassova et al., 2021). Hence, sustainable technologies are essential to overcome the challenges of increasing agriculture production under rapidly changing climatic conditions. In the past three decades, a number of technical innovations have been proposed to boost crop production. In this regard, the development of plant biostimulants, including microorganisms and/or substances they produce, could be a promising approach for addressing these pressing issues (Yakhin et al., 2017; Chiaiese et al., 2018; Antar et al., 2021; Shah et al., 2021). Among biostimulants, microbial-derived compounds could provide plants with necessary features to develop and grow by enhancing access to nutrients (Matse et al., 2020; Tang et al., 2020), producing phytohormones (Cassán et al., 2014; Moon and Ali, 2022), improving antioxidant defense system functionality (Khalilzadeh et al., 2018; Neshat et al., 2022), and/or inhibiting harmful microorganisms (Suryadi et al., 2019; Xia et al., 2020; Aioub et al., 2022) under both non-stressful or stressful conditions. Regarding the extracellular substances of PGPMs, some of these can be bacteriocins, which can control the dynamics of the plant-associated microbial population (phytomicrobiome) by acting against microbial strains closely related to the producer strain. Numerous bacterial taxa, including those found in the rhizosphere microbiome, have been shown to produce at least one bacteriocin, and *Bacillus* species were one of the first groups to be examined for the production of various bacteriocins. *Bt* NEB17 was isolated from soybean root nodules by our laboratory, which was since determined to produce and excrete a bacteriocin named Thuricin 17 (Th 17); none of the other discovered bacteriocins have been studied as extensively as Th17 (Gray et al., 2006a). This signal molecule was partially sequenced by Gray et al. (2006b) and its full sequence was eventually determined (Lee et al., 2009). The molecular weight of Th17 is low, 3.162 kDa, and this compound is highly resistant to heat and pH (range of 1.0–9.25) with anti-microbial activity and plant growth promotion capacity, particularly under stressful conditions (Jung et al., 2011; Prudent et al., 2015; Schwinghamer et al., 2016; Subramanian et al., 2016; Nazari and Smith, 2020; Nazari et al., 2022). We should add that the most recent characterization of Th17 has revealed differences from our initial understating; hence we are considering renaming it Bacillin 20. The use of bacteriocins in the food industry is of great importance, but little research has been done on their agronomic potential. Therefore, we are here attempting to determine the potential role of Th 17 in growth and development of canola, a valuable crop producing one of the healthiest oils for human consumption, under drought and the combination of heat and drought. This study is the perquisite experiment to examine two concentrations and application methods of Th17 for further field studies to develop a biostimulant to make crops more resilient to climate change.

Materials and methods

Production and purification of Th17

Th 17 was extracted and purified according to Gray et al. (2006a,b). In brief, King's B medium was used to culture *Bt* NEB17 as previously mentioned (Gray et al., 2006a). Bacterial cells were collected from plated material and cultured in 250 ml flasks with 50 ml medium for the initial broth inoculum. The bacterium was grown for 48 h on an orbital shaker revolving at 150 rev min^{-1} at 28 ± 2°C. Next, 5 ml of subculture were added to 2 liters of broth as an initial culture, and the culture was grown under the same conditions on the shaker. Bacterial populations were measured after 96 h with a Pro UV/Visible Spectrophotometer at 600 nm. Bacterial cultures were grown to an $O.D._{600nm}$ of at least 1.4 or ∼5.5 log CFU (colony forming units) cells ml^{-1} (Gray, 2005). Bacterial culture samples were centrifuged at 13,000 g for 10 min, then cell-free supernatants (CFS) were used for analytical-HPLC identification. For partial purification of Th17, 0.8 L of butanol was added to 2 L of the culture for 12 h, after which the upper layer was collected for rotary evaporation. The resulting viscose extract was diluted with 12 ml of 30% acetonitrile (ACN). The sample was centrifuged at 13,000 g for 10 minutes, followed by serial fractionation with ACN and HPLC identification. The chromatographic conditions were set as follows: column—Vydac C18 reversed-phase column (0.46 ×25 cm; 5μ), 25°C temperature, 1 ml/min flow rate, 214 nm detector wavelength, and a gradient of 18%—95% throughout the 18-min run. By comparing the retention time of a standard Th17 sample, the corresponding peak to Th17

was found (Figure 1). According to chromatographs, the highest peak of Th17 was observed once the peptide collected in 60% ACN. This fraction was lyophilized and then stored at −20°C for dilution to the required levels. Here, Th17 at 10^{-9} M (Thu1) and 10^{-11} M (Thu2) were used in all experiments.

Plant growth experiments

Untreated canola seeds of cultivar (cv.) InVigor L233P were used; this hybrid is recommended for short to mid-length growing season zones. For seed disinfection, 20% bleach (6% sodium hypochlorite, NaOCl) was used; seeds were then rinsed with distilled water until odorless. Th17 levels were applied either as a pre-planting seed treatment followed by root drench or spray at the time of stress. For the seed treatment experiments, seeds were soaked in 10 ml of two concentrations of Th17, 10^{-9} and 10^{-11} M, suggested from germination data analysis (Nazari et al., 2022), and distilled water as a control, which was followed by weekly root drenching of the compound (three times in total) until stress induction time (based on the initial experiments), while Th17 solutions and distilled water were sprayed on leaves with an atomizer just prior to stress induction for foliar experiments. For leaf sprays, Tween 20 (0.01%) was added as a surfactant to treatment solutions, including the control. To prevent dripping of the treatment solutions onto the soil, vinyl plastic was placed over the top surfaces of the pots. Canola seeds were placed in 10 cm pots filled with AGRO MIX® G10 media. The plant growth chamber (Conviron R, Canada) conditions were as follows: 22/18°C day/night, photoperiod of 14/10 h light/darkness cycle, 60%−70% relative humidity, and photosynthetic irradiance of 350–400 μmol m^{-2} s^{-1} (Nazari et al., 2022). The plants were trimmed to one seedling per container after a week. Plants were grown until the end of the third week and regularly watered using half strength Hoagland's solution. Then, 3-week-old plants were exposed to four levels of the stressors, where one level was the control. To induce uniform water stress, polyethylene glycol 8000 (PEG) was used; levels were control −0.25 MPa (half strength Hoagland solution for control), mild drought −0.5 MPa (197 g L^{-1} PEG), moderate drought −0.9 MPa (270 g L^{-1} PEG), or severe drought −1.3 MPa (330 g L^{-1} PEG) (Michel, 1983). For foliar experiments, the leaves were sprayed with Th17 solutions and water as the control, until the leaves were uniformly wet at the onset of stress induction. The temperature levels were 22/18°C day/night for drought stress experiments, whereas for the heat and drought combination, the same levels of drought stress were applied, but at 30/18°C day/night (Elferjani and Soolanayakanahally, 2018). In each case, the plants were allowed to grow for 2 weeks following the onset of stress treatment, and then sampled for data collection. For determination of plant physiology responses to the treatments, photosynthetic rate, transpiration rate, and stomatal conductance inside the leaves were measured (LI-COR 6400 portable photosynthesis meter at a constant CO_2 concentration of 400 ppm and light intensity of 500 μmol m^{-2} s^{-1}); readings were taken from the upper-most fully expanded leaf of each plant 1 day before stress onset, 1 day after, 1 week after, and just prior to harvesting. Harvested plants at the end of the experiment were used for leaf area, fresh weight, dry weight, and root trait data collection. For measuring root variables, after harvesting, soil was gently shaken off from roots followed by washing and using WinRHIZO™ in order to scan and measure total root length, total root surface, root volume, and root diameter.

Determination of antioxidant enzyme levels

Five hundred milligrams of fresh leaf tissue were ground to a fine powder in liquid nitrogen and then homogenized in 1.2 ml of 0.2 M sodium phosphate buffer (pH 7.8 with 0.1 mM EDTA). The samples were centrifuged at 13,000 g × for 10 min at 4°C and the supernatant was then subjected to enzyme activity assays. All spectrophotometric analyses were conducted on an Ultrospec (4,300 pr) UV/visible spectrophotometer. SOD activities were using the method employed by Giannopolitis and Ries (1977). The reaction mixture contained 50 ml of extract, 50 mM Tris-HCl (pH 8.0), 63 mM NBT, 1.3 mM riboflavin, 13 mM methionine, and 0.1 mM EDTA. One unit of SOD was determined as the amount of enzyme required to produce 50% inhibition of the rate of nitro blue tetrazolium photoreduction measured at 560 nm. POD activities were determined using the Britton and Mehley (1955) method; the reaction mixture contained 0.1 ml of extract, 50 L of 20 mM tetraguaiacol, and 2.8 ml of 50 mM Tris-HCl buffer (pH 8.0). By adding 20 μl of 40 mM H_2O_2 the reaction started, and after recording the change in absorbance at 470 nm for 1 min, POD activity was calculated using the extinction coefficient of tetraguaiacol. One unit of activity was determined by the required amount of enzyme for the formation of 1 μmol of tetraguaiacol min^{-1}. The CAT assay method described by Hugo and Lester (1984) were used to measure enzyme activities by monitoring oxidation of H_2O_2. The enzyme activity was calculated using extinction coefficient of H_2O_2 (40 M^{-1} cm^{-1} at 240 nm), which is expressed as the amount of enzyme activity required to decompose 1 μmol of H_2O_2 per minute.

Data analysis

The experiments were organized following a factorial randomized design with four replicates. Each experiment was repeated twice, and the data pooled for analysis using SAS 9.4; differences between means were considered statistically significant at $p < 0.05$, using Tukey's test. All analyses were conducted separately for seed treatment plus root drenching and foliar spray experiments since comparison of application methods is not a goal for this study. Two-way variance analysis was conducted, where Th17 treatment was one factor and stressful conditions was another. When the interaction of the compound and stressors was absent, one-way ANOVA was performed to indicate the main effects. For physiological variables, two-way repeated measures ANOVA was applied at each stress level.

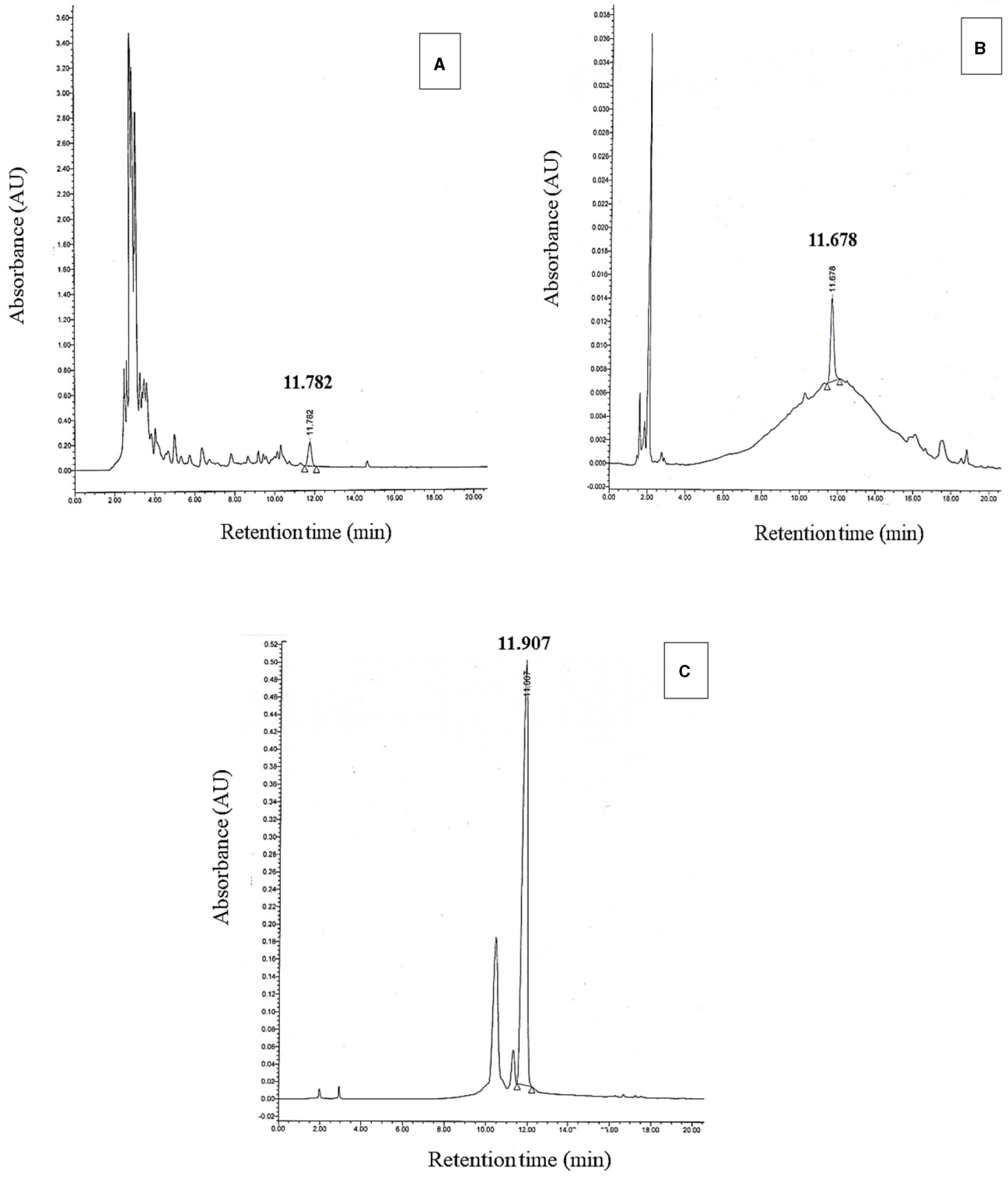

FIGURE 1
HPLC chromatograms for butanol extraction of the bacterial culture **(A)** Th17 standard **(B)** and Th17 collected in 60% ACN **(C)**.

Results

Morphological trait responses

Leaf area

Leaf area is an important indicator of vegetative growth and strongly responded to Th17 and stressors. For seed treatment plus root drenching experiments, leaf area significantly decreased across all drought levels for controls without Th17 treatment, where it dropped by ∼50% under severe drought, whereas significant reductions for Th17-treated plants occurred only under severe stress. Under moderate and severe drought stress alone, Th1-treated plants had 12.5 and 14.5% more leaf area than controls, respectively (Figure 2A). Similarly, spraying Th1 significantly increased leaf area by 13% compared to non-sprayed plants under severe drought (Figure 2B). Under non-stressful conditions, no stimulatory effects of Th17 were reported. For heat and drought experiments, significant decreases in leaf area occurred across heat and drought combinations. Seed treatment plus root drenching of Th2 significantly enhanced leaf area (by 27%) under the combination of moderate drought and heat stress (Figure 2C). Interestingly, the interaction of spray application and stressful conditions was significant ($p = 0.03$) in that foliar spraying of Th2 could significantly offset decreases in leaf area, by ∼19%, when moderate drought and heat occurred simultaneously (Figure 2D). However, neither seed treatment nor foliar spray could substantially offset leaf area reduction under severe drought and heat combination.

Fresh biomass

The interaction of drought stress and Th17 seed treatment plus root drenching was significant ($p = 0.04$) in that Th17 increased fresh biomass accumulation under either stressful or normal conditions. The highest fresh biomass resulted from seed treatment with Th17 plus root drenching, for Th2, at 37 g under normal conditions; however, severe drought decreased fresh biomass accumulation by 40% compared to optimal conditions, while Th1 offset this reduction by 27% (Figure 3A). Similar results were observed for plants sprayed with Th1; it significantly increased fresh biomass accumulation by 23 and 21% under moderate and severe drought conditions, respectively (Figure 3B). A drastic decline of fresh biomass was observed when a combination of drought and heat stress was imposed, although application of Th2 had significant stimulatory effects, resulting in 24 and 28% more biomass accumulation under moderate stresses for seed treatment plus root drenching (Figure 3C) and foliar spray (Figure 3D) experiments, respectively. Moreover, those plants grown with Th17 treatments, both application methods, could produce more fresh biomass under heat stress than controls, but the differences were not significant (Figures 3C, D).

Dry biomass

For seed treatment plus root drenching experiments, significant decreases in biomass accumulation were observed under severe drought stress while application of Th1 caused 16 and 14% more biomass production under conditions of moderate and severe drought, respectively, compared to the controls (Figure 4A). However, the interaction of drought stress and Th17 leaf spraying was significant ($p = 0.02$); Th1 increased biomass accumulation with greater increases at more severe drought stress levels (Figure 4B). When the two stressors were combined, considerable biomass reductions were reported across all combinations. However, Th17 applications, either seed treatment plus root drenching (Figure 4C) or spraying (Figure 4D), could enhance dry biomass compared to controls, although those increases were not depicted statistically significant, except for the effect of spraying of Th2 where dry biomass increased by 19% compared to control under moderate drought and heat combination.

Root system architecture

The interaction of Th17 seed treatment plus root drenching and drought stresses was significant for all root variables (Figure 5) except diameter: root length ($p < 0.04$), root surface ($p < 0.01$), and root volume ($p < 0.01$) were significantly increased. At increased drought stress levels, Th1 remarkably increased root length, presumably to help plants gain better access to water; this variable ranged from 2,882 cm for untreated seeds under well-watered conditions to 5,090 cm in response to Th1 seed treatment plus root drenching under severe drought conditions. Similarly, the greatest root volume (2.9 cm^3) and root surface (410 cm^2) were reported for plants grown with Th1 seed treatment plus root drenching. However, neither seed treating plus root drenching nor leaf spraying resulted in significant impacts under control (unstressed) conditions. Likewise, spraying Th17 did not significantly stimulate root development under drought conditions; roots merely responded to drought levels in which the greatest level of root attributes occurred under severe drought conditions (Table 1). Root diameter did not respond to growing conditions or Th17 applications.

In contrast to individual stresses, root variables considerably decreased under the combination of both stresses. Table 2 indicates that increasing the severity of stresses had negative effects on root growth and development where neither watering with Th 17 supplements nor foliar spray caused significant changes compared to controls. The greatest amounts, 3,664 cm for root length, 306 cm^2 for surface, and 2.13 cm^3 for volume, were for Th2 seed treatment plus root drenching under the combination of mild drought and heat. On the contrary, Th17 seed treatment plus root drenching treatments greatly assisted in developing better under-ground development under heat conditions; root length, root surface, and root volume increased by 30%, 27%, and 25%, respectively, for 10^{-9} M Th17 (Th1) seed treatment plus root drenching. In terms of root diameter, no significant changes were reported across all treatments and stressful circumstances.

Biochemical responses

Antioxidant enzymes

Plants under PEG-induced drought stress showed significant increases in the amount of SOD, POD, and CAT. For seed treatment

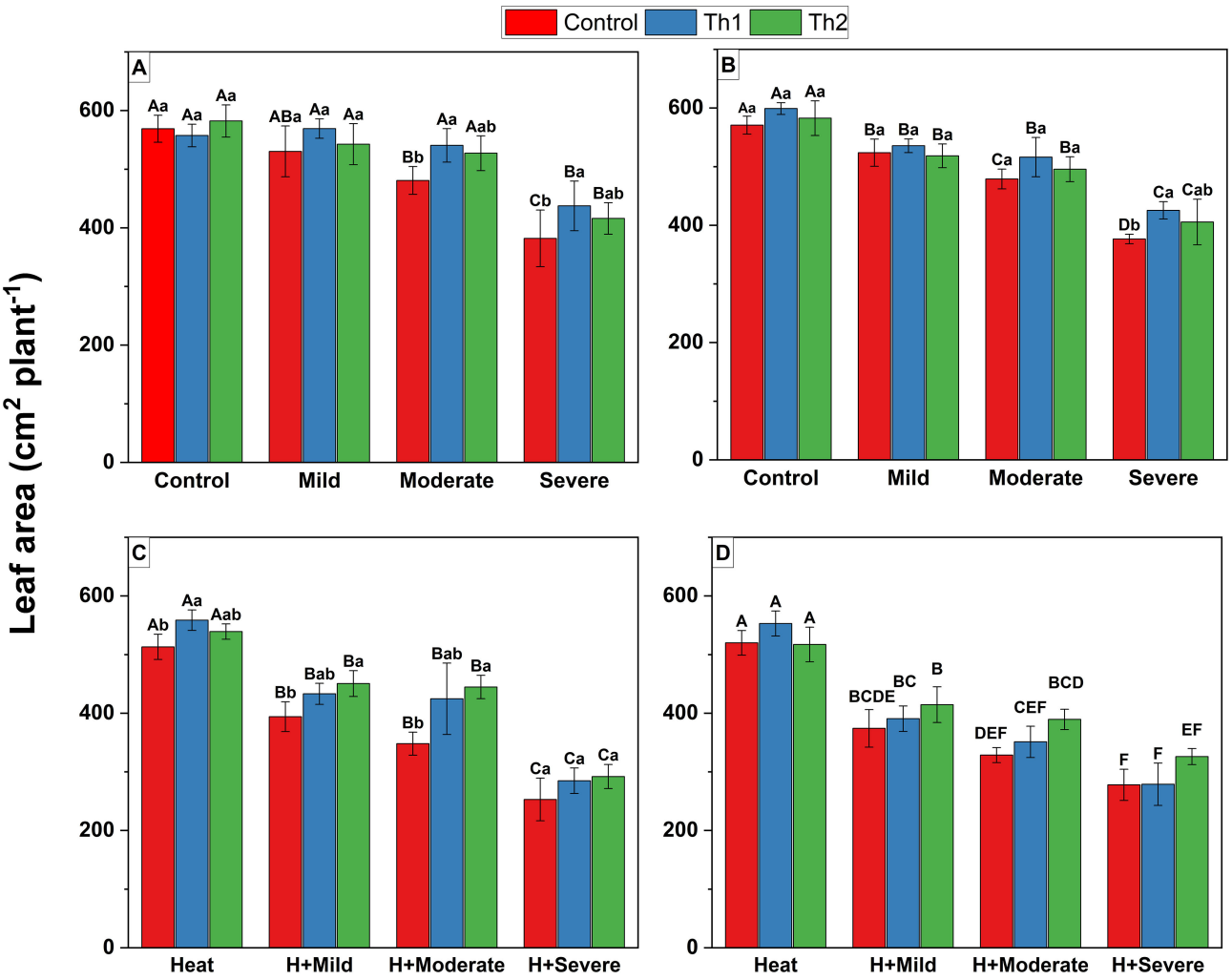

FIGURE 2
Effect of Th17 application on leaf area under drought **(A, B)** and combination of drought and heat **(C, D)**. **(A, C)** Seed treatment plus root drenching and **(B, D)** foliar spray of distilled water (Control), Th1 (10^{-9} M) and Th2 (10^{-11}M). Combination of lower and capital letters represents one-way ANOVA results for main effects; capital letters represent differences between different growth conditions at the same level of Th17 whereas lower letters indicate differences between different levels of Th17 at the same growth conditions. The absence of lower and capital letter combination indicates significant interaction of Th17 and stressful conditions by two-way ANOVA. Each bar represents mean ± standard error ($n = 8$). Means with the same letters are not significantly different ($p < 0.05$).

plus root drenching experiments, antioxidant enzyme contents were considerably affected by drought stress levels; the greatest numbers, 181 unit mg^{-1} fw for SOD (Th2), 119 units mg^{-1} fw for POD (Control), and 24 units mg^{-1} fw for CAT (Th2), were recorded under severe drought stress levels (Table 3). However, for foliar spray, the interaction of spraying the compound and drought levels was significant for SOD ($p < 0.05$), POD contents ($p < 0.02$), and CAT ($p < 0.04$); they were increased by 56%−29%, 31%−18%, and 56%−58% respectively in Th1 foliar sprayed plants compared to controls, under moderate to severe drought levels. When drought and heat were combined, antioxidant enzyme contents enhanced by increasing the severity of drought level, where the highest number of enzymes were reported under the combination of severe drought and heat conditions for both Th17 application experiments. Specifically, the antioxidant enzyme levels in plants grown from treated seeds plus root drenching were quite similar to untreated plants in a way that stress levels seem to be the stimulant for changes. For foliar experiments, enzymes did not meaningfully respond to Th17 under heat or the combination of heat and mild drought but did respond under the moderate and severe combination with heat (Table 4). Accordingly, the greatest increases, 33 and 20%, were observed with Th2 foliar sprayed plants over the controls for SOD, once heat was combined with moderate and severe drought, respectively. Similarly, spraying Th2 significantly increased the amount of POD and CAT by 26 and 56% under the combination of heat and severe drought stress, respectively.

Physiological responses

Photosynthetic rate

Figure 6 indicates that photosynthetic rate was considerably reduced by all stresses; drought aggravated it to a greater extent

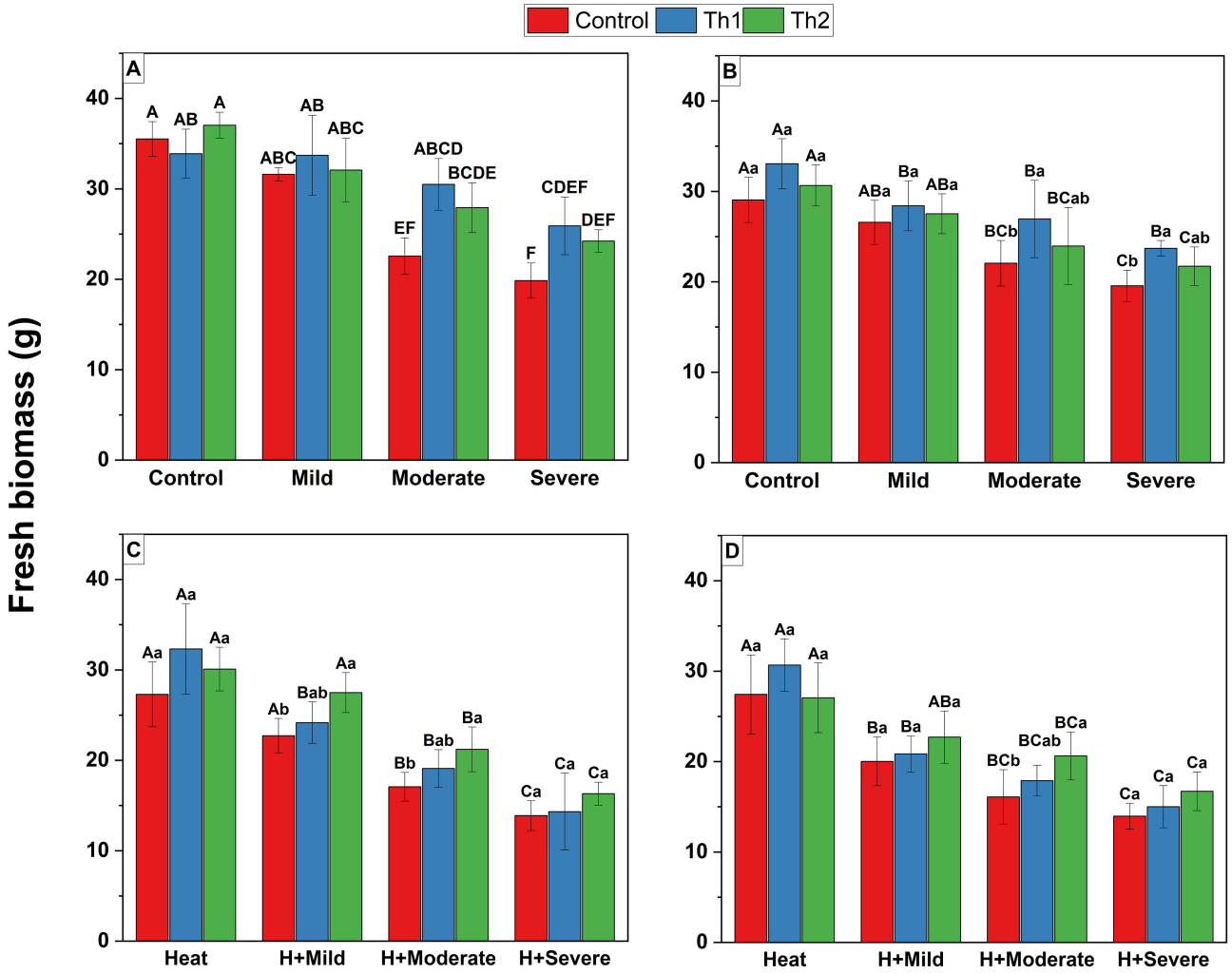

FIGURE 3
Effect of Th17 application on fresh biomass under drought **(A, B)** and combination of drought and heat **(C, D)**. **(A, C)** Seed treatment plus root drenching and **(B, D)** foliar spray of distilled water (Control), Th1 (10^{-9} M) and Th2 (10^{-11} M). Combination of lower and capital letters represents one-way ANOVA results for main effects; capital letters represent differences between different growth conditions at the same level of Th17 whereas lower letters indicate differences between different levels of Th17 at the same growth conditions. The absence of lower and capital letter combination indicates significant interaction of Th17 and stressful conditions by two-way ANOVA. Each bar represents mean ± standard error (n = 8). Means with the same letters are not significantly different ($p < 0.05$).

than heat, and it was greatly inhibited by the heat and drought stress combination. Under optimal growing conditions, no significant effect of treatments, either seed treatment plus root drenching or foliar spraying of Th17, was observed. Photosynthetic rate of plants grown from treated seeds and drenched roots did not show meaningful changes compared to untreated plants under drought and the combination of drought and heat (Figures 6A, C). However, spraying Th17 could alleviate reductions related to stresses compared to unsprayed plants. Assimilation rates in those plants which were supplementary sprayed with Th1 demonstrated increases during all measurement times across drought levels; particularly mean photosynthetic rate significantly increased by 14%, 23%, and 32% compared to controls under mild, moderate, and severe drought conditions, respectively, 1 day after spraying, whereas this significant stimulatory effect lasted until 1 week after spraying under severe drought conditions (Figure 6B). Likewise, spraying the supplement, in particular Th2 one day after spraying, caused meaningful responses in carbon assimilation rate under heat stress alone and the combinations of heat with drought levels (Figure 6D). Interestingly, the effect of the sprayed supplements could significantly last until 2 weeks and 1 week under individual heat and combination of mild drought and heat stresses, respectively.

Stomatal conductivity

Stomatal conductivity was greatly decreased by drought, but the decreases were smaller when both stresses were applied simultaneously; instead, heat stress increased stomatal conductance. No statistically significant changes in the stomatal conductivity resulted from Th17 applications either when the supplement was applied to seeds plus root drenching or spraying

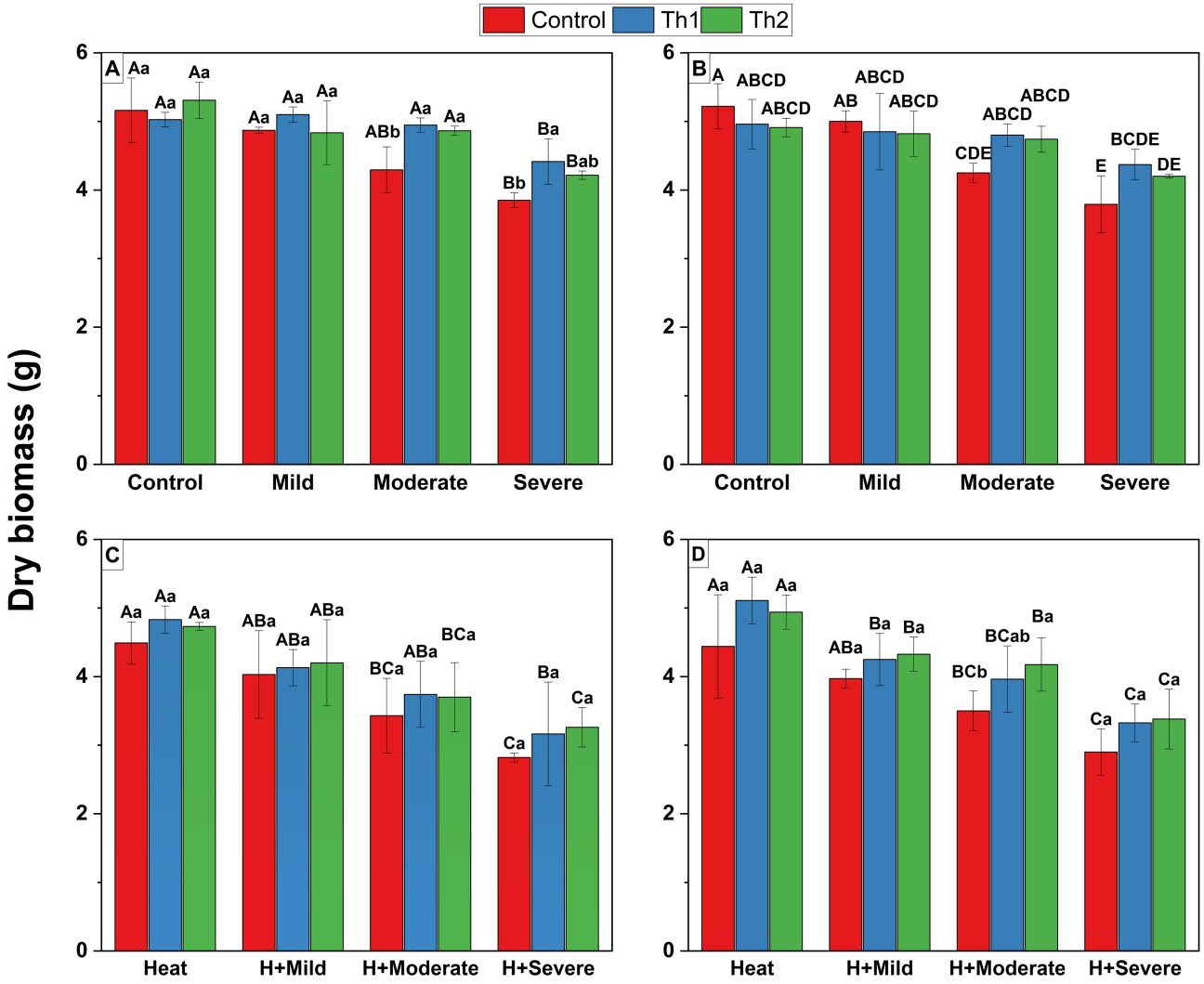

FIGURE 4
Effect of Th17 application on dry biomass under drought **(A, B)** and combination of drought and heat **(C, D)**. **(A, C)** Seed treatment plus root drenching and **(B, D)** foliar spray of distilled water (Control), Th1 (10^{-9} M) and Th2 (10^{-11} M). Combination of lower and capital letters represents one-way ANOVA results for main effects; capital letters represent differences between different growth conditions at the same level of Th17 whereas lower letters indicate differences between different levels of Th17 at the same growth conditions. The absence of lower and capital letter combination indicates significant interaction of Th17 and stressful conditions by two-way ANOVA. Each bar represents mean ± standard error (n = 8). Means with the same letters are not significantly different ($p < 0.05$).

across all conditions. Specifically, plants developed from Th17 treated seeds plus root drenching had insignificantly greater stomatal conductivity during almost all measurements for drought, heat and the combination of heat and drought (Figures 7A, C). Equally, for foliar experiments, stomatal conductance for sprayed plants was slightly higher than controls, particularly for Th1 under drought and Th2 when drought stress was accompanied by heat stress, but they were not significant (Figures 7B, D).

Transpiration rate

The results of measuring transpiration rate at four different times for both seed treatment plus root drenching and spraying experiments demonstrated that compared to optimal conditions, it was slighlty reduced by heat while sharp declines were observed when the plants were subjected to drought and a combination of drought and heat stresses; however, the decreases were greater under drought alone (Figures 8A, B) where the lowest transpiration rate resulted from severe drought stress. Both treated plants, either seed plus root drenching (Figures 8A, C) or sprayed ones (Figures 8B, D), had marginally higher transpiration rates than controls across all stressful conditions, although they were not statistically distingushable.

Discussion

Plants will be encountering higher average temperatures and more extreme drought episodes due to climate change, which will potentially cause marked declines in their productivity. Hence, studies regarding the discovery of potential approaches to assist

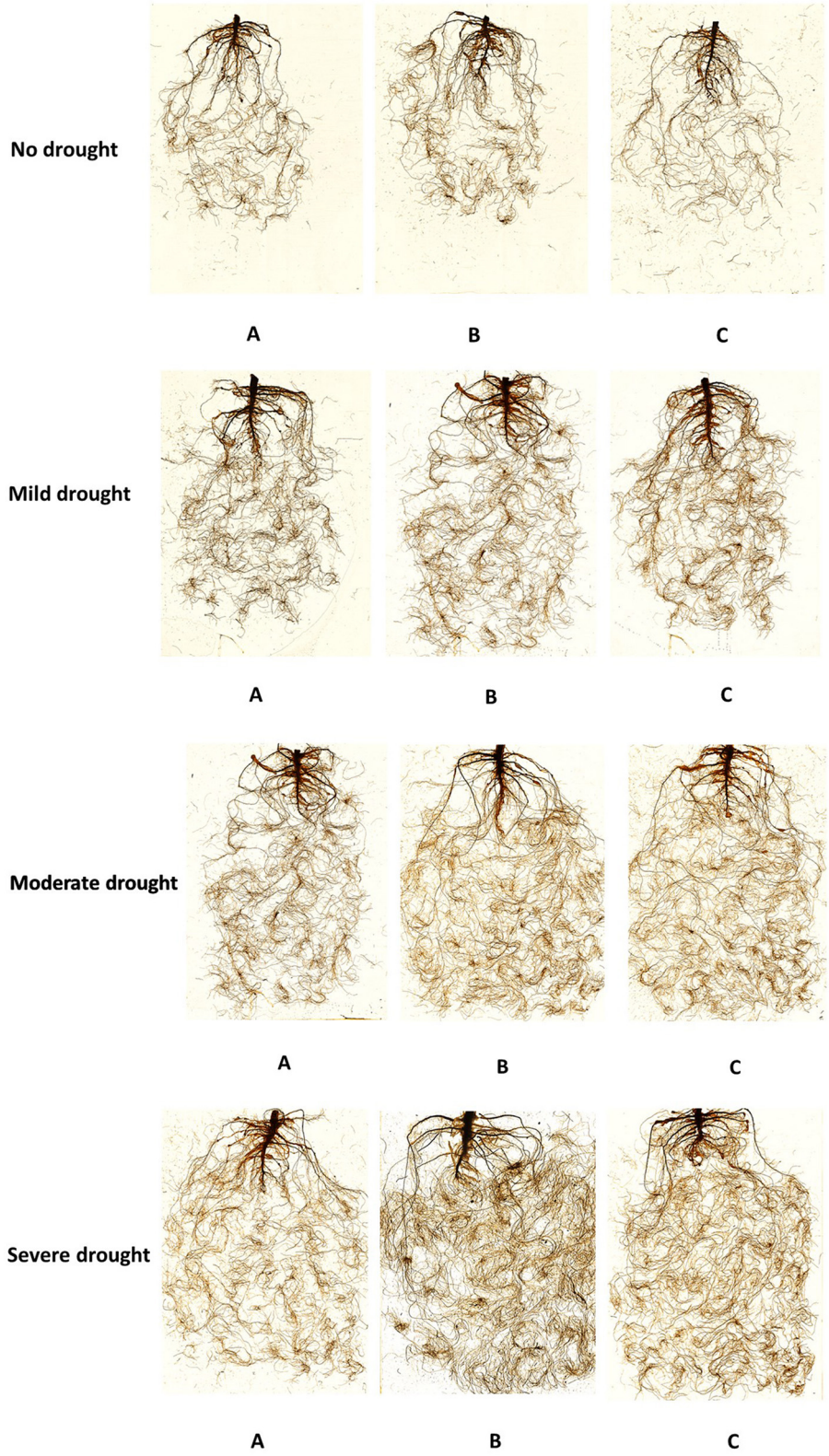

FIGURE 5
5-week-old Canola root images of seed treatment plus root drenching with distilled water **(A)**, 10 $^{-9}$ M Th17 **(B)**, C: 10^{-11} M Th17 **(C)** under control, mild, moderate, and severe drought stress, respectively.

TABLE 1 Effect of Th17 application on root variables under drought stress levels.

Stressors	Th17 treatments	Total root length (cm)	Total root surface (cm^2)	Root diameter (mm)	Root volume (cm^3)
Th17 seed treatment plus root drenching experiment					
Control	Control	$2,882 \pm 249^{G}$	240 ± 11^{E}	0.26 ± 0.2^{Aa}	1.53 ± 0.08^{E}
	Th1	$3,051 \pm 276^{FG}$	256 ± 22^{F}	0.253 ± 0.03^{Aa}	1.67 ± 0.13^{E}
	Th2	$3,170 \pm 49^{EFG}$	263 ± 15^{EF}	0.259 ± 0.03^{Aa}	1.73 ± 0.09^{E}
Mild drought	Control	$3,676 \pm 403^{EFG}$	301 ± 16^{DE}	0.25 ± 0.1^{Aa}	2.14 ± 0.1^{D}
	Th1	$3,973 \pm 265^{CDE}$	330 ± 14^{CD}	0.272 ± 0.02^{Aa}	2.37 ± 0.08^{CD}
	Th2	$3,871 \pm 272^{CDEF}$	317 ± 19^{D}	0.264 ± 0.01^{Aa}	2.29 ± 0.12^{CD}
Moderate drought	Control	$3,820 \pm 412^{DEFG}$	323 ± 18^{CD}	0.31 ± 0.6^{Aa}	2.37 ± 0.1^{CD}
	Th1	$4,879 \pm 327^{AB}$	393 ± 17^{AB}	0.267 ± 0.01^{Aa}	2.65 ± 0.14^{AB}
	Th2	$4,589 \pm 217^{ABCD}$	362 ± 13^{BC}	0.268 ± 0.01^{Aa}	2.46 ± 0.1^{BC}
Severe drought	Control	$4,218 \pm 405^{BCDE}$	337 ± 14^{CD}	0.3 ± 0.2^{Aa}	2.4 ± 0.1^{C}
	Th1	$5,090 \pm 217^{A}$	410 ± 11^{A}	0.285 ± 0.01^{Aa}	2.9 ± 0.12^{A}
	Th2	$4,618 \pm 154^{ABC}$	381 ± 23^{AB}	0.267 ± 0.01^{Aa}	2.72 ± 0.07^{AB}
Th17 foliar spray experiment					
Control	Control	$2,873 \pm 231^{Ba}$	234 ± 9^{Ca}	0.251 ± 0.01^{Aa}	1.43 ± 0.09^{Ca}
	Th1	$2,906 \pm 192^{Ca}$	244 ± 9^{Ca}	0.257 ± 0.01^{Aa}	1.50 ± 0.11^{Ba}
	Th2	$3,085 \pm 369^{Ba}$	238 ± 26^{Ca}	0.254 ± 0.01^{Aa}	1.48 ± 0.13^{Ca}
Mild drought	Control	$3,128 \pm 210^{Ba}$	261 ± 18^{BCa}	0.250 ± 0.01^{Aa}	1.77 ± 0.07^{Ba}
	Th1	$3,306 \pm 120^{BCa}$	278 ± 16^{Ba}	0.256 ± 0.02^{Aa}	1.92 ± 0.1^{Aa}
	Th2	$3,185 \pm 216^{Ba}$	266 ± 14^{BCa}	0.262 ± 0.02^{Aa}	1.82 ± 0.06^{Ba}
Moderate drought	Control	$3,614 \pm 186^{Aa}$	302 ± 27^{ABa}	0.262 ± 0.02^{Aa}	2.11 ± 0.13^{Aa}
	Th1	$3,577 \pm 345^{ABa}$	292 ± 10^{ABa}	0.265 ± 0.01^{Aa}	2.06 ± 0.14^{Aa}
	Th2	$3,736 \pm 294^{Aa}$	305 ± 17^{ABa}	0.252 ± 0.005^{Aa}	2.01 ± 0.12^{ABa}
Severe drought	Control	$3,819 \pm 215^{Aa}$	316 ± 25^{Aa}	0.254 ± 0.01^{Aa}	2.17 ± 0.04^{Aa}
	Th1	$3,783 \pm 192^{Aa}$	308 ± 18^{Aa}	0.257 ± 0.01^{Aa}	2.03 ± 0.1^{Aa}
	Th2	$3,885 \pm 369^{Aa}$	320 ± 15^{Aa}	0.254 ± 0.01^{Aa}	2.21 ± 0.16^{Aa}

Control: distilled water, Th1: 10^{-9} M of Th17, Th2: 10^{-11} M of Th17. Each value represents mean ± standard error ($n = 8$). Combination of lower and capital letters represents one-way ANOVA results for main effects; capital letters represent differences between different growth conditions at the same level of Th17 whereas lower letters indicate differences between different levels of Th17 at the same growth conditions. The absence of lower and capital letter combination indicates significant interaction of Th17 and stressful conditions by two-way ANOVA. Means with the same letters are not significantly different ($p < 0.05$).

crops in combating stressful conditions are a primary concern. Various research findings have proven the stimulatory effect of PGPMs under stressful conditions (Sarkar et al., 2018; Khan et al., 2019; Lin et al., 2020; Shah et al., 2021; Mellidou and Karamanoli, 2022); however, studies regarding the efficacy of microbial-derived materials such as signal molecules or cell-free supernatants are limited. Signal compounds are secreted into the producer strains growing medium, which, after being filtered, will still maintain the metabolites that possess plant growth stimulatory effects. Here, our experiments regarding the application of a bacterially produced compound, Th17, under stressful conditions highlights that compound concentration, stress level, and application method play pivotal roles in the effectiveness of Th17 as a plant growth biostimulant. The responses of plants to a single stress can be completely different from the conditions in which several stresses coincide. In this regard, the interaction of Th17 application with growing conditions was significant for several variables, while Th17 treatments caused different reactions at different stress levels. Plant morphological, physiological, and biochemical features were considerably affected by the induction of stresses where signal molecule treatments could partially assist in mitigating stress-associated damage. Above-ground morphological traits, including leaf area and fresh and dry biomass, were enhanced by the application of Th17 under stressed conditions; however, no distinguishable impacts were observed under optimal growth conditions. Specifically, the bacterial compound produced better results under moderate and severe drought stresses alone, but when the two stressors were combined, their effectiveness was reduced, particularly under the combination of severe drought and heat, which could be due to reasons such as high intensity of PEG induced-drought level or prolongation of stresses. This implies that increasing the PEG level with heat decreased the efficacy of

TABLE 2 Effect of Th17 application on root variables under drought and heat combination.

Stressors	Th17 treatments	Total root length (cm)	Total root surface (cm^2)	Root diameter (mm)	Root volume (cm^3)
Th17 seed treatment plus root drenching experiment					
Heat	Control	$3,432 \pm 252^{Ab}$	280 ± 26^{Ab}	0.253 ± 0.02^{Aa}	1.92 ± 0.15^{Ab}
	Th1	$4,481 \pm 234^{Aa}$	355 ± 48^{Aa}	0.259 ± 0.03^{Aa}	2.4 ± 0.2^{Aa}
	Th2	$4,220 \pm 170^{Aa}$	322 ± 30^{Aab}	0.257 ± 0.03^{Aa}	2.3 ± 0.14^{Aa}
Heat + mild drought	Control	$3,247 \pm 213^{Aa}$	254 ± 23^{ABa}	0.257 ± 0.01^{Aa}	1.82 ± 0.25^{ABa}
	Th1	$3,595 \pm 439^{ABa}$	304 ± 33^{ABa}	0.272 ± 0.02^{Aa}	2.1 ± 0.14^{ABa}
	Th2	$3,664 \pm 421^{Ba}$	306 ± 34^{Aa}	0.264 ± 0.01^{Aa}	2.13 ± 0.17^{ABa}
Heat + moderate drought	Control	$2,607 \pm 372^{Ba}$	221 ± 23^{Ba}	0.31 ± 0.06^{Aa}	1.54 ± 0.2^{ABa}
	Th1	$3,104 \pm 197^{Ba}$	255 ± 23^{Ba}	0.267 ± 0.00^{Aa}	1.76 ± 0.23^{BCa}
	Th2	$3,302 \pm 478^{BCa}$	265 ± 17^{Ba}	0.268 ± 0.01^{Aa}	1.93 ± 0.17^{BCa}
Heat + severe drought	Control	$2,359 \pm 399^{Ba}$	229 ± 29^{Ba}	0.3 ± 0.02^{Aa}	1.4 ± 0.18^{Ba}
	Th1	$2,654 \pm 349^{Ca}$	243 ± 33^{Ba}	0.267 ± 0.01^{Aa}	1.57 ± 0.2^{Ca}
	Th2	$2,817 \pm 594^{Ca}$	253 ± 24^{Ba}	0.285 ± 0.01^{Aa}	1.66 ± 0.1^{Ca}
Th17 foliar spray experiment					
Heat	Control	$3,532 \pm 419^{Aa}$	295 ± 17^{Aa}	0.25 ± 0.1^{Aa}	2.10 ± 0.1^{Aa}
	Th1	$3,374 \pm 233^{Aa}$	276 ± 25^{Aa}	0.252 ± 0.01^{Aa}	1.93 ± 0.2^{Aa}
	Th2	$3,516 \pm 307^{Aa}$	291 ± 22^{Aa}	0.251 ± 0.01^{Aa}	2.06 ± 0.15^{Aa}
Heat + mild drought	Control	$2,718 \pm 214^{Ba}$	230 ± 22^{Ba}	0.25 ± 0.1^{Aa}	1.36 ± 0.1^{Ba}
	Th1	$2,884 \pm 341^{ABa}$	241 ± 33^{ABa}	0.250 ± 0.03^{Aa}	1.42 ± 0.16^{Ba}
	Th2	$2,738 \pm 250^{Ba}$	234 ± 33^{Ba}	0.262 ± 0.02^{Aa}	1.51 ± 0.31^{Ba}
Heat + moderate drought	Control	$2,457 \pm 255^{Ba}$	210 ± 39^{Ba}	0.26 ± 0.1^{Aa}	1.35 ± 0.19^{Ba}
	Th1	$2,429 \pm 200^{Ba}$	215 ± 27^{Ba}	0.256 ± 0.01^{Aa}	1.3 ± 0.16^{Ba}
	Th2	$2,576 \pm 265^{Ba}$	219 ± 20^{Ba}	0.266 ± 0.02^{Aa}	1.43 ± 0.1^{Ba}
Heat + severe drought	Control	$2,176 \pm 264^{Ba}$	200 ± 26^{Ba}	0.253 ± 0.1^{Aa}	1.26 ± 0.1^{Ba}
	Th1	$2,217 \pm 165^{Ba}$	208 ± 18^{Ba}	0.25 ± 0.01^{Aa}	1.22 ± 0.14^{Ba}
	Th2	$2,204 \pm 201^{Ba}$	210 ± 23^{Ba}	0.267 ± 0.02^{Aa}	1.28 ± 0.21^{Ba}

Control: distilled water, Th1: 10^{-9} M of Th17, Th2: 10^{-11} M of Th17. Each value represents the mean ± standard error (n = 8). Combination of lower and capital letters represents one-way ANOVA results for main effects; capital letters represent differences between different growth conditions at the same level of Th17 whereas lower letters indicate differences between different levels of Th17 at the same growth conditions. The absence of lower and capital letter combination letters indicates significant interaction of Th17 and stressful conditions by two-way ANOVA. Means with the same letters are not significantly different ($p < 0.05$).

Th17 compared to its effectiveness under less stressful conditions. Interestingly, plants respond to the higher concentration of the compound, Th1, for induvial stresses, either drought or heat; however, a lesser concentration (Th2) is required to alleviate the effect of stress combinations. A similar conclusion, distinct responses to different concentrations of bacterial compounds, was reached by other studies (Schwinghamer et al., 2015, 2016; Naamala et al., 2022). A clear example of this was observed in our previous study when three different concentrations of Th17, 10^{-7}, 10^{-9}, and 10^{-11} M, were applied, and nanomolar of Th17 was the most effective in enhancing seed germination, seedling growth, and vegetative growth under stressfully high temperatures (Nazari et al., 2022). Our findings are consistent with the work of Schwinghamer et al. (2016) regarding the application of different levels of two microbial compounds, LCO and Th17, which indicated concentration-dependent behavioral responses under various growth conditions and development stages. Similarly, previous work showed that specific concentrations of Th17 can enhance soybean [*Glycine max* (L.) Merr.] and corn (*Zea mays* L.) growth (Lee et al., 2009).

Changes in the root system

Drought stress broadly inhibits above-ground growth and increases dry matter allocation to the root portion of the plant, which results in a lowering of the shoot-to-root ratio. Additionally, PEG-induced osmotic stress mediates premature differentiation of the root apical meristem outgrowth of lateral roots. Through a highly complex process comprising an integrated network of antioxidants, metabolisms, redox, and hormonal regulation, plants can increase the water absorption area by encouraging

TABLE 3 Effect of Th17 application on antioxidant enzymes under drought stress levels.

Stressors	Th17 treatments	SOD (unit/mg fw)		POD (unit/mg fw)		CAT (unit/mg fw)	
		Seed treatment	Foliar spray	Seed treatment	Foliar spray	Seed treatment	Foliar spray
Control	Control	56 ± 10^{Ca}	66 ± 8^{DE}	56 ± 10^{Ba}	54 ± 10^{E}	8.5 ± 3^{Ca}	8.9 ± 3^{D}
	Thu1	60 ± 7^{Ca}	67 ± 7^{DE}	57 ± 9^{Ba}	60 ± 8^{E}	8.6 ± 2^{Ba}	12 ± 2^{D}
	Thu2	55 ± 8^{Ba}	64 ± 6^{E}	60 ± 8^{Aa}	54 ± 6^{E}	7.7 ± 2^{Ba}	10 ± 3^{D}
Mild drought	Control	68 ± 9^{Ca}	79 ± 7^{DE}	75 ± 8^{ABa}	79 ± 7^{DE}	10.7 ± 3^{BCa}	11 ± 2^{D}
	Thu1	82 ± 10^{Ca}	101 ± 11^{CDE}	89 ± 10^{ABa}	96 ± 11^{BCD}	13.5 ± 2^{Ba}	16 ± 5^{CD}
	Thu2	76 ± 11^{Ba}	92 ± 7^{DE}	73 ± 11^{Aa}	90 ± 13^{CD}	12 ± 3^{Ba}	12 ± 3^{D}
Moderate drought	Control	124 ± 18^{Ba}	129 ± 19^{BCD}	87 ± 12^{ABa}	91 ± 10^{CD}	16.5 ± 5^{ABa}	16 ± 4^{CD}
	Thu1	136 ± 15^{Ba}	202 ± 29^{A}	95 ± 13^{Aa}	120 ± 17^{AB}	18 ± 3^{Aa}	25 ± 5^{BC}
	Thu2	133 ± 26^{Aa}	187 ± 22^{AB}	82 ± 9^{Aa}	115 ± 13^{ABC}	18 ± 8^{ABa}	22 ± 5^{BCD}
Severe drought	Control	168 ± 29^{Aa}	165 ± 20^{ABC}	114 ± 20^{Aa}	117 ± 12^{ABC}	22 ± 4^{Aa}	24 ± 7^{BC}
	Thu1	173 ± 22^{Aa}	213 ± 21^{A}	110 ± 23^{Aa}	138 ± 29^{A}	22 ± 7^{Aa}	38 ± 6^{A}
	Thu2	181 ± 24^{Aa}	200 ± 18^{A}	114 ± 19^{Aa}	128 ± 20^{A}	24 ± 4^{Aa}	33 ± 7^{AB}

Control: distilled water, Th1: 10^{-9} M of Th17, Th2: 10^{-11} M of Th17. Each value represents mean ± standard error (n = 8). Combination of lower and capital letters represents one-way ANOVA results for main effects; capital letters represent differences between different growth conditions at the same level of Th17 whereas lower letters indicate differences between different levels of Th17 at the same growth conditions. The absence of capital and lower letter combination indicates significant interaction of Th17 and stressful conditions by two-way ANOVA. Means with the same letters are not significantly different ($p < 0.05$).

TABLE 4 Effect of Th17 application on antioxidant enzymes under drought and heat combination.

Stressors	Th17 treatments	SOD (unit/mg fw)		POD (unit/mg fw)		CAT (unit/mg fw)	
		Seed treatment	Foliar spray	Seed treatment	Foliar spray	Seed treatment	Foliar spray
Heat	Control	66 ± 13^{Ca}	76 ± 13^{Ca}	63 ± 10^{Ba}	74 ± 8^{Ba}	17 ± 3^{Ba}	18 ± 3^{Ba}
	Th1	72 ± 23^{Ca}	85 ± 16^{Ba}	70 ± 12^{Ba}	85 ± 9^{Aa}	15 ± 2^{Ca}	22 ± 4^{Ba}
	Th2	63 ± 9^{Ca}	81 ± 12^{Ba}	68 ± 13^{Ba}	89 ± 6^{Ba}	18 ± 4^{Ba}	22 ± 2^{BCa}
Heat + mild drought	Control	91 ± 7^{Ca}	91 ± 11^{Ca}	$74 \pm 10A^{ABa}$	79 ± 6^{ABa}	18 ± 3^{ABa}	19 ± 4^{Ba}
	Th1	93 ± 11^{Ca}	102 ± 18^{Ba}	$79 \pm 13B^{ABa}$	87 ± 14^{Aa}	19 ± 4^{BCa}	24 ± 5^{Ba}
	Th2	85 ± 28^{Ca}	108 ± 14^{Ba}	$82 \pm 8B^{ABa}$	97 ± 12^{ABa}	17 ± 3^{Ba}	20 ± 5^{Ca}
Heat + moderate drought	Control	137 ± 25^{Ba}	144 ± 25^{Bb}	80 ± 13^{ABa}	83 ± 8^{ABa}	29 ± 7^{ABa}	29 ± 6^{ABa}
	Th1	140 ± 12^{Ba}	177 ± 21^{Aab}	82 ± 8^{ABa}	93 ± 12^{Aa}	27 ± 6^{ABa}	35 ± 8^{Ba}
	Th2	149 ± 14^{Ba}	192 ± 14^{Aa}	84 ± 9^{ABa}	103 ± 13^{ABa}	25 ± 5^{Aa}	32 ± 5^{Ba}
Heat + severe drought	Control	193 ± 11^{Aa}	182 ± 17^{Ab}	85 ± 14^{Aa}	92 ± 9^{Ab}	31 ± 7^{Aa}	37 ± 7^{Ab}
	Th1	196 ± 24^{Aa}	207 ± 19^{Aab}	92 ± 13^{Aa}	106 ± 10^{Aab}	33 ± 9^{Aa}	51 ± 9^{Aab}
	Th2	206 ± 17^{Aa}	218 ± 12^{Aa}	95 ± 15^{Aa}	116 ± 10^{Aa}	32 ± 6^{Aa}	58 ± 10^{Aa}

Control: distilled water, Th1: 10^{-9} M of Th17, Th2: 10^{-11} M of Th17. Each value represents mean ± standard error (n = 8). Combination of lower and capital letters represents one-way ANOVA results for main effects; capital letters represent differences between different growth conditions at the same level of Th17 whereas lower letters indicate differences between different levels of Th17 at the same growth conditions. The absence of capital and lower letter combination indicates significant interaction of Th17 and stressful conditions by two-way ANOVA. Means with the same letters are not significantly different ($p < 0.05$).

the development of long, extensive root hairs and lateral roots under drought stresses (Ober and Sharp, 2003; Duan et al., 2010; Ji et al., 2014; Zia et al., 2021). Consistent findings are seen in our experiments; root variables, including total root length, total surface area, and volume, were increased with increasing PEG levels, whereas declines in root development were observed in the presence of heat and drought simultaneously. The interaction of seed treatment plus root drenching with Th17 and drought levels was significant and resulted in a more developed root system than that of controls, particularly at moderate and severe drought levels as well as under heat stress alone.

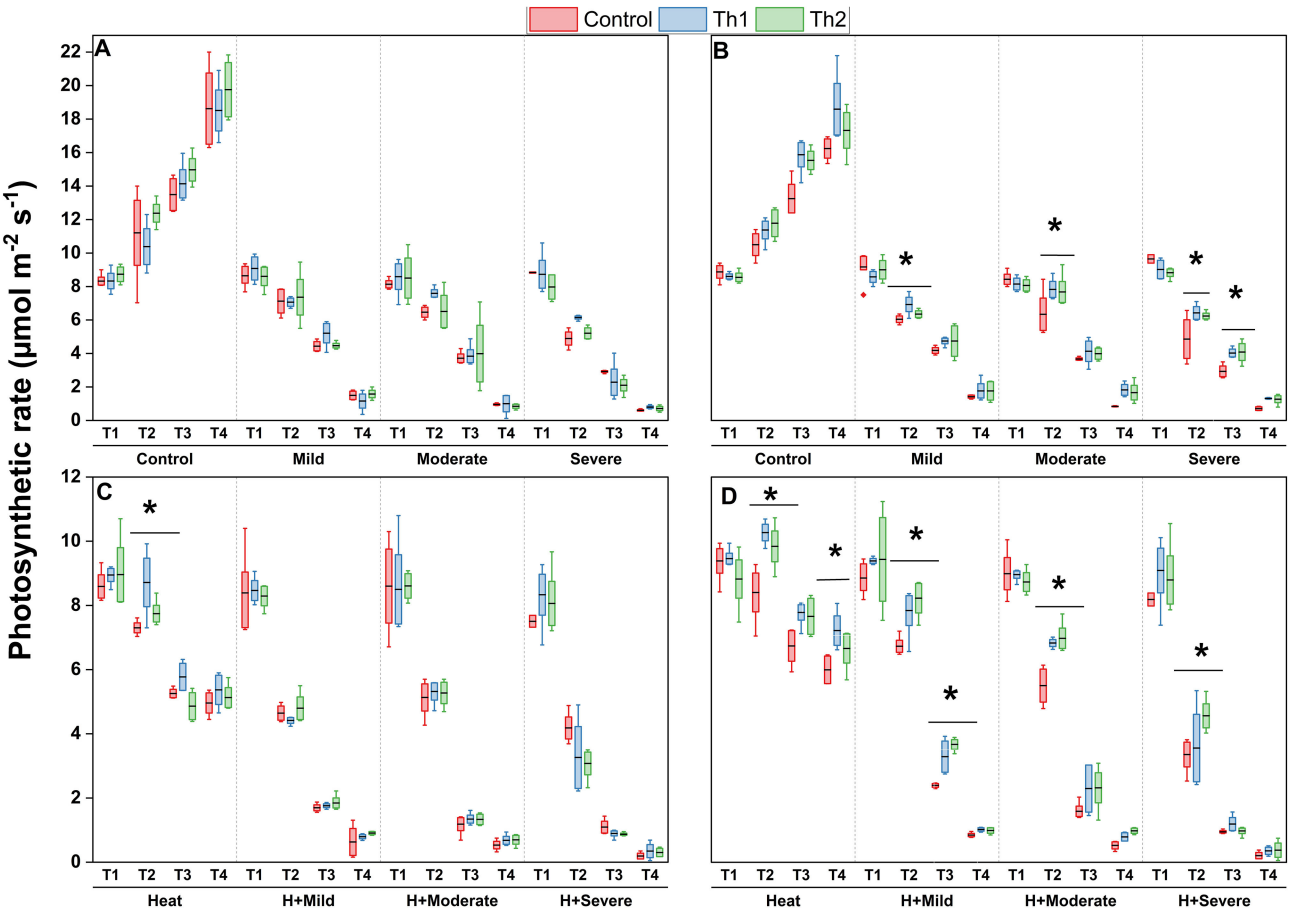

FIGURE 6
Effect of Th17 application on photosynthetic rate under drought **(A, B)** and combination of heat and drought **(C, D)** at 1 day before stress induction (T1), 1 day (T2), 1 week (T3), and two weeks (T4) after stress induction. **(A, C)** Seed treatment plus root drenching and **(B, D)** foliar spray of distilled water (Control), Th1 (10^{-9} M) and Th2 (10^{-11}M). A black line within the box marks the mean ($n = 8$), respectively. Boxes with an asterisk are significantly different at each measurement time and stress level ($p < 0.05$).

In contrast, no significant effects of the seed treatment plus root drenching were seen when drought and heat were combined, which could be due to the extreme stress levels leading to morphological, biochemical, and physiological damage, and consequently lower carbohydrate assimilation into roots. Additionally, for foliar experiments, across all conditions, no meaningful changes were observed due to the application of the compound. Our previous studies have indicated positive effects of treating canola seeds and root drenching with 10^{-9} and 10^{-11} M Th17, which caused germination increases, germination time reduction, and longer radicals under moderately high temperature and optimal conditions (Nazari et al., 2022). Similar root development effects on soybean have been reported for this plant growth regulator under salt stress and this was associated with changes in the level of ABA (Prudent et al., 2015). This might be one of the possible underlying mechanisms of root stimulation by Th17. As such, ABA is produced in the roots and transported to above-ground parts through xylem; it assists in stomatal closure, suppression of shoot growth, and maintenance of root elongation by crosstalk communication with phytohormones (Turan et al., 2021; Parwez et al., 2022). In another work, levels of auxin, which actively functions in cell division and lateral root development, and salicylic acid were increased in *Arabldopsis thaliana* rosettes in response to 10^{-9} M Th17 (Subramanian, 2014). All these findings are suggestive of a shift in the balance of plant hormones related to roots due to seed treatment and root drenching, which could be a possible explanation of the root system modification by Th17 application.

Biochemical responses to stressors and Th17

Plants are equipped with a compelling array of defenses, comprised of enzymatic and nonenzymatic systems, to reduce the accumulation of reactive oxygen species (ROS), which cause oxidative damage to lipids, proteins, and DNA, under stressful growing conditions. Responses of enzymatic activities to various stressors, either alone or in combination, differ. In this regard, PGPMs and their active compounds have been shown to aid plants in scavenging excessive ROS species (Zhou et al., 2016,

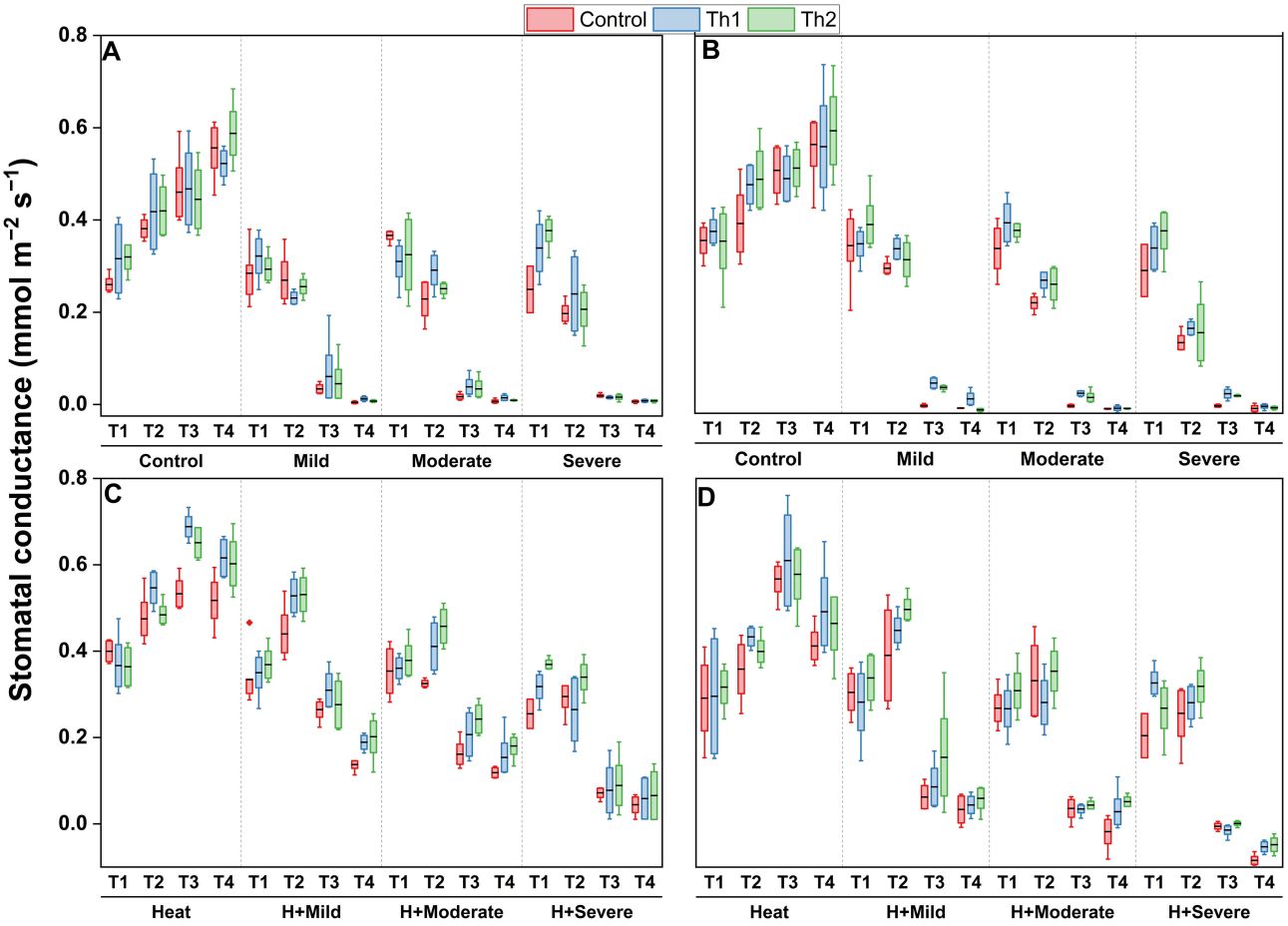

FIGURE 7
Effect of Th17 application on stomatal conductivity under drought **(A, B)** and combination of heat and drought **(C, D)** at 1 day before stress induction (T1), 1 day (T2), 1 week (T3), and two weeks (T4) after stress induction. **(A, C)** Seed treatment plus root drenching and **(B, D)** foliar spray of distilled water (Control), Th1 (10^{-9} M) and Th2 (10^{-11}M). A black line within the box marks the mean ($n = 8$), respectively. Boxes without an asterisk are not significantly different at each measurement time and stress level ($p < 0.05$).

2019; Gowtham et al., 2022; Neshat et al., 2022; Sati et al., 2022). Likewise, we found that exposure to stressful conditions increased levels of antioxidant enzymes compared to optimal growth conditions. Rather than the single effect of stress stimuli, very interestingly, foliar spray of the microbial compound demonstrated significant positive interactions with drought stress levels; the greatest numbers of SOD, POD, and CAT were recorded in Th1 sprayed plants under severe drought stress whereas spraying a lesser concentration of the compound (Th2) produced better results when drought and heat were combined. Nevertheless, treating seeds plus root drenching with the supplement could not meaningfully assist plants in the production of more antioxidant enzymes compared to untreated plants. In another study, the effect of two types of PGPR-produced bacteriocins, BF4 and Th17, on the plant defense system illustrated that antioxidant enzyme levels considerably increased in treated soybean leaves compared to untreated ones (Jung et al., 2011). Similarly, results of omics studies highlighted the role of two microbial signal molecules, LCO and Th17, in stress tolerance of *A. thaliana*, in which energy and antioxidant pathway proteins were increased in treated plants, causing salt stress mitigation as opposed to controls (Subramanian, 2014). At this stage of understanding, we propose that boosting the antioxidant enzyme system could be one of the possible underlying mechanisms of stress alleviation, however, it seems certain that there are still many things to learn about this compound.

Physiological responses to stressors and Th17

Under unfavorable conditions, considerable changes in plant physiological mechanisms occur, beginning with perception of stress signals, which trigger a series of molecular events, resulting in various levels of responses. We found that cooccurrence of heat and drought stresses led to a higher reduction in photosynthetic rate than either drought or heat stress alone, and drought stress caused declines to a larger extent than heat stress. Additionally, the negative effects of drought stress on carbon assimilation may contribute to stomatal closure, which resulted

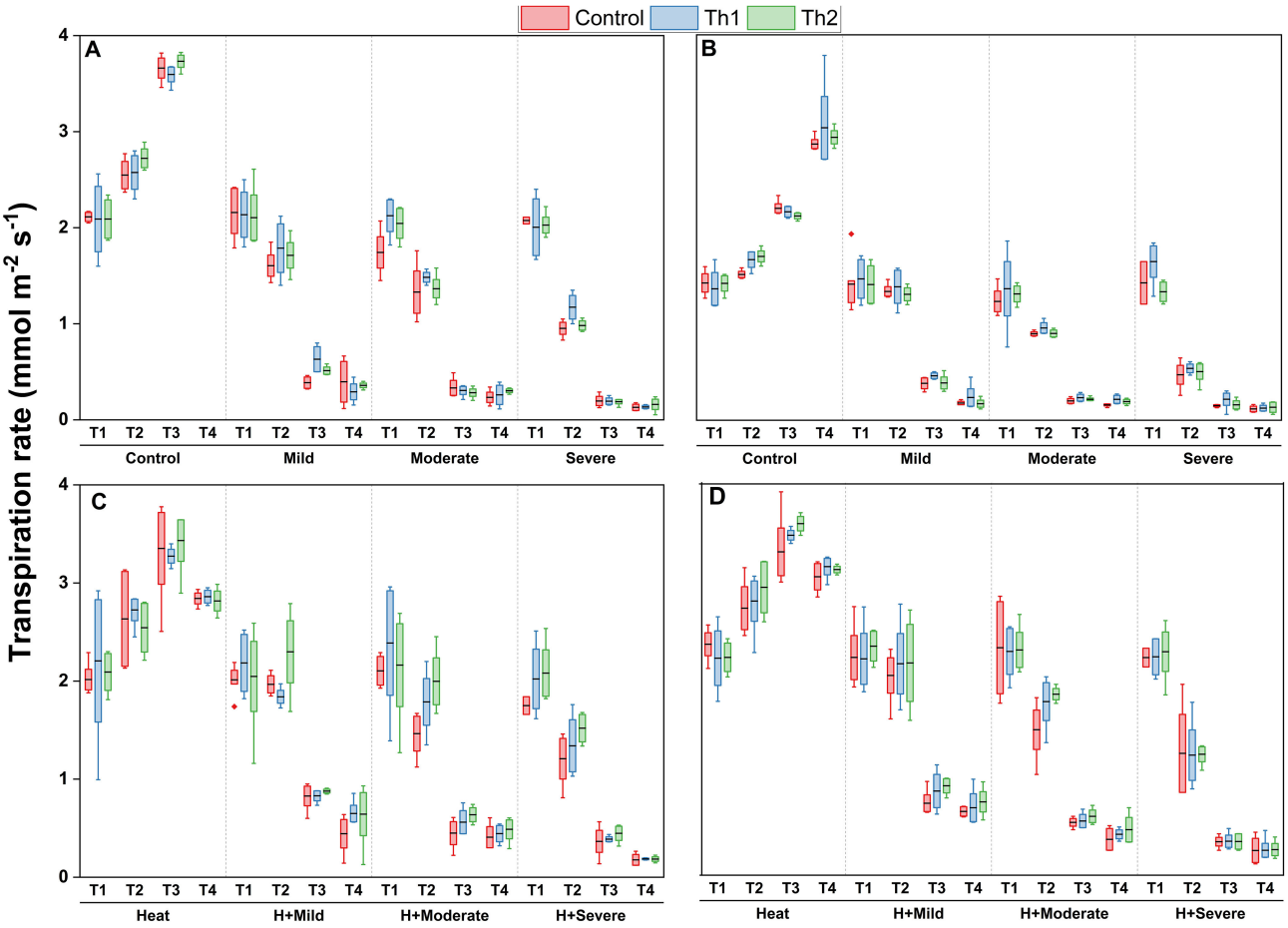

FIGURE 8
Effect of Th17 application on transpiration rate under drought **(A, B)** and combination of heat and drought **(C, D)** at 1 day before stress induction (T1), 1 day (T2), 1 week (T3), and two weeks (T4) after stress induction. **(A, C)** Seed treatment plus root drenching and **(B, D)** foliar spray of distilled water (Control), Th1 (10^{-9} M) and Th2 (10^{-11}M). A black line within the box marks the mean ($n = 8$), respectively. Boxes without an asterisk are not significantly different at each measurement time and stress level ($p < 0.05$).

in decreased intercellular CO_2; however, heat stress increased the conductivity of stomata and CO_2 diffusion into leaves. They have implied the existence of different underlying mechanisms in photosynthesis reduction by drought and heat stress. Our observations suggest that the stomatal factors under drought stress and non-stomatal factors, including, but not limited to, damage to the photosynthetic apparatus, inactivation of some enzymes under heat stress, and the combination of stressors, caused declines in photosynthesis (Wang et al., 2010, 2018; Carmo-Silva et al., 2012; Distéfano et al., 2017; Fatma et al., 2021; Lal et al., 2021). Heat stress also increases transpiration, to cool leaves, reducing leaf water potential and exacerbating plant conditions under heat and drought combinations. Likewise, lower leaf temperatures were observed in lipo-chitooligosaccharides (LCO) treated plants, related to enhanced transpiration and to stomatal conductance or membrane permeability (Schwinghamer et al., 2016). Our findings link well with previous studies wherein similar physiological behaviors in response to stressors were depicted (Elferjani and Soolanayakanahally, 2018; Wang et al., 2018; dos Santos et al., 2022). However, treating plants with Th17 could compensate for photosynthesis reduction compared to controls. We found that spraying Th1 onto leaves could enhance carbon assimilation across all drought levels and heat stress alone in such a way that the greatest effect of the compound was detected, at a statistically significant level, 1 day after treatment. Under the combination of heat and drought, the positive impact of the lesser concentration, Th2, was noticeable one day after spraying across all stressors and combinations. In contrast, plants grown from Th17 treated seed plus root drenching showed inconsistent results. No significant effects were observed from these treatments, except for the notable impact of Th1 under heat stress alone 1 day after stress induction. Accordingly, plants with higher carbon assimilation rates showed slightly higher stomatal conductance and transpiration, but their effects were not significantly different from the controls. From this point, it could be suggested that non-stomatal factors more actively contributed to resulting higher efficiency of the photosynthesis than stomatal effects. In this regard, higher levels of antioxidant enzymes, as described earlier for those plants, could be one of the possible explanations for reduced levels of ROS, and consequently, cause less damage to photosystems and

enzymes. Others have shown that leaf spray or root drenching of Th17 increased photosynthetic leaf rates and leaf greenness of soybean (Lee et al., 2009). In line with previous studies, Th17-treated plants had greater photosynthetic rates due to having more developed root systems and improved functions related to carbon and nitrogen acquisition (Prudent et al., 2015). The efficacy of Th17 in stimulating the photosynthetic rate was consistent with what has been found in our previous studies; the carbon assimilation enhanced in response to Th17, both Th1 and Th2, at moderately high temperatures (Nazari et al., 2022). Regardless of all positive responses, further agricultural field studies would be our next step to see if statistically different results are biologically different or not.

Conclusions

We found clear responses to Th17 concentrations related to growth conditions where plants reacted differently to the simultaneous prevalence of heat and drought as compared with individual stresses. Precisely, regardless of application method, 10^{-11} M treated plants could better tolerate the combination of heat and drought stress than the other concentrations and control; conversely, more promising results were caused by 10^{-9} M under individual stresses. Our results demonstrated that both application methods could assist plants in combating stressful conditions by varying mechanisms; however, no significant effects were seen for optimal growth conditions. Stimulation of the root system to uptake more water and nutrients might be one of the possible modes of action for seed treatment plus root drenching. Additionally, we speculate that spraying the compound increased the most important antioxidant enzymes contents, which scavenged excessive ROS and reduced associated damage to cells, consequently redcuing cell damage and increasing the efficiency of the photosystems. Collectively, Th17 has the potential to be a plant growth regulator, plus these results should be translated into real agricultural field setting. Further studies are aslo required to invistigate the signal's mode of action at molecular levels.

Data availability statement

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

Author contributions

MN designed the experiment, collected data, and wrote the manuscript. DS advised on the scientific approach and provided editorial input and intellectual context as well as funding. All authors approved the submitted version.

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Conflict of interest

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

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Plant-microbiome interactions under drought—insights from the molecular machinist's toolbox

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Plants face numerous challenges in novel and harsh environments, including altered precipitation regimes, salinity, extreme temperatures, increased atmospheric CO_2, nutrient deficiency, heavy metals, and oxygen. Drought remains a major constraint to crop productivity and meeting food demand, with the frequency, intensity, and duration of drought expected to raise in the coming century. The "cry for help" hypothesis proposes that timely recruiting of the microbiome by plants may confer benefits in stress alleviation, plant growth, fitness, and health. The root-associated microbiome harbors 10–100 times more functional genes than the host, which can significantly stimulate the metabolic and genetic potential of plant–microbiome assembly. However, cross-talk among drought and the root-associated microbes, and among the root-associated microbiome and the host-plant, is less well understood. Understanding the molecular aspect of multiple mechanisms by which microbes associate with plants during drought stress is of fundamental importance in plant biology and agriculture. In this review, we examine the progress in research on the response of plant and its microbiome assemblages and interactions to drought stress, including the impact of drought and root exudates on host resilience. We delve into the potential of 'omics' technologies to unravel the signaling networks underlying these interactions and the multiway interactions that occur among the host and its associated microbiome. We then discuss the shortfalls, challenges, and future research directions in this field. Overall, we argue that harnessing/manipulating the crop microbiome presents a promising strategy for improving agricultural systems in the face of global climate change.

KEYWORDS

plant-associated microbiome, biostimulants, plant resilience, 'omics', signaling networks, water deficit, root exudates, multiway interactions

1 Drought-tolerant microbiome: a growing field of scientific interest

Plants are continuously exposed to harsh environmental conditions, including water scarcity, salinity, heat waves, elevated CO_2 levels, heavy metals accumulation, and soil poverty, which challenge their adaptability and resilience (Shen et al., 2020). Drought remains a serious impediment to crop productivity and food security, especially with the increase in its frequency,

duration, and intensity under global climate change (Canarini et al., 2021; Anli et al., 2022). It disrupts plant metabolism, reduces photosynthetic activity, and induces electrolyte disturbances and reactive oxygen species (ROS) accumulation, causing cell apoptosis and plant death (Boutasknit et al., 2021; Benaffari et al., 2022; Toubali et al., 2022).

Drought stress affects plant functioning, which in turn alters plant metabolism and root exudates, influencing the plant rhizosphere microbiome. Plant exudates, such as sugars, vitamins, organic acids, amino acids, fatty acids, flavonoids, carboxylic acids, benzoxazinoids, and ethylene (ET), play a crucial role in selectively recruiting rhizospheric microbial communities (Vives-Peris et al., 2020). Phytohormones, such as jasmonic acid (JA) and salicylic acid (SA), and their signaling pathways significantly impact plant microbiome structure (French et al., 2019). Drought may also impact plant microbiome association, putting selective pressure on its components to endure stressful conditions. Inducing glycerol-3-phosphate (G3P) synthesis during drought, for example, favors Actinobacteria in the rhizosphere, which promotes plant fitness and health under water deficit (Xu et al., 2018b). Drought also induces a decline in SA production, which significantly impacts the formation of both exo- and endogen microbiome (Lebeis et al., 2015). Many investigations have revealed that during drought stress, plants promote monoderm bacteria (or gram-positive) over diderm bacteria (or gram-negative) in the rhizosphere (Naylor et al., 2017; Naylor and Coleman-Derr, 2018). Drought reduces iron and phytosiderophore availability in the rhizosphere, favoring Actinobacteria that may thrive in such environment and promote plant performances (Xu et al., 2021). A recent study by Santos-Medellín et al. (2021) revealed that drought can have long-lasting impact on the rhizospheric microbiome. They discovered that the rice root-associated microbiome structure was severely altered during a short period of water deficit but retrieved to its predrought state after recovery. However, extended water stress had serious and long-term impact on the endosphere community, which were not completely recovered even after rewatering. According to the same study, the abundance of Actinobacteria recorded a significant increase after prolonged drought, accounting for >80% of the bacterial community after the drought period. In the same vein, water shortage intervals the establishment of sorghum root microbial communities during the early development and plays a role in restructuring the root microbiome by increasing enrichment in monoderm bacteria and their activity (Xu et al., 2018b). Various studies have demonstrated that drought has a substantial impact on the activity and composition of plant root-associated bacteria in a manner that is remarkably preserved regardless of host species and sites (Karlowsky et al., 2018; De Vries et al., 2019; Williams and De Vries, 2020). The analysis of co-cited references in Figure 1 identified emerging trends and research hotspots in the field. Nine clusters were identified, with each cluster corresponding to a specific line of research. All clusters, except for "Cluster #1: Antibiotic resistance genes," were closely related to the topic of plant-microbe interactions under drought. "Cluster #0: Water deficit" contained the majority of the nodes and has been widely reported. "Cluster #1: Antibiotic resistance genes" and "Cluster #2: Abiotic stress tolerance" were the most active areas of research. Recent research has focused on plant tolerance to water stress and their associated microorganisms, as seen in "Cluster #0: Water deficit," "Cluster #2: Abiotic stress tolerance," "Cluster #4: Microbial communities," "Cluster #5: Plant responses," and "Cluster #7: Crop resiliency". These results emphasize the significance of understanding the role of plant-microbe interactions for mitigating the effects of drought and enhancing plant resilience. The evolving trends and research priorities in this field underscore the important role performed by plant-associated microorganisms.

The root-associated microbiome, including plant growth-promoting fungi (PGPF) and plant growth-promoting bacteria (PGPB), may promote plants' resistance and adaptation to water deficiency (Xu and Coleman-Derr, 2019; Table 1). Plant tolerance to drought is increased either directly by members of the root microbial community or indirectly via microbe-microbe interactions (Hartman and Tringe, 2019; Abbott et al., 2021). Microbiome may help host resist water deficit by activating transcriptional reprogramming of multiple genes and transcription factors (TFs) regulating plant defense. Microbes, for instance, have recently been shown to induce various genes expression under drought stress.

Although the composition of the plant-associated microbial communities has been studied in a great number of plant species, less interest has been paid to the effect of water shortage on the root-associated microbiota. There is a lack of understanding of the 'cross-talk' among drought, the root-associated microbiome, as well as between the root-associated microbial communities and the host-plant. Understanding the molecular patterns that orchestrate the assemblage of microbial communities with plants under drought stress is critical for the plant survival and fitness. Thus, knowledge of plant-microbe-drought regulation will be necessary for the design of environmentally-friendly crop management strategies and sustainable agriculture under changing environments. This review summarizes the research advances on the shaping of plant microbiome under water deficit conditions and the mechanisms by which microbiome could alleviate the adverse impact of drought on plants. Finally, we highlight the gaps, challenges, and perspectives of future research in this 'ménage à trois' that could help harnessing the microbiota to improve drought outcomes.

2 Plant-mediated changes in microbiota during drought trends

2.1 Plant-microbiome communication under drought trends: host plant churn out valuable chemicals for regulating microbial functions

Plants continuously release exudates into their surroundings, in the form of liquid, solid, or gaseous compounds, through their leaves, shoots, or roots. Plants exuded c. 11% of the net fixed C or 27% of the C assigned to roots to the rhizosphere. The amount of these compounds varies depending on multiple factors, such as plant age, species, and nutritional performance (Bais et al., 2006; Jones et al., 2009; Nakayama and Tateno, 2018). Root exudates contain various substances, including primary and secondary metabolites and phytohormones, such as ET, JA, SA, indole 3-acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA), and cytokinins (CKs). Primary metabolites consist of sugars, amino acids, and organic acids, while secondary metabolites include flavonoids, glucosinolates, phytoalexins, triterpenes, and benzoxainoids (Badri et al., 2008; Pang et al., 2021). For instance, tomato plants exude a mixture of

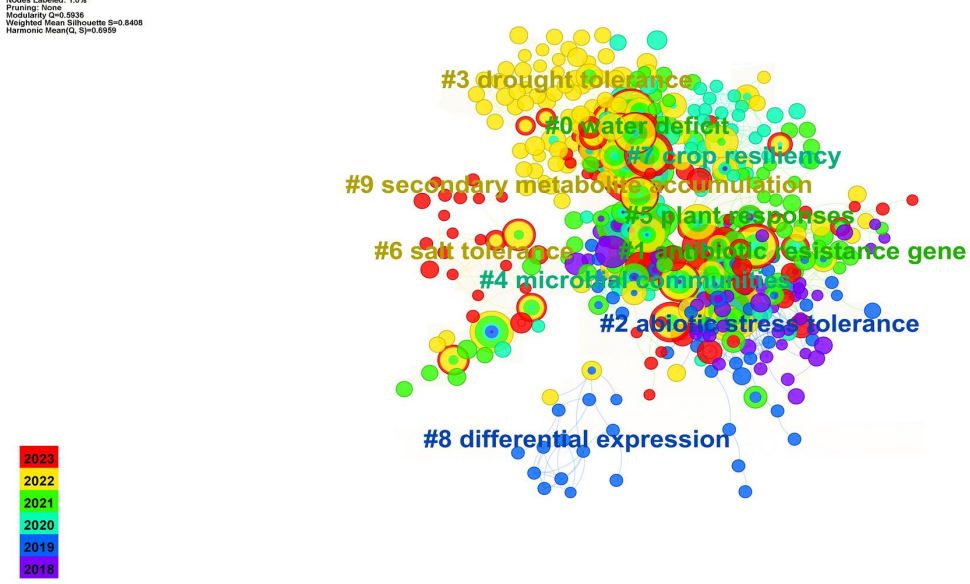

FIGURE 1
Co-cited references of keyword co-occurrence clustering analysis in the study of plant–microbiome interactions under drought from 2018 to 2023.

metabolites, including organic acids, steroidal glycoalkaloids, acylsugars, and hydroxycinnamic acid derivatives, into the rhizosphere (Korenblum et al., 2020).

Microbes diverge in their functioning and metabolism, and their sensitivity to water availability varies. Thus, drought can directly impact the assembly of plant microbiome. Plant surface microbiome, i.e., phyllosphere, is likely to be significantly altered by drought since environmental conditions vary more rapidly than those inside plant tissue (i.e., endosphere), which is more stable (Trivedi et al., 2020). Most of the microbes from the bulk soil (a source of potential microbes to colonize plant roots) are directly influenced by external climatic factors, including drought. On the other hand, rhizosphere microbiome are directly altered by these factors and indirectly by plant responses, such as changes in host morphology, physiology, immune system, and root exudation (Figure 2). Previous studies suggest a consistent response of the host-associated microbiome to water deficiency (Vescio et al., 2021; Wipf et al., 2021; Aslam et al., 2022; Tebele et al., 2023). Under water limitation, several plant species recruit monoderm bacteria that are resistant to dryness owing to their thicker cell walls and reduce diderm bacteria in the roots and rhizosphere (Naylor et al., 2017; Naylor and Coleman-Derr, 2018).

Understanding how drought stress affects plant–microbiome assemblage is still a challenging task owing to the multilayers and complex interconnections that orchestrate this 'triangle' interaction. Chemical signals exchange heavily influences plant-microbiome communication under water scarcity (Figure 2). For example, under stressful environments, plants have evolved a 'cry for help' mediated exudation, resulting in the recruitment of stress-mitigating microbes (Liu et al., 2020). Plants have a complex microbial recruiting system to select the most beneficial microorganisms to incorporate into plant tissues, discriminating between friendly and beneficial or hostile and harmful interactions (Hacquard et al., 2017; Teixeira et al., 2019; Zhang et al., 2023). The host plant possesses protective mechanisms to perform this selection: (i) pattern recognition receptors (PRRs), the initial mechanism of defense to identify microbe-associated molecular patterns (MAMPs), such as fungal chitin or bacterial flagellin, leading to MAMP-triggered immunity (MTI) and (ii) nucleotide-binding leucine-rich repeat (NLR) proteins as a second defense mechanism to identify pathogen effectors, inducing effector-triggered immunity (ETI). Changes in plant immunity caused by drought could structure the plant-microbiome assembly, especially inside host plant tissues. Suppression of ETI may interfere with host-mediated management of microbe colonization and may result in microbial dysbiosis inside plant tissues. The suppression of ETI can also be a new pattern used by plants to lessen their defense mechanisms, allowing beneficial microbes to colonize roots and promote stress mitigation-related gene transcripts.

The mutual communication involving the plant defense system and the microbiome structures the plant-microbiome assemblage. Plants control immune response in rapidly changing environmental conditions using active but tightly controlled modifications in different hormone pathways (i.e., ET, SA, ABA, and JA; Li et al., 2021; Ait-El-Mokhtar et al., 2022). Lebeis et al. (2015) reported that drought reduces SA production, which is involved in the assembly of both endophytic and epiphytic microbiome. SA can promote or inhibit microbial community growth directly on microbiome members or through established signaling pathways based on hormonal cross-talk. For instance, ABA-induced production under water scarcity interferes with the SA-mediated immune pathway. Drought-mediated alterations in plant hormones may differ depending on the plant developmental stage and tissue type. For example, maize grown under water limitation boosts benzoxazinoid defense system in plant aboveground part while stimulating terpenoid phytoalexins in the belowground tissues (Vaughan et al., 2018). Alterations in the

TABLE 1 Role of microbiome in drought tolerance: summary of reported studies.

Microbe inoculation	Plant	Inoculation method	Tolerance strategy	Ref.
Bacillus thuringiensis AZP2	Wheat	Drenching	Induced the production of VCs.	Timmusk et al. (2014)
Pantoea alhagi	Wheat	Drenching	Enhanced the production of siderophores, EPS, IAA, soluble sugars, ammonia, and protease and decreased chlorophyll degradation.	Chen et al. (2017)
Bacillus licheformis K11	Pepper	"watered in" via irrigation	Upregulated stress-related genes (*Cadhn*, *VA*, *sHSP* and *CaPR-10*) and proteins.	Lim and Kim (2013)
Sphingomonas	Soybean	"watered in" via irrigation	Enhanced biomass, proline, glycine, glutathione, glutamine, ABA, and JA content.	Khan et al. (2014) and Asaf et al. (2017)
B. amyloliquefaciens FZB42	Arabidopsis	Added to inoculated soil	Induced the production of proline and enzyme activity (i.e SOD and POX) and the overexpression of *RD17*, *RD29A*, *ERD1*, *LEA14* genes.	Lu et al. (2018)
B. amyloliquifaciens	Grape	Drenching	Enhanced melatonin secretion and reduced H_2O_2, O_2^-, and MDA concentration.	Jiao et al. (2016)
B. cereus AR156, *B. subtilis* SM21, and *Serratia* sp. XY21	Cucumber	Applied directly to roots	Induced the production of monodehydro-ascorbate, proline, and antioxidant enzymes, and altered the expression of *cAPX*, *rbcL*, and *rbcS* genes.	Wang et al. (2012)
Trichoderma and *Pseudomonas*	Rice	Coated onto seeds	Increased the transcript levels of multiple genes involved in the antioxidant enzymes and phenylpropanoid biosynthesis pathway.	Singh D. P. et al. (2020)
Pseudomonas spp.	Soybean Arabidopsis	Drenching	Induced the expression of *DREB/EREB*, *PIP*, *TIP*, *P5CS*, and *GOLS* genes. Induced the expression of *RD29A* and *RD29B* genes.	Vaishnav and Choudhary (2019) and Liu et al. (2020)
Bacillus and *Pseudomonas*	Sorghum	Applied directly to roots	Higher production of signaling molecules (i.e., IAA, CK, SA, GA, JA, BRs, sphingosine, psychosine), osmolytes, and antioxidants, and reduced ET content.	Carlson et al. (2020)
Sinorhizobium sp.	Barrel clover	Drenching	Upregulated JA pathway translation and downregulated ET biosynthesis.	Staudinger et al. (2016)
Bacillus spp.	Guinea grass	Drenching	Higher proline production and lower MDA concentration and GR activity.	Moreno-Galván et al. (2020)
Streptomyces spp.	Tomato	Added to inoculated sand	Improved growth and leaf water content, antioxidant enzyme activity, proline and H_2O_2 accumulation, and reduced *ERF1* and *WRKY70* gene expression.	Abbasi et al. (2020)
Bacillus subtilis	Arabidopsis Mustard	Applied directly to roots	Increased the expression of *RD29B*, *RAB18*, *RD20*, and *NCED3* genes. Increased the expression of *DREB1D*, *WRKY7*, and *CSD3* genes.	Woo et al. (2020)
Enterobacter cloacae, *Enterobacter* sp., *Ochrobactrum* sp., and *Microbacterium* sp.	Sorghum	Coated onto seeds	Improved growth fitness, osmotic adjustment, and proline accumulation, with the upregulation of proline biosynthesis genes *SbP5CS 1* and *SbP5CS 2*.	Govindasamy et al. (2020)
Bacillus subtilis and *Paenibacillus illinoinensis*	Pepper	"watered in" via irrigation	De-regulation of vacuolar H^+ pyrophosphatases gene expression.	Vigani et al. (2019)
Piriformospora indica	Barley	Drenching	Induced the expression of genes involved in regulating stress signaling molecules, including proteins and enzymes orchestrating pivotal metabolic pathways, transporters, autophagy and up-regulation of plant oxidative stress-associated proteins.	Ghaffari et al. (2019)

(Continued)

TABLE 1 (Continued)

Microbe inoculation	Plant	Inoculation method	Tolerance strategy	Ref.
Pseudomonas indica	Rice	Coated onto seeds	Improved growth performances, biomass accumulation, mineral nutrition (zinc and phosphorus), and proline accumulation, with the upregulation of *P5CS* genes.	Saddique et al. (2018)
Trichoderma sp.	Rapeseed	Applied directly to roots	Downregulation of ET genes (*ACO1* and *ERF1*) and upregulation of *NCED3* and *PYL4*.	Poveda (2020)
Rhizophagus irregularis	Apple	Applied directly to roots	Enhanced *MdMAPK7-1*, *MdMAPK16-2*, *MdMAPK17*, and *MdMAPK20-1* transcripts.	Huang et al. (2020)
Funneliformis mosseae	Trifoliate orange	Applied directly to roots	Increased the antioxidant enzyme (CAT and SOD) genes and activity and upregulation of polyamine metabolism-associated gene expression.	Zhang et al. (2020)
Funneliformis mosseae	Trifoliate orange	Applied directly to roots	Increased expression of root tip aquaporins (*PtTIP1;2*, *PtTIP1;3*, *PtTIP4;1*).	Jia-Dong et al. (2019)
Glomus clarum, Acaulospora scrobiculata, and *Gigaspora rosea*	Bean	Applied directly to roots	Increased expression of eight aquaporin-associated genes.	Recchia et al. (2018)
Funneliformis mosseae, F. geosporus, Claroideoglomus claroideum, Glomus microaggregatum, and *Rhizophagus irregulari*	Rice	Applied directly to roots	Increased P and IAA concentrations	Chareesri et al. (2020)
Glomus mosseae	Wheat	Applied directly to roots	Upregulated proteins involved in cell wall integrity and carbohydrate production and downregulated stress-associated molecules (i.e., ET biosynthesis enzymes)	Bernardo et al. (2017)

ABA, abscisic acid; ACO1, aconitase1; APX, ascorbate peroxidase; BRs, brassinosteroids; CAT, catalase; CK, cytokinin; CSD3, copper/zinc superoxide dismutase 3; DREB, dehydration-responsive element-binding protein; EPS, exopolysaccharides; EREB, ethylene responsive element binding protein; ERF1, ethylene response factor; ET, ethylene; JA, jasmonic acid; HSP, heat shock protein; IAA, indole-3-acetic acid; GA, gibberellic acid; GOLS, galactinol synthase; LEA, late embryogenesis abundant; MAPK, Mitogen-activated protein kinase; MDA, malonyldialdehyde; NCED3, 9-cis-epoxycarotenoid dioxygenase3; P, phosphate; P5CS, delta1-pyrroline-5-carboxylate synthase; POX, peroxide oxidase; PR10, pathogenesis-related protein 10; PYL, pyrabactin resistance-like; rbcL, ribulose bisphosphate carboxylase large chain; rbcS, ribulose bisphosphate carboxylase small chain; RD29/A, desiccation-responsive protein 29A; SA, salicylic acid; SOD, superozide dismutase; TIP, tonoplast intrinistic protein; VA, vacuolar H + -ATPase; VCs, volatile compounds.

distribution or allocation of diverse signaling molecules or defense metabolites as a result of drought may have an additional impact on microbiome assembly.

It is worth noting that in host-microbiome research, core-and-hub microbiota concepts are gaining traction (Singh B. K. et al., 2020). These refer to the microbiota that exist in a specific species regardless of environmental conditions, growing season, or management practices and perform critical host functions (Trivedi et al., 2020). Given their significance, it is crucial to understand how drought affects the 'core-and-hub' microbiota that may organize community-scale functions in plant-microbiome communications. An enlarged understanding of the ecological vectors that orchestrate microbiome response to water scarcity will promote our knowledge of microbiome traits that boost plant performances under changing environments.

2.2 Plant-mediated reshaping of microbiota composition in response to drought

Plant and microbiota have a two-way communication, both underground and aboveground, that enables them to sense and respond to stress conditions. In response to stress, plants release a range of metabolites that attract specific microorganisms capable of enhancing their tolerance to stress (Bai et al., 2022). According to the 'cry for help' hypothesis, plants recruit particular microbiome communities that aid them in managing stress (Liu et al., 2020). This concept was first observed when plants grown in soils deficient in phosphorus and nutrient-supplying arbuscular mycorrhizal fungi (AMF), and nitrogen recruited nitrogen-fixing rhizobia (Carbonnel and Gutjahr, 2014; Nishida and Suzaki, 2018). The 'cry for help' assumption is also applicable to plants experiencing drought stress, as the microbiome composition in roots significantly changes by promoting actinobacteria and other gram-positive bacteria over gram-negative ones (Timm et al., 2018). Plants may selectively recruit drought-tolerant microbes that have evolved from repetitive drought periods, resulting in beneficial and efficient plant-microbiome interactions that enhance the performance of both host and microbes (Naylor and Coleman-Derr, 2018). Terhorst et al. (2014) demonstrated that drought-stressed *Brassica rapa* plants exhibit higher and more diverse bacterial richness around their roots in comparison to the controls.

Santos-Medellín et al. (2021) revealed that the above- and below-ground microbiome undergo a plant-driven change in response to water limitation, resulting in an increase in drought-tolerant endophytic monoderm bacteria that may help mitigate drought. The

same authors demonstrated how persistent drought can permanently impede plant endophytic microbiome growth. This effect persists even after the drought constraint is alleviated. Their research revealed how long-lasting drought stress may shift microbial community composition, which may affect plant fitness. They also identified promising candidates for microbiome engineering to create performant microbial assemblages against water deficit, including drought-resistant endophytic microorganisms that increased in abundance in the endosphere after drought stress. Active microorganism recruitment under stressful conditions appears to be a common evolutionary mechanism to promote plant performances. Nonetheless, the strategies that allow plant host to incorporate external signals during symbiotic microbes' recruitment and the host genetic characters controlling this recruitment are still under investigations. These processes are orchestrated by multilayer components involving plants, microorganisms, soil, and environmental traits that shape the final result. The accumulation of stress-related factors in plant roots during drought, including G3P and pipecolic acid, has been linked to actinobacteria enrichment in the rhizosphere (Knight et al., 2018; Caddell et al., 2020; Table 2). Understanding the underground signals communication is still in its early stages, particularly in drought-stressed hosts that shape their tolerant microbes.

A dynamic understanding of the role of metabolites and their genomic features is essential to comprehend the multilayered complexity of the 'cry for help' theory in host microbe assembly before and after drought conditions. This will provide new insight into creating drought-tolerant microbial consortia for sustainable agriculture. Recently, Bai et al. (2022) proposed a model based on the 'cry for help' hypothesis for plant microbiome recruitment under water scarcity (Figure 2). Under drought conditions, plants undergo molecular and metabolic readjustments and produce specific root metabolites. The root exudates may promote the reprogramming and restructuring of the microbiome by recruiting selective drought-tolerant microorganisms with a vast arsenal of functional enzymes. Subsequently, drought-resistant microbiota can mitigate stress impact and deliver nutrients to host plants via multiple direct and indirect mechanisms.

2.3 Microbes that get the scoop—plant genes, signaling integrators, and metabolic changes co-ordinating rhizosphere microbiome under drought

The plant and its associated microbiome form a harmonized and functional entity known as a holobiont (Bordenstein and Theis, 2015), in which evolutionary selection occurs not only between the host and the associated microbes but also among the microbes (Ait-El-Mokhtar and Baslam, 2023). To maintain the harmony of this association, systems of coordination among the microbial communities and with the host plant are necessary. Our understanding of the acquisition of microbes in the environment and the rules governing their association in the plant holobiont is very limited. It is widely believed that during root penetration into bulk soil, the soil microbiome progressively differentiates into the rhizosphere microbiome via contact with rhizodeposits, which have a significant effect on the microbiota composition (Tian et al., 2020; Figure 2). After this initial community shift, the microbiota composition is finely adjusted in specific compartments (rhizosphere, rhizoplane, or endosphere; Bulgarelli et al., 2013). Plant genetic factors, including root exudate quality and quantity and root morphology, have been known to shape the rhizospheric microbial communities (Sasse et al., 2018). Other factors, including the plant developmental stage, plant immune system, and season, have also been shown to play a significant role in shaping the rhizosphere microbiome (Hassani et al., 2018). The presence of a core microbiota in some crops, irrespective of fertilization management or soil origin, suggests that these communities are partly assembled and selected by plants. In general, it is believed that the plant selects its microbial associates via the action of its root exudates. Yet, this unidirectional recruitment is being questioned (Uroz et al., 2019) because the co-evolution of the plant-microbe holobiont suggests

TABLE 2 Plant-mediated reshaping of rhizosphere microbiota composition in response to drought.

Regulator/Genes/ Key factors	Host plant	Pathways/Signals molecules shaping the microbiota	Effect on the microbiota	Effect of shifted microbiota on the host plant	Ref.
Unclear	*Sorghum bicolor*	Glycerol-3-phosphate	Monoderm bacteria (especially Actinobacteria)	Root growth promotion	Xu et al. (2018b)
Unknown	*Sorghum bicolor*	Pipecolic acid	Monoderm bacteria (Actinobacteria)	Root growth reduction	Caddell et al. (2020)
Unclear	*Oryza sativa*	Unclear	Actinobacteria (Streptomyces)	Root growth promotion	Santos-Medellín et al. (2021)
Unclear	*Oryza sativa*	Unclear	Actinobacteria and Chloroflexi	Root growth promotion	Santos-Medellín et al. (2017)
Unknown	*Populus deltoides*	Unknown	Proteobacteria, Actinobacteria, and Verrucomicrobia	-	Timm et al. (2018)
Unknown	18 plant species belonging to the Poaceae family	Unknown	Actinobacteria	-	Naylor et al. (2017)
Unknown	*Brassica rapa*	Unknown	Increased bacterial richness	-	Terhorst et al. (2014)

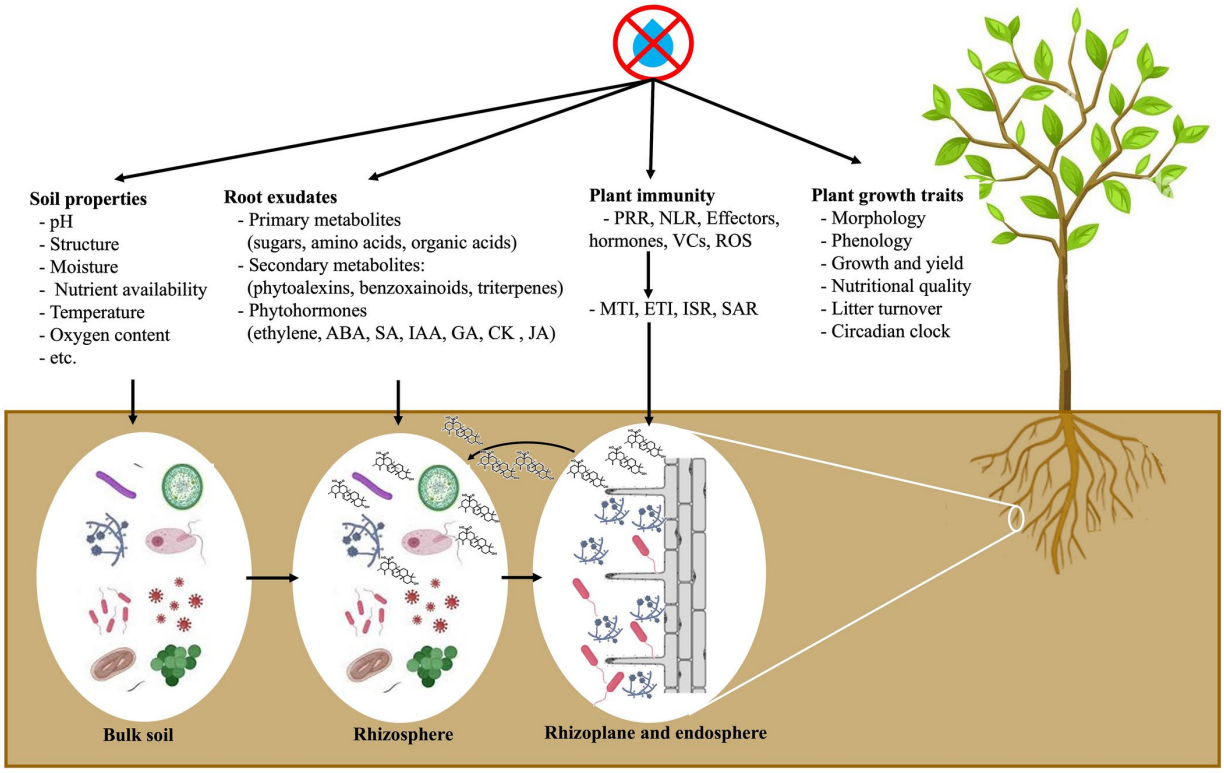

FIGURE 2
Drought-mediated rhizosphere microbiota recruitment: roots actively regulate rhizosphere microbial structure by secreting specific chemicals. ABA, abscissic acid; CK, cytokinin; ETI, effector-triggered immunity; GA, gibberllic acid; IAA, indole-3-acetic acid; ISR, induced systemic resistance; JA, jasmonic acid; MTI, microbe-associated molecular patterns (MAMP)-triggered immunity; NLR, leucine-rich repeats; PRR, plant pattern-recognition receptors; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; VCs, volatile compounds.

bidirectional interactions, particularly in terms of rhizosphere microbiome shaping (Figure 3).

Conventionally, root exudate secretion was thought to be a passive process facilitated by various pathways, such as diffusion across the root cell membrane, ionic channels, and vesicle transport (Baetz and Martinoia, 2014). The chemical characteristics of the exuded molecules determine the type of the secretion pathway. Diffusion is involved in the exudation of metabolites with low molecular weight, such as amino acids, sugars, carboxylic acids, and phenolics. This process is caused by the difference in the concentrations of compounds between root cell cytoplasm and rhizosphere, and it may be influenced by root membrane permeability, root cell integrity, and compound polarity (Badri and Vivanco, 2009). Ionic channel pathways are involved in the exudation of carbohydrates and particular carboxylates, including oxalate and malate (released in large amounts), which are transported through membranes via a transport mechanism performed by proteins rather than diffusion. Two types of anionic channels are involved in this process: SLow Anion Channels (SLACs), which are activated in many seconds, and QUick Anion Channels (QUACs), which take a few milliseconds to be activated (Dreyer et al., 2012). The third form of passive transport pathways is vesicle transport (exocytosis) responsible for the exudation of high molecular weight metabolites kept inside vesicles (Badri and Vivanco, 2009). Exuded compounds are produced by the endoplasmic reticulum or the Golgi apparatus and aid in pathogen defense (Weston et al., 2012). In contrast, the active transport pathway of root-secreted metabolites is mediated by plasmatic membrane proteins (Baetz and Martinoia, 2014): ATP-Binding Cassette (ABC) and Multidrug and Toxic Compound Extrusion (MATE) (Kang et al., 2011). Protein-mediated root exudation may take three forms and depends on their specificity: transporters exuding multiple compounds, compounds secreted via various membrane transporters in the rhizosphere, and metabolites secreted by a single transporter. ABC transporters are classified as primary transporters owing to the use of ATP hydrolysis for the necessary energy to transport varied solutes (Jones and George, 2002; Orelle et al., 2018). The gene names encoding these transporters have evolved over time. Nevertheless, another classification has been established based on the organization of TMD and NBD domains, assembling the various members into nine families termed with letters ranging from A to I, despite the fact that family H does not exist in plants (Verrier et al., 2008). Some studies have looked into the involvement of ABC transporters in root exudation and highlighted their significance in this process (Badri et al., 2008, 2009; Olanrewaju et al., 2019).

Recent studies have shown that shifting from naturally occurring microbiome to adapted microbial communities to the stressful condition enables the plant holobiont to quickly adapt to changing environments (Pantigoso et al., 2022; Faist et al., 2023; Li et al., 2023). Rice provides an example of plant-mediated microbial abundance modulation, where under water deficiency, the microbiome composition shifts to a greater abundance of drought-resistant plant growth promoting rhizobacteria (PGPR) strains (Santos-Medellín

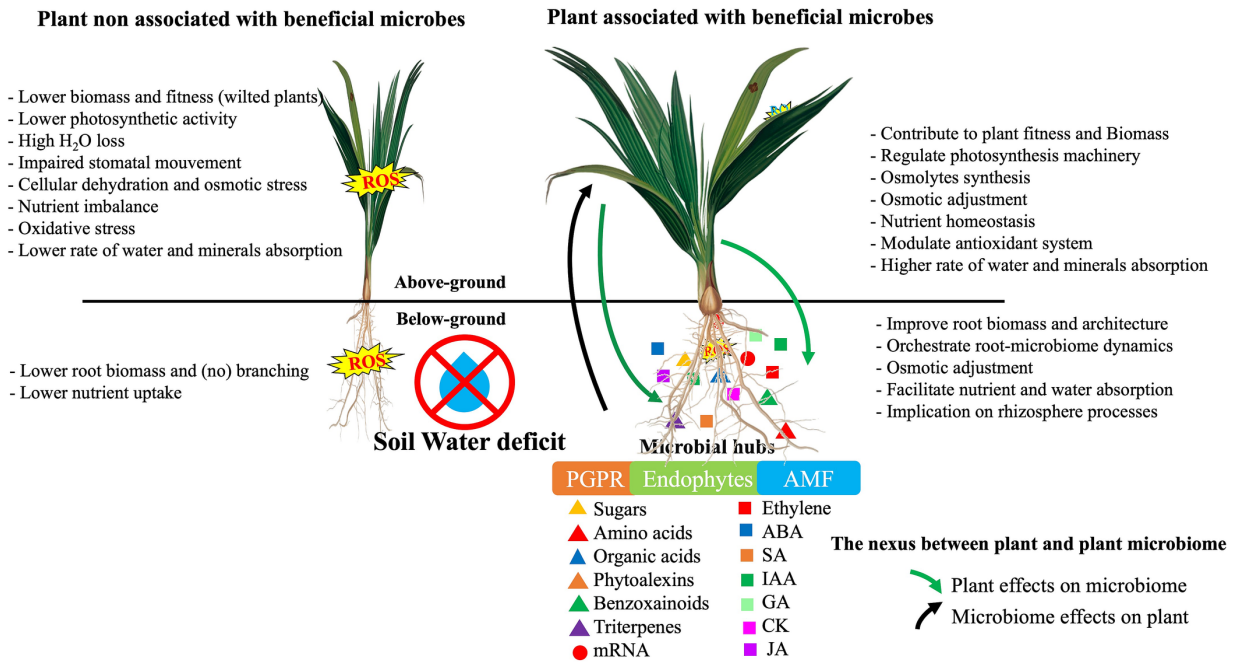

FIGURE 3
Overview of the mechanisms by which plant mediates rhizosphere microbiota to mitigate drought through signalling integrators and metabolic changes. ABA, Abscissic acid; CK, Cytokinin; GA, Gibberllic acid; IAA, Indole-3- acetic acid; JA, Jasmonic acid; mRNA, microRNA.

et al., 2017). Changes in root exudation caused by water shortage may be the source of the enhancement of several types of microorganisms. Stressful soil conditions, along with microbial composition changes, can influence rhizosphere microbial activity. The modulation of the rhizosphere microbiome is influenced not only by plants but also by microbes. By exuding phytohormones, antimicrobials, volatile compounds (VCs), and quorum-sensing, plants may regulate the plant environment and even benefit their hosts (Venturi and Keel, 2016). Under water limitation conditions, the rhizosphere microbiome is shaped by microbial interactions among members and their preferences for specific metabolites (Zhalnina et al., 2018). Microbial interactions may alter gene expression within communicating microbes (Sasse et al., 2018), indicating that microbial interactions affect both the function and shape of the microbiome. According to Uroz et al. (2019), both partners are ecological engineers of the holobiont because they regulate plant-associated microbes. Therefore, microbial communications (and plant-modulating microbiome) in the rhizosphere play a critical role in shaping plant-associated microbiome.

Using metatranscriptomic analysis to study the wheat holobiont under water deficiency, Pande et al. (2023) revealed that the microbial associates were the most responsive to water deficit. The majority of the differentially abundant (DA) genes were associated with bacteria in the rhizosphere and fungi in the roots when comparing drought-stressed wheat and control treatments. In the rhizosphere, Actinobacteria were overrepresented in positively regulated DA transcripts, while Acidobacteria and Proteobacteria were overrepresented in negatively regulated ones. These authors demonstrated that certain transcripts were more abundant in the roots and rhizosphere under severe water stress, including heat shock proteins (HSPs), as well as carbohydrate and amino acid transport and metabolism-related transcripts. Another metatranscriptomic study reported that drought stress promoted the transcripts of Proteobacteria and Bacteroidetes, while reducing those of Actinobacteria and Acidobacteria (Tartaglia et al., 2023). This study also indicated an over-expression of universal stress proteins by Proteobacteria and Bacteroidetes in water-stressed treatment compared to the control. Furthermore, Xu et al. (2018a,b) revealed greater enrichment in various Actinobacteria and Chloroflexi taxa and a decline in the abundance of different Acidobacteria and Deltaproteobacteria taxa under drought conditions. This change was coupled with an increase in G3P-related transcripts in Actinobacteria.

In this cross-kingdom communication, there has been a recent surge of interest in small RNAs, specifically microRNAs, as active 'hormone-like' mediators in cell-to-cell communication (Leitão et al., 2020). *Arabidopsis* and *Botrytis cinerea*, a fungal pathogen, were the first to demonstrate the bi-directional cross-kingdom *RNAi* via sRNA trafficking (Wang et al., 2016). Still, multiple studies are emphasizing the function of microRNA exchanges in non-pathogenic associations with a focus on the host-microbiota communication in the gut (Liu et al., 2016; Bi et al., 2020; Casado-Bedmar and Viennois, 2022). Intestinal epithelial microRNAs have been reported to influence the composition of the gut microbiome by penetrating specific bacteria and controlling the transcription of a large number of genes involved in sugar degradation and housekeeping, thereby influencing the bacterial fitness (Gao et al., 2019). After their ingestion, plants secrete extracellular vesicles (EVs) in the gut, where specific bacteria can uptake them with their microRNA, causing an alteration in their gene expression (Mu et al., 2014; Teng et al., 2018). As a result, their metabolite production and secretion could be modified, resulting in differential growth of bacterial strains that interact with these specific bacteria. Following these advances, Middleton et al. (2021) propose

that plants and their associated rhizosphere microbes interact through microRNAs, that modulate the composition and activity of rhizospheric microbiota. Strikingly, hundreds of microRNAs have been found in host root tissues (Breakfield et al., 2012). It is therefore assumed that some microRNAs, selected through co-evolution with nearby microorganisms, could be secreted through EVs structure in the rhizosphere.

Taking a step further, Escudero-Martinez et al. (2022) applied metagenomics data to map the plant genetic factors regulating microbiota in the rhizosphere of domesticated and wild barley genotypes. The authors identified a small number of loci that have a significant impact on the rhizospheric microbiome composition. One of those loci, called *QRMC-3HS*, emerged as a major determining factor of microbiota composition. A comparative root RNA-seq profiling of soil-grown sibling lines with contrasting *QRMC-3HS* alleles and presenting distinct microbiotas enabled the identification of three primary candidate genes: *Nucleotide-Binding-Leucine-Rich-Repeat* (*NLR*) gene and two other genes from *QRMC-3HS*. The *NLR* gene encodes an NLR protein, one of two types of immune system receptors involved in the microbe proliferation recognition through effector recognition (Jones and Dangl, 2006). The first gene of *QRMC-3HS* is differently regulated in sibling lines with different microbiotas, and it is unclear how it mechanistically contributes to rhizospheric plant-microbiota interactions because it encodes an unknown protein. The second gene encodes a xyloglucan endotransglucosylase/hydrolase (XTH) enzyme involved in the cleavage and/or rearrangement of xyloglucans (Yang et al., 2019), which are the most abundant hemicellulosic compounds in plant primary cell walls (Ezquer et al., 2020). The *XTH* gene may still be involved in microbiota shaping through cell wall polysaccharide modification closely related to plant-microbe interactions (Vorwerk et al., 2004). In *Arabidopsis*, cell wall traits serve as recruitment cues for almost 50% of the endogenous root microbial communities (Bulgarelli et al., 2012). In addition, cell wall alterations underpin some of the gene ontology classes identified in genome-wide association mapping experiments carried out using this plant (Horton et al., 2014). Improved adaptation to soil physicochemical conditions may represent further involvement of *XTH* genes in plant-microbiome interactions. Han et al. (2017) previously reported that *XTH* genes are involved in drought stress tolerance.

2.4 Microbiome drivers of plant fitness benefits under drought

As previously discussed, soil microbes provide various benefits to plants, with root-associated microbiome playing a key role in determining host fitness and performance under various environments, including drought. Root microbiome composition is influenced by both plant and environmental factors. Plant-associated microbiome may boost host development under drought stress by stimulating different layers of plant tolerance mechanisms (Table 1). Among these microbes, PGPRs are abundant in the rhizospheric area and have been shown to be one of the most successful strategies for mitigating the adverse effect of water shortage on the host plant (Etesami and Jeong, 2018). PGPRs enhance crop plant resistance by increasing the production of osmolytes (including glycine betaine and proline), accumulating secondary metabolites, and modulating the expression of a myriad of host drought-related genes.

Vurukonda et al. (2016) revealed that PGPRs associated with stressed plants are crucial for inducing systemic tolerance (IST). A metabolome study of drought-stressed *Sorghum bicolor* primed with *Bacillus* and *Pseudomonas* isolates showed an induction of signature metabolic profiles and biomarkers related to IST in the host (Carlson et al., 2020). The findings revealed substantial treatment-related differential metabolic reprogramming among rhizobacteria-treated and control plants. This was correlated to the ability of the selected isolates to preserve host plants against drought stress by up-regulating IAA, CK, SA, GA, JA, brassinosteroïds (BRs), sphingosine, psychosine, osmolytes and antioxidants, and down-regulating ET production. Raheem et al. (2018) found that IAA and other auxins produced by PGPR bacteria enhance plant performance under drought stress, as demonstrated by *Bacillus amyloliquefaciens*, an auxin-producing bacteria isolated from *Acacia arabica* rhizospheric areas in arid climate. The production of exopolysaccharide by PGPRs is another cue inducing plant drought tolerance. Using a *Bacillus amyloliquefaciens* exopolysaccharide-deficient mutant (epsC), Lu et al. (2018) demonstrated that the *epsC* gene is a key gene involved in drought tolerance in Arabidopsis (Table 3). The authors highlighted the effect of ET and JA in inducing IST and up-regulating many drought-resilient genes (including *ERD1*, *LEA14*, *RD17*, and *RD29A*) in leaves. Staudinger et al. (2016) used proteomics to show that *Sinorhizobium* sp -inoculated *Medicago truncatula* grown under drought induced hormonal crosstalk and increased JA translational regulation, playing a role in increased leaf maintenance in nodulated plants during drought. PGPRs have been shown to accelerate the antioxidant enzymes and osmoprotectants biosynthesis in drought-stressed plants. By using various formulations of PGPRs isolated from *Megathyrsus maximus* rhizosphere in dry areas, Moreno-Galván et al. (2020) showed that the isolates improved *Megathyrsus* drought tolerance by up-regulating proline biosynthesis and down-regulating malondialdehyde (MDA) concentration and glutathione reductase activity. Recently, Singh D. P. et al. (2020) revealed that the overexpression of genes encoding enzymes responsible for phenylpropanoid has been linked to ROS scavenging in rice plants inoculated with *Pseudomonas* and *Trichoderma* and grown under water scarcity conditions. Similarly, upregulation of genes encoding *PiP*, *DHN*, and *DREB* contributes to the improvement of drought resistance in plants treated with PGPRs. Drought-stressed tomato plants treated with PGPRs yielded enhanced growth traits, water status, proline biosynthesis, antioxidant enzyme defense (i.e., ascorbate peroxidase, catalase, and glutathione peroxidase), and H_2O_2 accumulation compared to the controls (Abbasi et al., 2020). The authors demonstrated that PGPR-treated tomato plants showed altered stress signaling and regulatory networks controlling the expression of target genes related to plant response to drought, such as *ERF1* and *WRKY70*. Woo et al. (2020) reported that *Bacillus subtilis* boosted drought resilience in *Arabidopsis* and *Brassica* by up-regulating the expression of drought-sensor genes, including *NCED3, RAB18*, *RD29B*, and *RD20* in *Arabidopsis* and *WRKY7*, *CSD3,* and *DREB1D* in *Brassica*.

Several studies have shown that specialized microbiome may alleviate plant drought stress (Mathur and Roy, 2021; Aslam et al., 2022; Singh et al., 2023). Endophytes, microorganisms living in the endosphere without inducing disease symptoms, confer stress

TABLE 3 Key genes influenced by the microbiome in drought resistance and their primary functions.

Microbe inoculation	Host plant	Genes	Major function	ref.
Bacillus subtilis GOT9	Arabidopsis	*RD29B*, *RAB18*, *RD20*, *NCED3* *DREB1D*, *WRKY7*, *CSD3*	ABA pathways, resistant protein to biotic and abiotic stress	Woo et al. (2020)
Bacillus amyloliquefaciens	Arabidopsis	*epsC, RD17*, *RD29A*, *ERD1*, *LEA14*	EPS production and ET- and JA-mediated pathways	Lu et al. (2018)
Bacillus subtilis, Paenibacillus illinoinensis	Chili pepper	V-PPase genes	Osmoregulation	Vigani et al. (2019)
Ochrobactrum sp. EB-165, Microbacterium sp. EB-65, *Enterobacter* sp. EB-14, *E. cloacae* EB-48	Soybean	P5CS genes (*SbP5CS 1* and *SbP5CS 2*)	Osmoregulation	Govindasamy et al. (2020)
Pseudomonas simiae	Soybean	*P5CS*, *GOLS*, *DREB/EREB, PIP*, *TIP*	Osmoregulation, water transport	Vaishnav and Choudhary (2019)
Bacillus marisflavi CRDT-EB-1	Mustard	*ABA2*, *ABA3*, and *NCED3*	ABA pathway	Gowtham et al. (2021)
Trichoderma parareese	Rapeseed	*ACCO1*, *ERF1*, and *NCED3*	ABA and ET pathways	Poveda (2020)
Funneliformis mosseae	Trifoliate orange	AQP genes (*PIPs*, *TIPs*, *NIPs*)	Water transport	Zou et al. (2019)
Rhizophagus irregularis	Apple	*MdIAA24*, *MdD27*, *MdCCD7*, *MdCCD8a*, *MdCCD8b* and *MdMAXa*	Mycorrhization regulation, SL production	Huang et al. (2021)
Rhizophagus irregularis	Apple	MAPKs (*MdMAPK7-1*, *MdMAPK20-1*, *MdMAPK17*, *MdMAPK16-2*)	Signal transduction during biotic and abiotic stress	Huang et al. (2020)
Piriformospora indica	Rice	*P5CS* genes	Osmoregulation	Saddique et al. (2018)
Funneliformis mosseae	Trifoliate orange	*PtSPMS*, *PtCuAO1*, *PtCuAO2*, *PtCuAO6*, *PtCuAO8*, *PtADC1*, *PtADC2*, *PtPAO1*, *PtPAO2*, and *PtPAO3*	Polyamine metabolism	Zhang et al. (2020)
Funneliformis mosseae	Trifoliate orange	AQP genes (*PtTIP1;2*, *PtTIP1;3*, *PtTIP4;1*)	Water transport	Jia-Dong et al. (2019)
Glomus clarum, *Acaulospora scrobiculata*, *Gigaspora rosea*	Bean	*PvPIP2;3*	Water transport	Recchia et al. (2018)
Glomus intraradices	Lettuce	*Lsnced* gene	ABA biosynthesis pathway	Testerink and Munnik (2005)
Rhizophagus irregularis	Black locust	AQP *genes* (*RpTIP1;1; RpPIP1;3; RpTIP2;1; RpPIP2;1*) and *RpAPX, RpGR*	Water transport, antioxidant defense system	He et al. (2016)
Glomus intraradices	Maize	AQP genes (*ZmPIP1;1, ZmPIP1;3, ZmPIP1;4, ZmPIP1;6, ZmPIP2;2, ZmPIP2;4, ZmTIP1;2, ZmPIP2;5*)	Water transport	Li and Chen (2013)
Glomus intraradices	Chinese milkveth	*AsPT1, AsPT4*	P transport	Xie et al. (2013)
Glomus intraradices	Barrel medic	*AMT2;3*	P transport	Breuillin-Sessoms et al. (2015)
Funneliformis mosseae	Common hoptree	*PtAHA2*	Osmoregulation	Cheng H. Q. et al. (2021)
Rhizophagus irregularis	Black locust	*RpFe-SOD, RpMn-SOD, RpPOD, RpCAT1;*	Antioxidant defense system	He et al. (2017)
Rhizophagus intraradices	Tomato	*TFT2, TFT3*	ABA signaling pathway	Xu et al. (2018a)
Funneliformis mosseae	Barrel medic	*Fm201, Ri14–3-3, RiBMH2*	Mycorrhizae establishment	Sun et al. (2018)
Rhizophagus irregularis	Tomato	*SlCCD7*	SLs signaling pathway	Ruiz-Lozano et al. (2016)
Funneliformis mosseae	Common hoptree	*PtYUC3, PtYUC8, PtABCB19, PtLAX2, PtPIN1, PtPIN3*	IAA signaling pathway	Liu et al. (2018)
Funneliformis mosseae	Sweet orange	*CsCDPK20, CsCDPK22*	Signal transduction during biotic and abiotic stress	Shu et al. (2020)

ABA, abscisic acid; ABCB19, ATP-binding cassette transporter B19; ACCO1, 1-aminocyclopropane-1-carboxylic acid oxidase; ADC1; Arginine Decarboxylase 1; AHA2, H + -ATPase; AMT, ammonium transporter; APX, ascorbate peroxidase; CAT, catalase; CCD, carotenoid cleavage dioxygenase; CDPK, Ca^{2+}-dependent protein kinases; CSD, copper/zinc superoxide dismutase; CuAO1, Copper Amine Oxidase 1; D, Dwarf; DREB, dehydration-responsive element-binding protein; EPS, exopolysaccharides; ERD, early responsive to dehydration; EREB, ethylene responsive element binding protein; ERF, ethylene response factor; ET, ethylene; GR, glutathion reductase; JA, jasmonic acid; IAA, indole-3-acetic acid; Fm, Funneliformis mosseae; GOLS, galactinol synthase; LAX2, Like-Aux1 (AUX1) carrier 2; LEA, late embryogenesis abundant; MAX, more axillary growth; MAPK, mitogen-activated protein kinase; NCED, 9-cis-epoxycarotenoid dioxygenase; NIP, nodulin 26-like Intrinsic Protein; PAO1, Polyamine Oxidase 1; PIN, PIN-FORMED; PIP, prolactin induced protein; POD, peroxidase; PT, phosphate transporter; P5CS, delta1-pyrroline-5-carboxylate synthase; RAB, responsive to ABA; RD, desiccation-responsive protein; Ri, Rhizophagus intraradices; SLs, Strigolactone; SOD, Superoxide dismustase; SPMS, Spermine Synthase; TFT, Tomato Fourteen-Three-three; TIP, tonoplast intrinistic protein; V-PPase, vacuolar proton pumps H^+-PPase. YUC3, YUCCA3.

resilience to host species and perform a significant function in the survival of some plants in high-stress environments. Various bacterial and fungal endophytes have been reported to increase plant drought resilience. Bacterial endophytes isolated from drought-stressed maize roots improved plant fitness due to biosynthesis of IAA, GA, and CK, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and siderophore biosynthesis (Sandhya et al., 2017). Drought-stressed sorghum plants treated with a consortium of root bacterial endophytes (*Enterobacter cloacae*, *Enterobacter* sp., *Ochrobactrum* sp., and *Microbacterium* sp.) resulted in higher growth and osmotic adjustment (Govindasamy et al., 2020). Drought tolerance mediated through microbial inoculation has been associated with enhanced proline accumulation and proline-related biosynthesis genes, *SbP5CS 1* and *SbP5CS 2*. Interestingly, Vigani et al. (2019) found that root endophytic bacteria conferred drought resistance in *Capsicum annuum* by modifying vacuolar H^+ pyrophosphatases (VPP) expression, which aided in preserving osmotic balance. Strains of *Bacillus* endophytes found in *Lepidium perfoliatum* roots were responsible for the formation of biofilm on roots, resulting in drought resistance and seedling germination (Li et al., 2017). *Piriformospora indica*, a well-known root fungal endophyte, colonizes the roots of many plant species and provides multifaceted amenities, including drought alleviation (Gill et al., 2016). Metabolome and proteome profiling analysis of barley plants inoculated with *P. indica*- grown under drought stress showed that inoculation redistributes resources, maintains the aquaporins (AQPs) presence, and promotes energy modulation, photorespiration protective proteins and transporters production, primary metabolism, and autophagy in water-stressed plants (Ghaffari et al., 2019). Inoculation of rice roots with *P. indica* increased growth, biomass accumulation, mineral nutrition (Zn and P), and upregulated *P5CS* genes in drought-stressed plants (Saddique et al., 2018). González-Teuber et al. (2018) reported that inoculating the roots of *Chenopodium quinoa* with the fungal endophyte *Penicillium minioluteum* primarily conferred drought tolerance through significant adjustments in below-ground biomass, photosynthesis, water-use efficiency (WUE), and photochemical efficiency.

AM symbiosis is one of the most complex and mutualistic interactions that plants have evolved to cope with droughts. Following a molecular dialog between the two partners involving "branching factors," "mycorrhizal factors," and common symbiotic signaling pathways, the AMF enters the plant roots and establish arbuscule formations ensuring nutrient exchange. Plant hormones act as central regulators of the development of the plant-AMF interaction (Santner et al., 2009; Charpentier et al., 2014; Etemadi et al., 2014), and auxins have potential key role in this interaction (Takeda et al., 2015; Jin et al., 2016). The modulation of AMF-mediated host drought tolerance is an extremely intricate process implicating multiple metabolites and pathways. Particularly, AMF can directly improve nutrients and water absorption and transport, boost host osmotic regulation, and increase plant gas exchange capacity, WUE, and antioxidant defense (Osakabe et al., 2014; Ruiz-Lozano et al., 2016). Zhang et al. (2019) found that a combination of *Funneliformis mosseae*, *Rhizoglomus intraradices*, and *Diversispora versiformis* improved the survival of *Zenia insignis* subjected to water scarcity through regulating osmolytes accumulation, antioxidant enzyme activity, and plant N and P uptake. Drought-stressed trifoliate oranges inoculated with *F. mosseae* have less oxidative stress via the increase of H_2O_2 efflux from the root system (Santner et al., 2009; Jin et al., 2016). In addition, the regulation of polyamine metabolism-associated gene transcripts by AMF has been identified to play a pivotal role in drought resilience (Zhang et al., 2020). Taking a step further downstream, several AM-specific host genes and proteins have been reported to play a key role in promoting plant water stress resilience. The different mechanisms involved in conferring drought resilience to mycorrhizal plants have been previously reviewed (Cheng S. et al., 2021; Wang et al., 2023). Drought-induced genes and compounds can be divided into: functional genes playing a direct role in stress [i.e., late embryogenesis abundant (LEA) proteins, AQPs, sugar, and proline] and regulatory genes implicated in the regulation of gene expression and the transduction signals (i.e., stress-related TFs) and signal molecules, such as calmodulin-binding protein. Many aspect of the molecular regulatory network linking AM symbiosis and drought stress have been elucidated, including the identification of several gene/protein functions (Bahadur et al., 2019; Ho-Plágaro and García-Garrido, 2022). Various TFs, such as *AP2/ERF* family, *GRAS* family, and *MYB* family have been found to be specifically induced by AMF under drought conditions, thereby contributing to the modulation of stress by phytohormones and other molecular signaling pathways (Wang et al., 2023). Jia-Dong et al. (2019) revealed that AM symbiosis significantly activated the expression of root tip *AQPs PtTIP1;2*, *PtTIP1;3*, and *PtTIP4;1* in drought-stressed trifoliate orange. In the same vein, it has been reported that drought stress induced the up-regulation of eight *AQP*-associated genes in AMF-treated plants (Recchia et al., 2018). An interactive impact of AMF and drought was recorded on the over-expression of *MAPK* pathway gene that triggers an improvement in photosynthetic efficiency, osmolyte production, and antioxidant defense (Huang et al., 2020). Testerink and Munnik (2005) showed the role of ABA in up-regulating resistance genes expression to mitigate drought-induced damages. Under water limitation, lettuce inoculated with *G. intraradices* exhibited high expression of the *Lsnced* gene, which encodes a pivotal enzyme in the production of ABA. In contrast, the expression of this gene in roots was not affected by the exogenous application of ABA. This suggests that mycorrhizal plants adjust the endogenous ABA levels more efficiently and quickly than non-AM plants, leading to a more appropriate equilibrium between water acquisition and leaf transpiration under water deficit (Aroca et al., 2008). In a proteomic study on wheat, differential expression proteins involved in cell wall integrity and carbohydrate production were observed, while most stress-associated molecules, including enzymes involved in ET biosynthesis, were downregulated (Bernardo et al., 2017).

Recently, Poveda (2020) found an increase in the secretion of the fungal enzyme chorismate mutase by using mutant strains of *Trichoderma parareese*. This increase conferred tolerance to rapeseed plants grown under drought conditions by increasing gene expression (i.e., *NCED3* and *PYL4*) related to the hormonal pathways of ABA. Notably, colonization of *Trichoderma* sp. on rapeseed roots increased under stressed condition, initiating a myriad of host metabolic pathways. Comparative qRT-PCR analyzes with the chorismate mutase-silenced strain connected this enzyme to drought-tolerant mechanisms owing to its involvement in *ACCO1* (1-Aminocyclopropane-1-carboxylic acid oxidase) and *ERF1* downregulation, and ABA pathway genes *NCED3* upregulation. In addition, Bashyal et al. (2021) used transcriptome profiling of droughted- *Trichoderma harzianum*-inoculated rice and revealed the upregulation of 1,053 genes and the downregulation of 733 genes in stressed *T. harzianum*-inoculated plants. Most photosynthetic and

antioxidative genes, including plastocyanin, PSI subunit Q, PSII subunit PSBY, small chain of Rubisco, proline-rich protein, osmoproteins, stress-induced proteins, AQPs, and chaperonins, were exclusively expressed in stressed *T. harzianum*-inoculated rice. Using the enrichment analysis, the same authors showed that the metabolic (38%) and pathways involved in the synthesis of secondary metabolites (25%), phenyl propanoid (7%), carbon metabolism (6%), and glutathione metabolism (3%) were the most enriched pathways.

The co-inoculation with different members of plant microbiome was also reported to enhance host drought tolerance. Eshaghi Gorgi et al. (2022) studied the effect of the dual application of PGPR and AMF on biomass accumulation, water status, photosynthetic pigments, and proline content of drought-stressed *Melissa officinalis*. They demonstrated that the combined microbial treatment increased all these parameters under drought stress. The same authors also reported that leaves chemical composition of secondary metabolites was altered by PGPR+AMF inoculation. Another study showed that the co-inoculation of Common myrtle with *Funneliformis mosseae* and *Rhizophagus irregularis* AMF strains and *Pseudomonas fluorescens* and *P. putida* PGPR strains boosted seedlings survival, growth fitness, and (non-)enzymatic antioxidant accumulation while reducing electrolyte leakage, and MDA and proline concentrations under drought stress (Azizi et al., 2021). In the same vein, the combined application of *Glomus versiforme* and *Bacillus methylotrophicus* recorded significant drought resistance through improving growth and photosynthetic performances, nutrition status, phenols and flavonoids accumulation, antioxidant enzymatic system and ABA and IAA concentrations in tobacco plants under drought stress (Begum et al., 2022). Singh D. P. et al. (2020) revealed that *Trichoderma* and *Pseudomonas* primed rice seeds induced significant increase in the transcript levels of multiple genes involved in the antioxidant enzymes and phenylpropanoid biosynthesis pathway in seedlings grown under drought stress.

Several studies have revealed the significant beneficial effects of combining microbes and others biostimulants in mitigating drought stress. The application of AMF and organic amendment improved the tolerance of pistachio seedlings to drought stress through enhancing soil physicochemical and biological traits, as well as plant nutrient uptake (Paymaneh et al., 2023). Soussani et al. (2023) demonstrated that the application of AMF and/or compost promoted tomato growth, yield and fruit bioactive compounds, while reducing oxidative stress and enhancing the efficiency of the antioxidant enzyme system under water stress. The combined effect of AMF, PGPR, and compost boosted tomato growth fitness, fruit yield and quality, while increasing drought stress tolerance (Tahiri et al., 2022). A two-year field experiment showed that the application of different biostimulants such AMF, PGPR, and seaweed extract increased wheat root volume, membrane stability index, leaf relative water content (RWC), and photosynthetic pigment content, which promoted plant resilience under water shortage conditions (Najafi Vafa et al., 2022).

3 Concepts and mechanisms of plant-microbial adaptation to drought trends

The rhizosphere microbiome induces a profound influence on host physiology, and both monocrop and rotational crops exhibit significant variations in the regulation of different genes related to phytohormones and plant defense system (Li et al., 2021). Managing aboveground biodiversity can increase the diversity of plant-associated microbiome and plant immunity, resulting in substantial economic and ecological benefits. Substantial progress has been made in understanding how drought affects the molecular machinery that drive the connection between aboveground and belowground diversity. In fact, drought can promote plants to support root biomass production or form novel and stronger associations with AMF to provide distant water from the rhizospheric area to the plants. These phenotypic and biotic alterations have the potential to significantly affect soil physical properties. Elucidating how combined changes in abiotic components affect plant adaptation allows for anticipating resilience and productivity. However, it will be necessary to understand the spatial and temporal dynamics of ecosystems and to consider multiple ecosystem dimensions, especially physical properties.

Plants possess cognitive capacities and may obtain, process, and memorize information that may adjust their behavior to (upcoming) natural signals (Michmizos and Hilioti, 2019). The cognitive abilities of plants can be extended to their surrounding environment either through root functions or beneficial symbiotic microorganisms (Parise et al., 2020). However, the operation of a plant cognitive system is still largely unknown given the lack of a nervous communication system, as is the case with animals. Well-identified learning and memory mechanisms at cellular and molecular levels may provide a plausible pathway toward adaptation built on prior stimuli such as prolonged water stress. However, determining the role of plant gnosophysiology–a newly emerging scientific field challenges conventional perspectives and delves into the concept that plants possess the ability to respond to stimuli, and learn from them, and make decisions ensuring their survival–and its relative contribution to drought resilience will require substantial theoretical and experimental evidence.

Due to the importance of plant and soil-rhizosphere microbiome diversity in boosting host defense and resilience to environmental constraints, a drought-related decrease in biodiversity has the potential to impact the abiotic resilience of plant communities with serious implications for adaptation. Enhanced plant diversity offers various PRR reservoirs capable of recognizing a variety of MAMPs, thereby boosting immunological defense mechanisms. Systemic signals can then be transmitted from one plant species to another via VCs (produced by microbes and plants) or root exudates, which impact primary productivity and host tolerance (Weisskopf et al., 2021).

By inducing the transcriptional reprogramming of numerous genes and TFs involved in a range of plant defense systems, microbial communities can assist host-plants endure water deficiency (Figure 4). A recent study revealed that under stressful conditions, microorganisms may produce ABA or ABA analogs and trigger essential genes that produce ABA, such *ABA2*, *ABA3*, and *NCED3* (Gowtham et al., 2021). ABA plays a variety of roles, from detecting environmental signals to activating transcription as an adaptive mechanism to regulating a range of developmental, physio-biochemical, and cellular, properties. According to Woo et al. (2020), *B. subtilis* strain GOT9 boosted water stress tolerance in *A. thaliana* by inducing the expression of several *NCED* genes, which control crucial ABA production-related enzymes. Additionally, a large

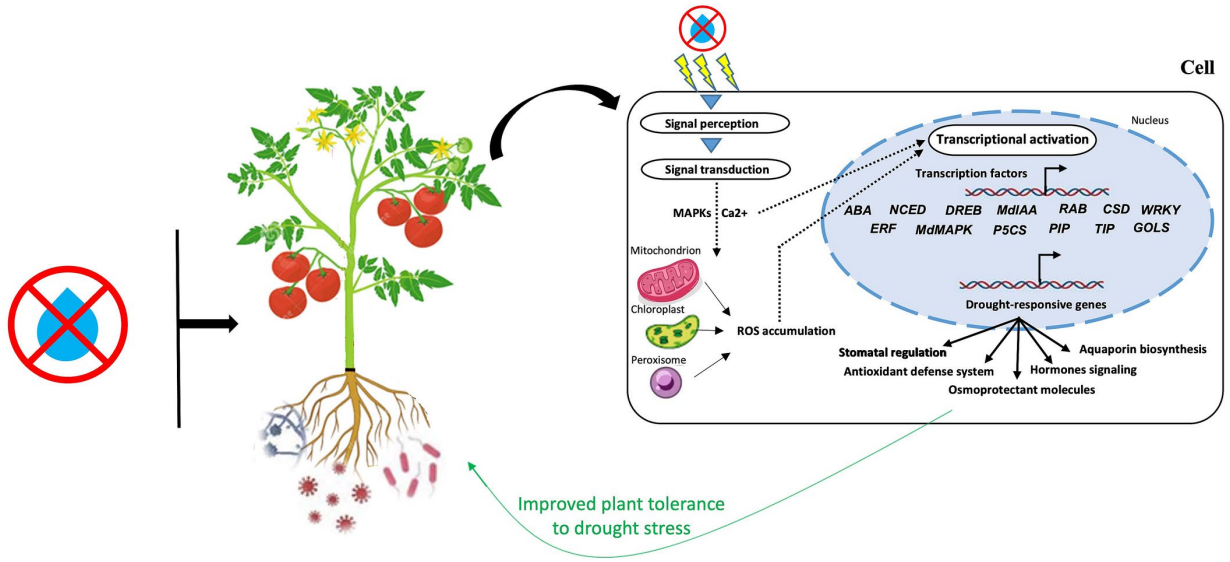

FIGURE 4
A schematic diagram highlighting the major signaling players involved in the microbiome-plant-drought axis. MAPKs, mitogen-activated protein kinases; ROS, reactive oxygen species.

number of genes implicated in the control of ABA drought signaling pathways (Takahashi et al., 2018), including *DREB1D*, *RAB18*, *RD20*, *RD29B*, *CSD3*, *WRKY7*, and *ERF1* are induced by plant-associated microorganisms (Abbasi et al., 2020; Woo et al., 2020).

Furthermore, the transcriptional activity of MAPKs genes, including *MdMAPK7-1*, *MdMAPK16-2*, *MdMAPK17*, and *MdMAPK20-1*, which are crucial for signal transduction during stress, have been reported to increase in AMF-treated apple seedlings (Huang et al., 2020). Ca^{2+}-dependent protein kinases (CDPKs) and MAPKs perform a significant function in the signals transduction to the nucleus during drought stress through multiple TFs (i.e., *DREB*, *ABRE*, *MYB/MYC*, *WRKY*, and *NAC*) involved in drought-tolerant genes regulation (Ali et al., 2022). In the same vein, a recent study in maize reported that *pyrroline-5-carboxylate synthase* (*P5CS*) genes, responsible of proline production, including *SbP5CS 1* and *SbP5CS 2,* were upregulated under water stress (Govindasamy et al., 2020). Similar findings were made by Saddique et al. (2018), who found that *P. indica*-treated rice showed *P5CS* genes upregulation and improved phosphate and zinc uptake in order to alleviate the negative impact of water deficiency. In addition, *Pseudomonas simiae*-inoculated soybean enhanced fitness and water stress resistance through improving the transcription of several important genes, including water transporters (*TIP* and *PIP*) and osmoprotectants (*P5CS*, *DREB/EREB*, *GOLS*) (Vaishnav and Choudhary, 2019). The aforementioned alterations in the expression of proline-related genes were strongly linked with plant morphological and physiological adaptation to water deficit effects.

AQPs are considered as a main actor in the cell transport system. Jia-Dong et al. (2019) have suggested that these proteins are frequently linked to the process of nutrient exchange during mycorrhizal symbiosis. Zou et al. (2019) proposed an AMF-boosted mechanism that enhances the tolerance of trifoliate orange to water deficiency by up-and-down regulating the expression of specific AQPs genes. This study confirms the essential and direct involvement of these fungi in host water stress resistance. In fact, *MdIAA24* gene overexpression, which controls apples mycorrhizal symbiosis by regulating strigolactones production, was associated with general plant drought tolerance, including improvements in RWC, stomatal conductance, and osmotic adjustment (Huang et al., 2021). Metabolomic and proteomic investigations have revealed the upregulation of multiple essential genes controlling transporters, signaling proteins, major metabolic enzymes, and oxidative stress-related proteins following the application of *P. indica* in barley under drought condition (Ghaffari et al., 2019). However, further research in other species is necessary to determine whether these gene expression patterns are universal.

In addition to plants inoculated with beneficial microorganisms, pathogen-infected plants have also shown improved tolerance to drought stress. For instance, rice infected with brome mosaic virus (BMV) and beet plants infected with cucumber mosaic virus (CMV) have shown enhanced production of various antioxidants and osmoprotectants, which promote their tolerance to drought stress (Xu et al., 2008). Another study demonstrated that the viral protein 2b, which impacts host ABA signaling and RNA silencing pathways, provided drought tolerance to *Arabidopsis* plants in the case of CMV (Westwood et al., 2013). When *Nicotiana benthamiana* was treated with Yellowtail flower mild mottle virus, its ability to withstand water stress was also enhanced (Dastogeer et al., 2018). The effects of two ascomycete fungal endophytes isolated from wild Australian *Nicotiana* growing in an arid area that were examined in conjunction with this virus on plant gene expression and osmolytes and antioxidants production under drought stress were comparable to the virus effect. These outcomes indicated that the responses of plants infected with viruses and fungi to water deficiency are somewhat similar.

4 Surviving drought with new microbiome research approaches and applications in modern agriculture

4.1 Research approaches and applications–paving the way to harness the power of microbes to survive droughts

The ubiquitous presence of the soil microbiome and its impact on various aspects of plant functioning under drought stress have made it increasingly challenging to isolate specific aspects of the microbiome-plant-drought axis. This highlights the need for multi-disciplinary interventions to uncover opportunities and strategies for enhancing crop drought resistance (Hartman and Tringe, 2019; Ali et al., 2022). Multidomain research approaches that combine plant resilience, microbiome recruitment, and the interactions among the different (a)biotic components of the ecosystem have proven to be valuable strategies. They establish connections that modulate microbiome composition and activity, leading to improved drought tolerance (Trivedi et al., 2022). Yet, the full potential of the host microbiome in sustainable agriculture is not yet fully realized due to multiple factors, including soil type, plant genotype, microbial interactions, agricultural practices, and the complex interplay among these components (Busby et al., 2017; Soman et al., 2017; Schmidt et al., 2019). Moreover, microbiome engineering faces challenges in achieving accurate and long-lasting positive effects on plants. Multiple interconnected factors, such as the richness and complexity of microbial communities and alterations in microbiota functioning during host development stages, contribute to the complexities that limit the effectiveness of microbiome engineering.

To maximize the benefits of plant microbiome assembly under drought stress, it is crucial to investigate the genetic complexity of both plants and their associated microbiome, the heterogeneity of their environment, and the metabolic patterns that influence the host microbiome. This requires the application of inclusive and systemic biological methods coupled with advanced multiomics techniques. While existing tools and methodologies have made significant advancement in understanding the effect of the microbiome on host resistance to water stress, there are still many gaps to be filled in order to make substantial progress toward microbiome-targeted approaches for enhancing crop production and resilience under drought stress.

Many approaches such as culture-dependent, culture-independent, and reductionist synthetic communities (SynCom) have been primarily used to study the root microbiome. However, reproducing a suitable natural conditions required for the development of different microbes in the laboratory is challenging, and a large number of them cannot be cultured, leading to a loss of microbiome information (Hill et al., 2000). However culture-dependent methods, including the reductionist SynCom method remain highly efficient for microbiome studies (Bai et al., 2015; Zhang et al., 2021). DNA fingerprinting and phospholipid fatty acid approaches, as culture-independent methods, focus on studying the composition and diversity of the entire microbiome community. However, these approaches provide less data in comparison to the current advances in metaomics, sequencing, and computational techniques (Jo et al., 2020). By utilizing advanced sequencing methods, studies have achieved unprecedented precision and comprehensiveness in understanding the composition of microbial communities under drought condition (Lundberg et al., 2012; Liu et al., 2019). Furthermore, the development of metagenomics, metatranscriptomics, and metaproteomics approaches enable a thorough understanding of microbiome function under drought stress (Liu et al., 2021).

The census method, which includes metagenomic and amplicons statistics, offers a comprehensive understanding of plant associated-microbes, which is essential for studying natural microbiome facts. On the other hand, the reductionist SynCom method links plant molecular biology to microbial ecology (Guttman et al., 2014; Mendes et al., 2014; Bulgarelli et al., 2015; Edwards et al., 2015; Liu et al., 2019). The census approach provides data for the reductionist investigation of plant-microbiome interactions. By combining this information with data on isolated strains, an extensive range of representative SynCom can be created, providing accurate genetic information (Bulgarelli et al., 2012; Vorholt et al., 2017). These SynComs are then used to simulate plant-microbiome interactions, allowing the study of the components that orchestrate microbial communities and validate their monitoring functions and molecular strategies throughout plant growth and development, even under stressful conditions, such as drought (Bai et al., 2015; Castrillo et al., 2017; Chen et al., 2020; Finkel et al., 2020).

Multiomics approaches offer a valuable tool for addressing the challenging task of translating plant alterations at the genetic, proteomic, or metabolomic levels. The integration of multiomics data-driven science has significantly advanced our understanding of microbiome composition and functional responses in intricate environments, such as the rhizosphere, where the complex network of microbial connections orchestrates plant behavior under stressful conditions. The combination of these approaches has led to remarkable progress in plant-microbiome-related research.

Each technique employed in multiomics approaches brings unique advantages, but no single approach can provide a comprehensive understanding of the mechanisms governing the assembly of plant-associated microbiome under drought stress. Therefore, it is crucial to integrate various research approaches to pave the way toward a comprehensive understanding and utilization of the microbiome in future studies, with the aim of developing targeted strategies to boost plant resilience to water deficit stress.

Unlocking the intricate interactions between plant and their microbiome under water limitation and their implications for plant fitness and productivity are crucial steps toward harnessing the potential of the microbiome to promote host adaptation to environmental perturbation. Exploring the extent of these interactions at evolutionary, ecological, biochemical, and molecular levels can provide valuable insights for advancing our system-level understanding and inform microbial approaches to improve host resistance and fitness. Several key research directions should be prioritized in the future:

Gain a complete comprehension of how drought impacts the assembly and functions of the plant microbiome across different temporal and spatial scales.

Investigate the effect of drought on plant fitness and defense system, elucidating the specific alterations in photosynthates, root exudates, and defense mechanisms, and their influence on the assembly and functions of the plant microbiome.

Determine the changes in major signaling cascades and metabolite profiles and their interactions, elucidating their roles in shaping host plant and microbial functions and fitness.

Increase our understanding of the biosynthetic pathways, genetics, and mechanisms of action of drought-responsive

phytohormones, as well as their effects on plant-microbiome and microbe-microbe interactions.

Identify the frequency and duration of drought necessary for the eco-evolutionary adaptation of plant microbiome and the establishment of drought resilience in host plant.

Acquire cutting-edge knowledge about the molecular interactions that orchestrate plant-microbe interactions under drought conditions.

Develop methods for *in situ* manipulation of the plant and associated-microbiome to mitigate the detrimental effects of drought on crop productivity.

4.2 Eying the future–microbiome could hold keys to mitigating drought

Microorganisms exhibit remarkable resilience to harsh environments, triggering various signaling cascades and metabolic processes that enable them to swiftly adapt to changing conditions. The soil microbiome, with its manifold benefits for plants, has the ability to mitigate the adverse impact of drought on crops (Anli et al., 2020; Boutasknit et al., 2020; Ben-Laouane et al., 2021; Meddich et al., 2021). For instance, certain ectomycorrhizal fungal (EMF) species in the rhizosphere have been found to colonize the roots of specific pinyon pine genotypes, enhancing their drought resistance (Lau and Lennon, 2012). The varying drought tolerance among different genotypes can be attributed to the presence of these EMF communities, which represent an extended genetic repertoire of the host plant and may serve as a key strategy for drought resilience. The association between host plant and diverse bacterial/fungal communities, possessing potent metabolic and biogeochemical functions, can facilitate plant adaptation to water scarcity. Through long-term field and greenhouse studies coupled with microbial community sequencing, researchers have uncovered links between host-determined EMF communities and disparities in plant performance. Moreover, they have discovered that drought-resistant genotypes exhibit more intense colonization by *Geospora* EMF species. Adaptation has been a subject of extensive discussion in evolutionary biology and ecology. Gehring et al. (2017) revealed that the adaptation does not necessarily involve drastic changes in phenotype but rather subtle variations that affect how the host forms associations with microscopic rhizospheric fungi. Many questions remain unanswered; (1) What are the host traits that control specific interactions with microbial communities? Are they morphological, chemical, or even phenological? Speculation can be made regarding the involvement of genes and chemical signaling molecules that underlie host-mycorrhizal interactions, (2) How significant are plant-associated soil microbial communities in drought adaptation? The fact that genotypic difference in drought resistance were observed only in pinyon pine associated with microbes suggests that the plant's ability to form specific microbial associations is the primary driver of drought resistance.

Given that host genotypes shape distinct soil microbial communities that perform various biogeochemical and metabolic functions (Edwards et al., 2015; Wagner et al., 2016), the potential adaptation of plant traits controlling plant-microbe interactions becomes significant. A comprehensive understanding of host-microbiome interactions at biochemical and genomic levels will enhance our knowledge of drought adaptation and contribute to improved models of host responses to water deficiency. Recognizing that genotypes exhibit differential interactions with aboveground and belowground microbes may revolutionize the strategies employed to enhance plant agronomic traits and sustain global food security in the face of increasing drought trends.

5 Conclusion

During periods of water deficiency, plants heavily rely on microbiome to perform essential functions, including nutrient uptake and stress adaptation. In this review, we have synthesized the current understanding of plant-microbiome interactions under drought stress, which ultimately shape the composition of root-associated microbes. However, there is a lack of research investigating the root-associated microbiota specifically under drought conditions. The limited knowledge about the intricate connections among microbes and their modulation under drought, as well as their complex communication with the host plant, highlight the need for further investigation. Therefore, it is imperative to conduct more research on the plant–microbiome-drought axis, with a particular emphasis on omics-based techniques that integrate genomics, metagenomics, proteomics and metabolomics. Such approaches will unravel the mechanisms orchestrating the adaptation of plant microbiome assembly to drought trends and provide insights to identify microbial communities that confer drought resilience and enhance plant performance under water deficient conditions.

Author contributions

MA: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. AM: Investigation, Supervision, Validation, Writing – review & editing. MB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Endophytic fungi: versatile partners for pest biocontrol, growth promotion, and climate change resilience in plants

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Plant-associated endophytic fungi (EFs) are emerging as a promising solution to advancing modern agriculture and fostering environmental sustainability, especially in the face of climate change scenarios. These fungi, either naturally residing in plants or introduced through artificial inoculation techniques, improve agricultural production due to their various roles in protecting and supporting host plants. The majority of EFs serve as natural biocontrol agents for a variety of agricultural pests, such as insects, phytopathogens, nematodes, and weeds. Notably, EFs produce secondary metabolites, trigger immune responses, modify plant defense gene expression, confer host plant resistance and/or tolerance, and regulate pest growth, populations, and survival to combat agricultural pests. Beyond controlling pests, EFs promote optimal plant growth, development, and resilience by aiding in the synthesis of vital compounds such as phytohormones and bioactive metabolites, nutrient acquisition, and fortifying plants against environmental stresses and climatic changes. Moreover, the mostly nonpathogenic nature of EFs, coupled with their high yield potential, environmental safety, and cost effectiveness, positions them as eco-friendly and economically viable alternatives to synthetic agrochemicals amidst rapid climate change scenarios. As a result, the promising horizon of EFs in agricultural production necessitates interdisciplinary study and microbial modulation approaches to optimize symbiotic plant-EF relationships and their potential for improved productivity. This review provides current and comprehensive insights into the practical applications and multifaceted benefits of EFs in pest management, plant growth promotion, and climate change resilience for future agricultural production improvements. The analysis reveals the potential of developing EFs into innovative bioformulations such as biofertilizers, biostimulants, and biopesticides, thereby paving the way for their integration into a sustainable and more resilient future agricultural system.

KEYWORDS

agricultural pests, colonization, growth promoters, mutualistic endophytes, nutrient acquisition

1 Introduction

Global food and nutrition security, in the face of rapid population growth and climate change, requires a sustainable food production approach addressing availability, access, utilization, and stability, ensuring livelihoods while preserving natural ecosystems and services (Giller et al., 2021). Agriculture, which is responsible for 90% of global food calories (Cassidy et al., 2013), will be at the forefront of transforming and/or reforming conventional practices by employing agroecological principles to address sustainability and environmental concerns within food systems. These concerns have led plant-associated microorganisms, particularly endophytic fungi (EFs), to emerge as a new frontier in agricultural production, achieving global demands for nutritious foods and eventually becoming fundamental to sustainable agriculture (Chitnis et al., 2020; Singh et al., 2021; Verma et al., 2022). In particular, EFs can reduce reliance on synthetic agrochemicals, which have a detrimental impact on agricultural productivity, ecosystems, and human health, thereby promoting environmentally friendly approaches for addressing food production challenges (Fadiji and Babalola, 2020; Rigobelo and Baron, 2021). Therefore, EFs are crucial for guaranteeing agricultural production and environmental sustainability.

The widespread application of EFs is revolutionizing the field of agriculture, primarily due to their remarkable capacity to support and protect host plants by producing beneficial bioactive molecules and acting as biocontrol agents to naturally regulate plant pest populations (Yan et al., 2019; Verma et al., 2022). These fungi colonize plants through root, stem, or leaf tissues and can occur naturally or be introduced into plants through seed coating, root dipping, and foliar spraying (Vega et al., 2008; Tefera and Vidal, 2009; Jain and Pundir, 2017). Most EFs are classified in the phyla *Oomucota*, *Ascomycota*, *Chytridiomycota*, and *Zygomocata* (Gul et al., 2014). Most of these important EFs are found in the orders *Hypocreales* (*Ascomycetes*) and *Entomophthorales* (Sharma et al., 2019). For instance, *Beauveria, Metarhizium, Paecilomyces, Aschersonia, Hirsutella,* and *Lecanicillium* are the most well-known fungal genera of these orders (Goettel, 2008). Mostly, they are formulated as *Beauveria bassiana*, *Metarhizium anisopliae*, and *Trichoderma* spp. propagules (Gul et al., 2014).

Endophytic functional groupings are diverse in ecological functions, host range, taxonomy, transmission, tissue specificity, and colonization patterns (Aamir et al., 2020). Based on ecological category/diversity/functional roles, EFs were grouped into two main categories: clavicipitaceous fungal endophytes (C-endophytes) and nonclavicipitaceous (NC-endophytes) fungal endophytes (Aremu et al., 2017). C-endophytes, also known as true endophytes, are host-specific and most common in the grass family Poaceae, which includes *Balansia* sp. and *Epichloë* sp., and often transmitted vertically through seeds, potentially resulting in an obligate association and higher rates of infection (Khiralla et al., 2016). These EFs are beneficial to the plant by improving plant growth and producing certain toxic chemicals against herbivory; however, they rely on the host plant species, environmental conditions, and genotype (Aremu et al., 2017). In contrast, NC endophytes are predominant and associated with both nonvascular and vascular plant species and are transmitted horizontally. These endophytes, including EFs such as *Lecanicillium lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae,* and *Isaria* spp., can have impacts on several agricultural pests by antagonizing plant diseases and promoting plant growth (Jain and Pundir, 2017). However, until now, only a few species of EFs have been isolated, identified, characterized, and implemented in pest management.

Mutualistic endophytes, which are mostly fungal microbes that survive within plant tissues without adversely affecting the host, can promote plant growth and development while efficiently reducing agriculturally important pests, especially insects (Vega et al., 2008; Agbessenou et al., 2020), plant pathogens (Yan et al., 2019), and nematodes (Tolba et al., 2021). The mechanisms of EF colonization to suppress potential pests differ depending upon the species of EFs and the host plants to be colonized, and they can be employed in either direct or indirect ways (Quesada-Moraga et al., 2014; Zhang et al., 2019; Rajani et al., 2021). Furthermore, EFs are ubiquitous and have been demonstrated to improve host plant growth and tolerance against various abiotic stresses (Lata et al., 2018; Morsy et al., 2020). As a result, there is significant and developing interest in implementing them in agriculture as biocontrol agents of pests and plant growth and adaptation promoters (Molina-Montenegro et al., 2016; Mantzoukas and Eliopoulos, 2020). The use of EFs is also considered an alternative to synthetic insecticides (Sinno et al., 2020), eco-safety and lack of pathogenicity to plants (Mantzoukas and Eliopoulos, 2020), to reduce the impact of pesticides on living organisms and resistance development (Hernández-Rosas et al., 2020), less harmfulness to nontarget organisms, cost-effectiveness, high yield, and absence of harm to the environment (Mantzoukas and Eliopoulos, 2020). According to Yung et al. (2021), EFs are mostly nonpathogenic, with potential applications in organic matter mineralization, plant nutrition, biocontrol agents, crop productivity improvement, and sustainability while reducing chemical inputs, making them environmentally friendly tools that require integration and/or rotation with other alternatives. In addition, EFs also protect plants from different environmental stresses, such as drought, salinity and temperature (Lata et al., 2018; Morsy et al., 2020). However, their success depends on many factors, including their interactions, target host, and environment (Morales-Sánchez et al., 2020). The specific relationships between host plants and EFs, as well as the environment, could change throughout the plant's lifecycle based on environmental and intrinsic factors such as leaf traits, host chemistry, and leaf chemical profiles (Sinno et al., 2020). In general, despite their numerous benefits, the utilization of EFs in developing countries by farmers is limited. Nevertheless, these EFs are not yet sufficiently produced and/or available to fulfill farmers' demands even in developed countries. Hence, this review provides recent and comprehensive information on plant-fungal endophyte associations, the efficiency of EFs as pest biocontrol agents, growth promoters, and climate change resilience partners in plants.

2 Plant-fungal endophyte associations and related regulating factors

All plants appear to be symbiotic with fungal endophytes in natural ecosystems, which survive within plant tissues either throughout their lives or during a particular period of their lifespans without producing obvious damage and/or changing the morphology of their hosts. This makes the plant-fungal endophyte interaction a balanced antagonism where fungi survive by consuming nutrients from the plants, providing various benefits, while the host plants

activate virulence mechanisms for colonization and host defenses (Baron et al., 2020). However, EFs inhabiting healthy plant tissues may convert from nonpathogenic to pathogenic modes when the plant is stressed (Galindo-Solís and Fernández, 2022). This diverse group of fungi can have a significant impact on plant families by conferring abiotic and biotic stress tolerance, boosting biomass while reducing water utilization, or decreasing fitness through resource allocation modifications (Khiralla et al., 2016). Through these positive relationships, plant growth, nutrition, and productivity can be improved (Behie and Bidochka, 2014) in various ways, such as providing nitrogen (N) to plants (Behie et al., 2015), acting as biocontrol agents (Akello and Sikora, 2012; Suryanarayanan, 2019; Huang et al., 2020), and improving tolerance to abiotic stress (Morsy et al., 2020). After successful colonization, EFs assist plants in producing plant hormones (phytohormones) and other bioactive compounds by promoting the production of secondary metabolites within plant tissues (Khan et al., 2019), enhancing tolerances to drought, salinity, and extreme temperature (Morsy et al., 2020), interfering with weed growth and germination and hindering the activities of insects, pathogens, and plant parasitic nematodes (Schouten, 2016; Sallam et al., 2021). What is interesting in a plant-fungal endophyte symbiotic relationship is that the plant itself helps fungal endophytes by offering shelter, nutrients, and seed spreading. In general, EFs are known to affect the biosynthesis of phytohormones, enzymes, and other bioactive compounds in plants (Satheesan and Sabu, 2020).

However, for EFs to be effective symbionts of different plants (pests), they need to be capable of infecting, occupying, and establishing themselves in both organisms (Branine et al., 2019). Colonization by EFs may be systemic (Quesada-Moraga et al., 2014), restricted to some plant tissues (Wearn et al., 2012), or distributed throughout the plant parts (Behie et al., 2015). Both gene duplication (Wang and St Leger, 2007) and horizontal gene transfer (Zhang et al., 2019) are involved in the penetration and establishment of EFs inside both host plants (pests). However, environmental conditions, inoculation methods, season of sample collection, species of fungal endophytes, geographical location, and genotype of the host plant itself can influence EF colonization of host plants (; Tefera and Vidal, 2009; Morales-Sánchez et al., 2020; Wu et al., 2020). Furthermore, depending on the method of inoculation (seed coating, foliar spray of conidia, conidial suspension injection, radicle dressing, soil drenching, and root and rhizome immersion), EFs can vary in their ability to colonize different plant parts (Tefera and Vidal, 2009). Wei Q. Y. et al. (2020) found that leaf spraying of conidial suspension on tomato plants resulted in greater colonization by *B. bassiana* than seed dressing. Leaf inoculation using *B. bassiana* conidial suspensions has also been described as the most efficient method of colonizing sorghum plants, successfully delivering the fungal endophyte into leaves (Tefera and Vidal, 2009).

Several factors related to geographic factors and the ecological function of the host plant also exert an influence on the diversity, specificity, and specialization of EF communities (Aamir et al., 2020). A report on native Hawaiian plants coexisting along an altitudinal gradient, for instance, revealed distinct patterns of EF diversity, host specificity, and interaction specialization across various elevations, with less specialization and greater diversity occurring at extreme altitudes (Cobian et al., 2019). This suggests that associations between host plants and EFs are more specialized under intermediate conditions and less specific under ecological extremes. A study by Wu et al. (2020) found that certain key chemical components and growth patterns in *Dendrobium catenatum* were influenced by the integration of cultivar genetics and species of fungal endophyte. He et al. (2019) studied the genetic variation in the forb host *Oxytropis ochrocephala*, its interaction with EF *Alternaria oxytropis*, and swainsonine mycotoxin production, revealing that host genotype and precipitation significantly influenced population-scale swainsonine production. Similarly, Hughes et al. (2020) observed significant impacts from the root EF *Lulwoana* sp. on phenotypic traits across genotypes of *Spartina alterniflora*, suggesting that plant-EF associations can exert community- and ecosystem-level impacts on plant species. A recent study also revealed that host plant genotype and tissue type have a significant influence on fungal endophytic communities in wheat, with the effects of host genetics mostly limited to leaves and roots (Latz et al., 2021). Hence, studying the factors driving plant-fungal endophyte associations is crucial for optimizing their development as agricultural solutions, with a focus on colonization and related mechanisms, as well as ecological effects on fungal endophyte community assembly and host interactions.

3 Efficiency of endophytic fungi in agricultural pest biocontrol

Agricultural pests, such as pathogens (e.g., fungi, bacteria, viruses, and nematodes), insect pests, and weeds, impede crop growth and yields, necessitating a comprehensive approach to addressing food production, as well as environmental conservation challenges. Management techniques that involve overreliance on synthetic agrochemicals have resulted in the resistance and resurgence of pests and negative environmental outcomes such as pollution (Waqas et al., 2015a; Verma et al., 2022). Alternative strategies such as biocontrol agents utilizing EFs are more effective, sustainable, and environmentally friendly approaches to plant protection (Ambele et al., 2020; Ahmad Y. et al., 2020; Mota et al., 2021). This section presents comprehensive analyses of the efficacy of endophytic fungi in the biocontrol of agricultural pests, including insect pests, plant diseases, parasitic nematodes, and weeds.

3.1 Endophytic fungi against insect pests

Biological control strategies utilizing beneficial organisms have increasingly attracted interest in recent decades as a means to reduce dependence on synthetic pesticides. Endophytic fungi in particular show promise due to their ability to form mutualistic relationships with host plants, alter metabolism, strengthen stress tolerance, and protect against insect pest damage (Aamir et al., 2020). The mechanisms through which EFs colonize and reduce insect herbivore damage vary based on the crops, fungal endophytes, application methods, and target insects. In insect pests, colonization (as a mode of action) of EFs begins with the bonding of fungi to the host plant surface, followed by penetration through the host cuticle and proliferation within the body cavity, which is the mode of entry for most EFs (Vega et al., 2008). During insect invasion, after efficient adherence, conidia develop to form hyphae, and conidial surface proteins, such as *Metarhizium* adhesin genes, *MAD1* and *MAD2* of

M. anisopliae, and hydrophobin proteins (*hyd1* and *hyd2*) of *B. bassiana,* are needed for plant and insect attachment and to recognize insect-specific compounds that then degrade the insect cuticles. For instance, Wang and St Leger (2007) reported that in *Metarhizium robestsii*, the adhesive *MAD1* is essential for insect cuticles and *MAD2* in plant conidial spore adhesions. Then, degradation of the insect cuticle/plant cell wall allows EF access to plant/insect tissues, which is accomplished by enzymatic activity such as that of various proteases and the mechanical pressure exerted by specific infections of hyphal structures (such as appressoria; Barelli et al., 2016). The EF needs to escape the insect immune system to efficiently parasitize insects and kill them. Once the EF pierces the insect cuticle, it distributes the insect's hemolymph, where it divides into blastospores (yeast-like asexual spores), which collect nutrients in the hemocoel and generate particular insecticidal metabolites, leading to insect death (Fan et al., 2017). The mechanisms/modes of action by which EFs reduce insect herbivore injury in their host plants comprise antibiosis or pest avoidance/feeding deterrence (Vega et al., 2008), reduction of insect fitness, maximizing risks of predation and parasitism (Shymanovich and Faeth, 2018), reduction of insect survival (Lopez and Sword, 2015; Rasool et al., 2021), retardation of insect growth (Rondot and Reineke, 2019; Ahmad I. et al., 2020) and modification of plant defense gene expression (Ahmad I. et al., 2020). A possible mode of action could be direct defense or indirect plant defense by enhancing plant odors and attracting additional olfactory foraging natural enemies (Fuchs and Krauss, 2019).

Reductions in insect pest-induced plant damage were documented in a number of agricultural crop plants that were treated with EFs following effective colonization (Table 1). For instance, *Tuta absoluta* (Meyrick; Lepidoptera: Gelechiidae), which causes serious damage to tomato and nightshade (*Solanum scabrum*), was reduced when these plants were treated with *Trichoderma asperellum*, *Beauveria bassiana* (Balsamo) vullemin (Ascomycota: Hypocreales), and *Hypocrea lixii* (Agbessenou et al., 2020). In tomatoes, the use of *B. bassiana* was also effective in reducing the damage caused by *Helicoverpa armigera* (Toffa et al., 2021). A similar reduction in damage caused by bean stem maggot (*Ophiomyia phaseoli*) was reported in common bean (*Phaseolus vulgaris*) when *M. anisopliae* ICIPE 78 was used as a potential EF (Mutune et al., 2016). Similarly, *B. bassiana* colonizing tomato leaves also resulted in a reduction in the longevity of *T. absoluta* larvae and caused 50% mortality of all larval instars (Klieber and Reineke, 2016). Furthermore, EFs can be employed along with other insect pest management techniques, such as biological control agents acting as natural enemies (Akutse et al., 2013). Jaber and Araj (2018) reported that EFs such as *B. bassiana* and *Metarhizium brunneum* could be combined with the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) for the control of the green peach aphid *Myzus persicae* Sulzer (*Homoptera*: Aphididae) in sweet pepper (*Capsicum annum*).

Several reports on insect pest damage reduction after the application of EFs to crops are also available (Quesada-Moraga et al., 2014; Muvea et al., 2015; Rondot and Reineke, 2019; Table 1). For example, the production of alkaloids by *Epichloë* species in diverse grass species accumulating in plants is toxic to numerous insect pests (Lugtenberg et al., 2016). Nodulisporic acid, produced by *Nodulisporium* sp., is another alkaloid molecule that is vital for preventing insect herbivory by activating glutamate, which results in the flow of chlorine ions through the chlorine channels of insect muscle and nerve cells, resulting in flaccid paralysis (Rigobelo and Baron, 2021). New insecticidal anthraquinone and chloramphenicol (Yuan et al., 2020) derivatives characterized from *Acremonium vitellinum* were reported to be effective against the cotton bollworm *Helicoverpa armigera* (Hübner; Lepidoptera: Noctuidae). In general, a wide range of secondary metabolites, such as polyketides exhibiting antibiotic activity, have been synthesized by fungal endophytes (Lugtenberg et al., 2016; Aamir et al., 2020). Therefore, insect pest management approaches could benefit from harnessing EFs given their low costs, high efficacy, safety for nontarget species and the environment, and potential to enrich agroecosystem biodiversity (Barelli et al., 2016).

3.2 Endophytic fungi against plant diseases and parasitic nematodes

Fungal endophytes can act as biocontrol agents by stimulating the plant's inherent defense molecules and defending themselves or by producing bioactive compounds with the ability to kill or prevent pathogen attacks on their own (Jaber and Araj, 2018; Huang et al., 2020). These bioactive compounds have antifungal and antibacterial properties and include terpenoids, flavonoids, alkaloids, quinols, chlorinated compounds, peptides, steroids, polyketides, phenols, and other VOCs (Moraes et al., 2020; Lu et al., 2021). As a result, several crop plants treated with EFs showed reductions in plant damage from a variety of plant diseases and plant-parasitic nematodes following effective colonization (Tables 2, 3). In a recent study, *Fusarium oxysporum* f. sp. *cucumerinum*, which causes wilt in cucumber, was treated with 30 species of EFs, of which *Penicillium* sp. and *Hypocrea* sp. were reported to be effective at hindering the mycelial colony growth of diseases and successfully suppressing wilt severity in cucumber (Abro et al., 2019). Recent studies have indicated that endophytic *Trichoderma* has the capacity to reduce the incidence of red root rot disease caused by *Ganoderma philippii* in *Acacia mangium* seedlings (Gafur, 2023; Gafur et al., 2023). Sallam et al. (2021) also reported the antagonistic activity of *T. longibrachiatum*, *T. asperellum*, and *T. atroviride* against *R. solani*a, and the three EF isolates produced pectinase and chitinase and solubilized phosphorus (P) in soybean plants. Similarly, *M. brunneum* and *B. bassiana* colonize wheat, leading to a reduction in disease incidence, severity, and development against *F. culmorum,* the causal agent of crown and root rot, following plant colonization (Jaber, 2018). Furthermore, Billar de Almeida et al. (2010) also reported the possibility of using EFs for the management of grapevine trunk diseases, which are very important and widespread fungal diseases impacting grapevines. Several other reports on plant–pathogen suppression following the treatment of crops with EFs are well documented (Hassanein et al., 2020; Zanudin et al., 2020; Aldinary et al., 2021; Mota et al., 2021; Table 2).

Much is unclear regarding the particular mode of action by which these fungi combat nematodes in most scenarios; however, they are most likely highly diverse. EFs can kill cells directly and impede cell development, obscure them when finding their host, immobilize and attack resources, repel nematodes, or use a combination of these modes of action (Schouten, 2016). For example, Swarnakumari and Kalaiarasan (2017) found that *Purpureocillium lilacinum* and *Pochonia chlamydosporia* can attach, penetrate, colonize, and finally condense egg contents in root-knot nematodes, *Meloidogyne* spp., and stop

TABLE 1 EFs used against agricultural insect pests described in diverse field crops.

Crop	Fungal endophytes	Application methods	Target insect	References
Theobroma cacao	*B. bassiana*	Seed soaking, soil drench and foliar spray	*Odontotermes* spp.	Ambele et al. (2020)
Solanum lycopersicum	*B. bassiana*	Leaf spray	*Bemisia tabaci*	Wei Q. Y. et al. (2020)
Vicia faba	*B. bassiana* and *Hypocrea lixii*	Seed inoculation	*Liriomyza huidobrensis*	Akutse et al. (2013)
S. lycopersicum and *Solanum scabrum*	*Trichoderma asperellum*, *B. bassiana* and *H. lixii*	Seed inoculation	*T. absoluta*	Agbessenou et al. (2020)
Phaseolus vulgaris	*M. anisopliae* and *B. bassiana*	Seed inoculation	*Ophiomyia* spp.	Mutune et al. (2016)
Corchorus capsularis	*B. bassiana*	Seed inoculation	*Apion corchori*	Biswas et al. (2013)
Gossypium hirsutum	*B. bassiana* and *Purpureocillium lilacinum*	Seed inoculation	*Helicoverpa zea*	Lopez and Sword (2015)
S. lycopersicum	*B. bassiana*	Injection, solid substrate root dip, and direct foliar application	*H. armigera*	Qayyum et al. (2015)
Zea mays	*B. bassiana*	Seed dressing, topical application and stem injection	*Sesamia calamistis*	Cherry et al. (2004)
Musa spp.	*B. bassiana*	dipping roots	*Cosmopolites sordidus*	Akello et al. (2007)
Coffea arabica	*B. bassiana*		*Hypothenemus hampei*	Vega et al. (2008)
Allium cepa	*Clonostachys rosea*, *Trichoderma asperellum*, *Trichoderma atroviride*, *Trichoderma harzianum*, *Hypocrea lixii* and *Fusarium* sp.,	Seed or seedling inoculation	*Thrips tabaci*	Muvea et al. (2015)
G. hirsutum	*B. bassiana*, *Lecanicillium lecanii*	Leaf discs immersion	*Aphis gossypii*	Gurulingappa et al. (2010)
V. faba and *P. vulgaris*	Beauveria, Hypocrea, Gibberella, Fusarium and Trichoderma isolates	Seed inoculation	*Liriomyza huidobrensis*	Akutse et al. (2013)
Triticum aestivum	*B. bassiana*, *Aspergillus parasiticus*	-	nymphs	Gurulingappa et al. (2010)
Wild barley	*Neotyphodium coenophialum*		*Rhopalosiphum padi* and *Metopopophium dirhodum* and *Mayetiola destructor*	Clement et al. (2005)
G. hirsutum	*Purpureocillium lilacinum* (Formerly *Paecilomyces lilacinus* and *B. bassiana*)	Seed inoculation	*Aphis gossypii*	Lopez and Sword (2015)
V. faba	*Trichoderma asperellum*, *Gibberella moniliformis* and *B. bassiana*, *M. anisioplaie* and *Hypocrea lixi*	Seed inoculation	*Acyrthosiphon pisum* and *Aphis fabae*	Akello and Sikora (2012)
Cucumis melo	*Fusarium oxysporum*	Seed inoculation	*Aphis gossypii*	Menjivar (2010)
Capsicum annum	*F. oxysporum*	Seed inoculation	*Myzus persicae*	Menjivar (2010)
T. aestivum	*M. brunneum* and *M. robertsii*	Seed inoculation	*Tenebrio molitor*	Keyser et al. (2014)
C. annum	*B. bassiana* and *M. brunneum*	Soil drenching	*Myzus persicae*	Jaber and Araj (2018)
S. lycopersicum	*B. bassiana*	Plant growth substrate	*Trialeurodes vaporariorum*,	Barra-Bucarei et al. (2020)
Vitis vinifera	*B. bassiana*	Leave inoculated feeding	*Aphis illinoisensis*	Sayed et al. (2020)

development at the gastrula stage. An additional example is the EF *Acremonium implicatum* from tomato root galls, which suppresses egg hatching and inhibits the development of root galls by the nematode *M. incognita* (Tian et al., 2014). Furthermore, an EF isolated from *Cucumis melo* root, which was characterized as *P. brefeldianum,* was also reported to be effective in reducing the severity and gall numbers caused by *M. incognita* (Miao et al., 2019). Apart from this, EFs can also produce different nematocidal secondary metabolites against different nematode species. For instance, chaetoglobosin A synthesized by *Chaetomium globosum* controls *M. incognita* (Khan

TABLE 2 EFs used against plant pathogens and their effects on plant diseases.

Crop	Fungal endophytes	Target pathogens	References
Cucumis sativus	*Penicillium* sp. and *Hypocrea* sp.	*Fusarium oxysporum* f. sp. *cucumerinum*	Abro et al. (2019)
G. hirsutum	*Fusarium solani*	*Verticillium dahliae*	Wei et al. (2019)
T. cacao	*Colletotrichum gloeosporioides, Clonostachys rosea and Botryosphaeria ribis*	*Moniliophthora perniciosa*, *Phytophthora palmivora* and *Moniliophthora roreri*	Mejía et al. (2008)
C. sativus	*Streptomyces rimosus*	*Fusarium oxysporum* f. sp. *cucumerinum*	Lu et al. (2016)
Musa spp.	*Streptomyces* sp.	*Fusarium oxysporum* f. sp. *cubense* Tropical Race 4	Wei Y. et al. (2020)
S. lycopersicum	*Streptomyces* sp.	*Ralstonia solanacearum*	Le et al. (2021)
Citrus aurantiifolia var. 'Bears'	*Xylaria adscendens*, and *Trichoderma atroviride*	*Colletotrichum acutatum*	Muñoz-Guerrero et al. (2021)
Musa spp.	*Streptomyces* sp.	*F. oxyspoum* f. sp. *cubense*	Zou et al. (2021)
C. sativus	*Fusarium, Chaetomium, Colletotrichum* and *Acrocalymma*	*Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Fusarium oxysporum* f. sp. *cucumerinum*	Huang et al. (2020)
G. max	*Trichoderma longibrachiatum S12, T. asperellum S11, and T. atroviride PHYTAT7*	*Rhizoctonia solani*	Sallam et al. (2021)
S. lycopersicum	*Aspergillus alabamensis, Aspergillus tubingensis and Aspergillus oryzae*	Fusarium wilt	Aldinary et al. (2021)
P. vulgaris	*Induratia* spp.	*Colletotrichum lindemuthianum*, *Sclerotinia sclerotiorum* and *Pseudocercospora griseola*	Mota et al. (2021)
T. aestivum	*Fusarium subglutinans*	*Fusarium semitectum*, *Aspergillus petrakii*	Hassanein et al. (2020)
T. aestivum	*B. bassiana, M. brunneum*	*Fusarium culmorum*	Jaber (2018)
Z. mays	*M. anisopliae, Trichoderma harzianum*	*Sugarcane mosaic virus/Maize lethal necrosis*	Kiarie et al. (2020)
P. vulgaris	*Trichoderma* spp.	*Fusarium solani*	Toghueo et al. (2016)
Hordeum vulgare	*Fusarium equiseti* and *Pochonia chlamydosporia*	*Gaeumannomyces graminis* var. tritici	Maciá-Vicente et al. (2009)
Traditional rice varieties	Absidia and Acremonium	*Magnaporthe grisea*, the causative agent of rice blast disease	Atugala and Deshappriya (2015)
Solanum melongena	*Helicomyces* spp., *Rhizopus* sp., *Mucor* sp., and *Penicillium* sp.	*F. oxysporum*	Nuraini et al. (2017)
Capsicum annuum	*Cercospora nicotianae, Curvularia* sp., *Fusarium* sp.	*Pepper yellow leaf curl virus*	Lestari et al. (2018)

et al., 2019), while the production of VOCs by *Daldinia cf. concentrica* affects the viability of *M. javanica* (Liarzi et al., 2016). As indicated in Table 3, several reports have revealed the positive impact of EFs on plant growth and their nematicidal effects on diverse species of nematodes (Yan et al., 2011; Liarzi et al., 2016; Bajaj et al., 2017; Zhou et al., 2018; Farhat et al., 2022).

The primary possible mechanisms of plant disease and plant-parasitic nematode suppression differ depending on the EF species. Several mechanisms through which these plant-EF associations benefit the host to shape resistance against various pathogens and nematodes have been suggested (Schouten, 2016; Swarnakumari and Kalaiarasan, 2017; Sallam et al., 2021). Mechanisms could occur through the direct inhibition of pathogens, such as competition for resources (space and nutrients), mycoparasitism and antibiosis (Rajani et al., 2021), and indirect inhibition by induced systemic resistance (ISR) and the induction of secondary metabolites (Rajani et al., 2021; Urooj et al., 2021). For instance, *B. bassiana* can endophytically inhabit a wide range of plant species and has been reported to diminish and limit the growth and development of various soilborne plant diseases *in vitro*, such as *Pythium*, *Rhizoctonia*, and *Fusarium*. *Beauveria* spp. are known to produce an array of bioactive metabolites, which in turn induce systemic resistance and limit the growth of fungal pathogens. Moreover, the defense responses of the EF *Piriformospora indica*, which causes sheath blight disease, against *Rhizoctonia solani* were able to disease the severity of the disease by limiting levels of hydrogen peroxide (H_2O_2) and increasing the activity of antioxidants such as superoxide dismutase (SOD) in rice crops (Nassimi and Taheri, 2017). Induced resistance by EF elicitors against early blight diseases caused by *Alternaria solani* in tomatoes was recently demonstrated by increasing certain enzymatic activities, such as lipoxygenase, peroxidase, polyphenol oxidase, and phenyl ammonia-lyase, when tomato seeds were treated with fungal colony-forming units (CFU)

TABLE 3 Effect of fungal endophytes against plant parasitic nematodes (PPNs) in diverse crops.

Crop	Fungal endophytes	Target PPN	Effects on PPN	References
S. lycopersicum	*Daldinia cf. concentrica*	*Meloidogyne javanica*	Reduced the viability of the second-stage juveniles (j2s) and decreased eggs hatching	Liarzi et al. (2016)
Musa spp.	*Fusarium oxysporum*	*Pratylenchus goodeyi* and *Helicotylenchus multicinctus*	Nematode population densities were reduced by >45%; Percentage root necrosis was reduced by >20% as a result improved yield	Waweru et al. (2014)
S. lycopersicum	*Trichoderma asperellum*	*Meloidogyne* spp.	Reducing root galling severity by 47% and nematode reproduction by 67%, respectively, and significantly inhibiting egg hatch by up to 85%	Affokpon et al. (2018)
C. sativus	Chaetomium Ch1001	*Meloidogyne incognita*	Produced compounds affect the motility of the second-stage juveniles of *M. incognita* reduced galls formed	Yan et al. (2011)
S. lycopersicum	*T. asperellum* and *F. oxysporum*	*M. incognita*	Reduced root-knot nematode egg densities by 35–46%	Bogner et al. (2016)
S. lycopersicum	*Acremonium implicatum*	*M. incognita*	96.0% of second-stage juveniles of *M. incognita* were killed by a culture, suppressed egg hatching, with only 36.3% of treated eggs hatching, inhibited the formation of root galls, reduced the nematode population in soil	Tian et al. (2014)
S. lycopersicum	*Pochonia chlamydosporia*	*M. incognita*	Enhanced defense gene expression	Tolba et al. (2021)
Piper nigrum	*Annulohypoxylon nitens, Daldinia eschscholtzii, Fusarium* spp., *Ceriporia lacerata, Diaporthe* sp. and *Phomopsis* sp.	*Phytophthora capsici, Radopholus similis*	The highest mortality of up to 60%	Sreeja et al. (2016)
Musa spp.	*F. oxysporum*	*R. similis, Pratylenchus goodeyi* and *Helicotylenchus multicinctus*	Higher nematode mortality	Van Dessel et al. (2011)
Glycine max	*Piriformospora indica*	*Globodera* spp. and *Heterodera* spp.	Egg density was significantly decreased and had a strong growth- and yield-promoting effect in soybean	Bajaj et al. (2017)
Solanum tuberosum	*P. fluorescens, P. putida, P. syxantha, P. aurantiacea*	*Globodera rostochiensis*	Nematode multiplication reduced by 40.7–42.2%	Trifonova et al. (2014)
Musa spp.	*F. oxysporum*	*R. similis*	Reduced root penetrations by *R. similis*	Vu et al. (2006)
Oryza sativa	*F. moniliforme*	*Meloidogyne graminicola*		Le et al. (2016)

and different concentrations of crude oligosaccharide (CO) of EF; as a result, the vegetative and reproductive parameters of tomato were enhanced (Sujatha et al., 2021). Several research findings have reported that EFs can induce resistance in various crops against insects (Kiarie et al., 2020), nematodes (Swarnakumari and Kalaiarasan, 2017), phytopathogens (Muvea et al., 2015; Galletti et al., 2020; Urooj et al., 2021), and abiotic stresses (Lata et al., 2018; Morsy et al., 2020; Singh et al., 2021).

Some filamentous fungi, such as *Trichoderma*, act on plant-parasitic nematodes directly and indirectly through antibiosis,

production of lytic enzymes, parasitism, paralysis, competition, improving nutrient and water absorption, modifying plant root morphology and/or rhizosphere interactions, and inducing resistance by initiating hormone-mediated responses, such as salicylic acid, jasmonic acid, and strigolactone, for plant defense (Poveda et al., 2020). As a direct mechanism, for instance, *Acremonium implicatum* antagonized the colonization of *Meloidogyne incognita* Chitwood nematodes in tomato (*Solanum lycopersicum*; Tian et al., 2014), and *Penicillium brefeldianum* antagonized the colonization of *M. incognita* nematodes in *Cucumis melo* (Miao et al., 2019). EFs can also produce different nematocidal secondary metabolites that can act indirectly against different nematode species. For instance, it was revealed that chaetoglobosin A formed by *Chaetomium globosum* acted against the root-knot nematode *M. incognita* (Khan et al., 2019), and volatile organic compounds (VOCs) released by *Daldinia cf. concentrica* acted against *M. javanica* in tomatoes (Liarzi et al., 2016).

3.3 Endophytic fungi as weed biocontrol agents

Weeds are among the undesired plants that grow alongside crops in the field, competing for nutrients and space, as well as hosting pathogens that attack crop plants and reduce production (Asim et al., 2022). Fungal endophytes have been shown to have bioherbicidal potential that suppresses and controls weeds while simultaneously stimulating crop plant growth, making them particularly suited for this use due to their dual benefits (Suryanarayanan, 2019; Ahmad Y. et al., 2020). Therefore, the use of EFs provides a more effective and environmentally sound approach to weed control than either synthetic herbicides, which pose risks to human and environmental health, or other biocontrol agents whose long-term efficacy relies upon persistence in introduced settings. The biocontrol of *Orobanche* using EFs such as *Ulocladium* spp. and *Fusarium* spp. resulted in 90% control in tomato and 90–97% control in watermelon, while other EFs, such as *Rhizoctonia*, *Alternaria*, and *Sclerotinia,* were also effective (Nemat Alla et al., 2008). Earlier studies have shown the effective biocontrol of *Convolvulus arvensis*, *Chenopodium album*, and *Avena fatua* through various EF species, with these weeds exhibiting substantial reductions in growth and germination when sprayed or inoculated with EF culture filtrates (Tunali et al., 2009; Akbar and Javaid, 2012).

Endophytic fungi exhibit multiple weed control mechanisms, impacting germination and subsequent growth stages and various physiological and biochemical processes. For example, absorbed fungal metabolites may damage seed cell membranes and disrupt vital processes such as amylase activity and cell division, delaying or preventing germination (Moura et al., 2020). Additionally, intercellular root colonization releases toxic substances, hindering germination and growth by suppressing photosynthesis and phytohormones while enhancing ROS and stress hormones such as ethylene and abscisic acid (Asim et al., 2022). For instance, *Diaporthe phaseolorum*, *T. spirale*, and *P. simplicissimum* have been shown to play crucial roles in reducing photosynthesis and growth in *Ipomoea grandifolia* and *Senna occidentalis* through different hydrolytic enzyme production (Moura et al., 2020). Daba et al. (2021) demonstrated that conidial suspensions of EFs exhibit varying herbicidal activities against weed germination and growth, with the herbicidal actions of applied conidia or exudates during fungal growth reducing germination and growth by up to 65%. Asim et al. (2022) also reported that *F. oxysporum* controlled *A. fatua* by reducing seed germination, vigor index, root length, shoot length, fresh weight, and dry weight by up to 95%, 100%, 64.21%, 62.5%, 73.68%, and 99%, respectively. Ahmad Y. et al. (2020) found that *Alternaria* spp. and *Drechslera* spp. reduced *C. album* germination by 57 and 75% and *A. fatua* germination by 44% and 31%, respectively. Bashir et al. (2018) also reported that secondary metabolites of *Aspergillus niger* significantly decreased the germination and shoot and root biomass of parthenium weeds by 90%, 57%, and 68%, respectively, when using original metabolites.

Endophytic fungi-secreted phytotoxic compounds such as holdysenterine and drechslerol-C, isolated from *Drechslera* spp. and cyclic tetrapeptide isolated from *Alternaria* spp. have reduced germination, growth, and chlorophyll content, leading to chlorosis, cell damage, and necrosis in *C. album, A. fatua, C. arvensis*, and *Rumex dentatus* (Akbar and Javaid, 2012; Ahmad Y. et al., 2020). Host-specific phytotoxic compounds called chenopodolans have been reported in the EF *Phoma chenopodiicola* for effective biocontrol of *C. album* (Ahmad Y. et al., 2020). These phytotoxic compounds usually cause the appearance of necrotic spots surrounded by chlorosis and cell damage on the leaves of target weeds and lack toxicity on nontarget cultivated and wild plants (Cimmino et al., 2015). Cyclic tetrapeptide and AAL toxins from *Alternaria* spp. also inhibited chlorophyll contents, caused cell damage and electrolyte leakage, and interfered with overall plant metabolism by producing reactive oxygen species, ultimately leading to chlorosis and cell death (Ahmad Y. et al., 2020). Studies have demonstrated that conidial suspensions of *Aspergillus* spp. have variable herbicidal activities against weed germination and growth, with applied conidia or exudates during fungal growth reducing germination and growth by up to 65% (Bashir et al., 2018; Daba et al., 2021).

Herbicidal compounds secreted by EFs have also been found to potentially compromise cell membrane integrity, resulting in increased permeability, solute leakage, and elevated electrolyte leakage, significantly impacting the overall health and functionality of the affected weeds. For instance, EF-produced phytotoxic compounds, such as bisanthraquinones, octahydronaphthalenes, alkaloids, and molecules possessing chemical scaffolds, are capable of absorbing electrons and affecting redox processes (Ahmad Y. et al., 2020; Moura et al., 2020). EF *F. oxysporum* also produces polyphenols that reduce *A. fatua* growth, including quercetagetin, isovitexin, calycosin, dihydroxy-dimethoxyisoflavone, naringenine, vitaxin, cis-cafatric acid, caffeoyl-D-glucose, and p-dyroxy benzoic acid (Asim et al., 2022). Some of these chemicals have also shown allelopathic and herbicidal activity against *A. retroflexus*, *P. oleracea*, *C. album*, and *Abutilon theophrasti* (Boselli et al., 2021). Cimmino et al. (2015) also found that nonproteic toxic amino acids from the culture of *Ascochyta caulina* cause cell harm and necrosis in the leaves of *C. album*, finally causing electrolyte leakage. Toxins such as indole 3-acetic acid (IAA) isolated from *A. alternata* were also found to damage cells, cause chlorosis and induce electrolyte leakage in various weed species, demonstrating their potential as natural herbicides (Ahmad Y. et al., 2020). Suryanarayanan (2019) reported that EFs produce metabolites that exhibit herbicidal activities and induce chlorosis, finally causing necrosis in *Lemna minor*. The herbicidal effects of various species of EFs on different weeds, as well as the compounds released by these EFs and their effects on the host weeds, are shown in Table 4.

TABLE 4 Use of endophytic fungi in weed control and management.

Crops	Fungal endophytes	Compounds released by the EFs	Target weeds	Effects on the weeds	References
T. aestivum	*F. oxisporum*	Isovitexin, calycosin, quercetagetin, and dihydroxy-dimethoxyisoflavone	*Avena fatua*	Inhibited the growth of *A. fatua* by the biomass of the fungus in the soil	Asim et al. (2022)
	Drechslera holmii, D. biseptata and D. australiensis	Metabolites such as holadysenterine, de-O-methyldiaporthin, drazepinone, Ophiobolin E, 8-epi-ophiobolin J, di-(2-ethyl-hexyl)-phthalate, and 2,4-dichlorophenoxyacetic acid	*Rumex dentatus*	Reduced germination, shoot and root growth, and shoot and root fresh and dry biomasses (For instance, metabolites of *Drechslera* spp. suppressed the weed germination, shoot length, shoot dry biomass, root length and root biomass by 12%–56%, 73%–85%, 72%–88%, 82%–94% and 77%–88%, respectively)	Akbar and Javaid (2012) and Akbar et al. (2020)
	Drechslera spp. (*D. hawaiiensis*, *D. holmii*, *D. biseptata*, and *D. australiensis*)	Metabolites and herbicidal constituents, like ophiobolin A, 6-epi-ophiobolin A, anhydro-6-epiophiobolin A, ophiobolin I, and drazepinone	*Chenopodium album* and *A. fatua*	Reduced germination and root and shoot growth and biomass	Akbar and Javaid (2010, 2012)
Parthenium hysterophorus	*Alternaria*, *Aspergillus*, and *Drechslera* spp.	Phytotoxic compounds like holadysenterine and drechslerol-C, nonproteic toxic amino acid, AAL toxins, cyclic tetrapeptide, reactive oxygen species (ROS), and other bioactive compounds	*C. album*, *A. fatua*, and *Convolvulus arvensis*	Reduced germination, shoot and root length, and plant biomass, and other growth parameters, phytotoxic effects, increased leaf relative electrolyte leakage, reduced biochemical and physiological processes like cellular respiration and chlorophyll contents, chlorosis, and necrosis	Ahmad Y. et al. (2020)
C. arabica	*Aspergillus niger* and *Trichoderma* spp. (*T. asperlium*, *T. atroviride*, *T. hamatum*, *T. harzanium*, *T. longibrachatum* and *T. viride*)	Phytotoxins and other compounds with herbicidal constituents and inhibitory potential found in fungal conidia suspensions	*Bidens pilosa* (Asteraceae)	Inhibited germination percentage and index, plumule and radicle length, seedling vigor index, and overall early growth of the weed	Daba et al. (2021)
Helianthus annus	*Aspergillus alliaceus*	Phenolic substances such as gallic acid, catechin, syringic acid, p-coumaric acid, syringic acid, caffeic acid, and abscisic acid	*Orobanche cernua*	Reduced synthesis and disrupted balances of hormones (abscisic acid, salicylic acid, and jasmonic acid), damaged free radicals and protein synthesis metabolism, inhibited antioxidant enzymes, and weakened apoptosis-based plant defense reactions eventually leading to a slow and continuous death	Aybeke (2020)
	F. oxysporum	Secondary metabolites such as phenols (catechin, syringic acid, caffeic acid and p-coumaric acid), flavonoids, ROS, and antioxidant enzymes such as Mn-superoxide dismutase and Zn-superoxide dismutase	*O. cernua*	Hormonal disorders, lethal physiological damages, and induced phenol synthesis, accumulation and oxidation causing typical symptoms of tissue browning finally killing the weed quickly	Aybeke (2017)
	Aspergillus alliaceus	Phenols, phytoalexins, mucilaginous substances (mucilage), mycotoxins (ocratoxin A), lignin-like reddish-brown droplets (safranin-stained droplets), and carbohydrate-derived products (nonesterified pectins)	*O. cernua*	Collapse of outer cells of various tissues, thickenings, lignification and disintegration of cell wall, breakdown and disintegration of organelles and cytoplasm, deformations of cellular and pollen grains, destruction of seed hilum, embryo and endosperm finally causing tissues disappearance, leaf necrosis, and reduction of weed attachments, tubercles, and emergent shoots	Aybeke et al. (2014)

(Continued)

TABLE 4 (Continued)

Crops	Fungal endophytes	Compounds released by the EFs	Target weeds	Effects on the weeds	References
V. faba and *S. lycopersicum*	*F. oxysporum* (Foxy I and Foxy II)	Microconidia and chlamydospores	Orobanche spp. (*O. crenata* and *O. ramosa*)	Decreased germination, attachments, tubercles, emerged shoots, shoot height and dry weight (For instance, application of isolate Foxy I and Foxy II, respectively, reduced *invitro* germination by 80 and 76% in *O. crenata* and by 77 and 76% in *O. ramosa*)	Nemat Alla et al. (2008)
S. lycopersicum, *Brassica oleracea*, and *Nicotiana tabacum*	*Fusarium* spp.	Various secondary metabolites	*Orobanche ramosa*	Reduced number, length, and fresh and dry weights of shoots (by approximately 60%) and number of tubercles attached to the roots of host plants (by more than 70%)	Boari and Vurro (2004)

4 Efficiency of endophytic fungi in plant growth promotion

4.1 Endophytic fungi as inducers of plant growth-promoting phytohormones

Fungal endophytes are microorganisms that enhance plant growth and development either directly through secreting or stimulating vast arrays of phytohormones (Bilal et al., 2018; Yan et al., 2019; Rajani et al., 2021). They can actively or passively regulate plant growth by enhancing the production of plant hormones such as auxin, gibberellic acid (GA), abscisins, ethylene, and IAA (Lata et al., 2018; White et al., 2019; Rigobelo and Baron, 2021). Due to their ability to produce enzymes and other bioactive compounds, EFs such as *Penicillium, Piriformospora indica, Sebacina vermifera*, and *Colletotrichum* have more potent plant growth-promoting abilities even in unfavorable environments (Kaur, 2020). A diverse range of phytohormones and phytohormone signaling pathways are utilized in mediating PGP, resulting in greater root growth and, ultimately, higher yield and biomass (Bilal et al., 2018; Abro et al., 2019; Fadiji and Babalola, 2020). Some common plant growth hormones produced by different fungal endophytes in different plants are presented in Table 5. Aamir et al. (2020) further delineated that the most common mechanisms by which EFs provide benefits to plants have been achieved by siderophore production, solubilization of phosphates, N fixation, phytohormones such as IAA production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Various mechanisms by which EFs benefit their host plants are presented in Figure 1.

Auxins, gibberellins, and cytokinins synthesize EF and are known to serve as chemical mediators and signaling molecules for plant growth (Baron et al., 2020). Endophytic fungi, such as *F. fujikuroi*, *Serendipita indica*, and *Piriformospora indica*, can produce phytohormones such as indole-3-acetic acid (IAA), cytokinin, and gibberellic acid, suggesting that they can regulate host signaling and influence physiological and metabolic activities (Liu et al., 2021). However, PGP is primarily driven by IAA, which positively impacts shoot and root development, including responses to tropism, cell division, cell elongation, vascular tissue differentiation, and root formation (Airin et al., 2023). Khan et al. (2008) found that bioactive gibberellins such as GA1, GA3, GA4, and GA7 in *P. citrinum* IR-3-3, EF isolated from dune plants, improved seedling length and growth in Waito-c rice dwarf mutant and sandy plant *Atriplex gmelinii*. Bader et al. (2020) studied *Trichoderma* strains from soils of Argentine Pampas, revealing high phytohormone production capacities and significantly improved plant height, fresh and dry biomass, chlorophyll content, and higher surface area in inoculated tomato seeds after 45 days. Baron et al. (2020) found that *P. lilacinum*, *P. lavendulum*, and *Metarhizium marquandii* can produce IAA in soybean, bean, and maize, promoting growth by improving parameters such as dry matter, attributed to endophytic colonization of the plants. As a result, sustainable agriculture could rely largely on endophytic fungi, which possess the unique ability to thrive and colonize host plant tissues, promoting the production of various phytohormones with biological activities.

4.2 Endophytic fungi as modulators of nutrient acquisition by plants

Fungal endophytes can also boost plant growth indirectly by assisting the plant in acquiring nutrients (Bilal et al., 2018; Yan et al., 2019; Rajani et al., 2021). In previous studies, EFs that dwell in host plants without developing obvious symptoms have been noted to play an important role in boosting plant growth by aiding nutrient absorption (Molina-Montenegro et al., 2016; Abro et al., 2019; Huang et al., 2020). These microorganisms are capable of enhancing agricultural productivity by increasing plant access to nutrients [N, P, K, Zn, iron (Fe), etc.] producing plant hormones, reducing ethylene, or increasing the water acquisition rate (Baron et al., 2018; Abro et al., 2019; Huang et al., 2020). Various nutrient transporters and processes of translocation have been confirmed in different plants infected with EFs. For instance, the nutrient acquisition mechanism employed by EFs includes the release of nutrients from insects that are decayed by microbes (Behie and Bidochka, 2014; White et al., 2019). This has been confirmed by Behie and Bidochka (2014), who reported the EF transfer of N from insects by some EF species, namely, *M. guizhouense, M. flavoviridae, M. robertsii, M. brunneum, M. acridum, Akanthomyces (=Lecanicillium) lecanii and B. bassiana*, *P. vulgaris* (common bean), *G. max* (soybean), *P. virgatum* (switchgrass), and *T. aestivum* (wheat). In this regard, Rigobelo and Baron (2021) showed that some species of EF can kill the larvae of insects and colonize plants endophytically, facilitating the transfer of nutrients from insects to these plants.

Fungal endophytes also help in the biodegradation of the litter of their host plants and decomposition of organic components,

TABLE 5 Examples of common phytohormones produced by EFs reported in various host plants.

Crop	Fungal endophytes	Phytohormone	References
S. lycopersicum	*Curvularia lunata* and *Nigrospora sphaerica*	Indole 3-acetic acid (IAA)	Saad and Badry (2020)
H. annuus	*Penicillium citrinum* and *Aspergillus terreus*	IAA and GAs	Waqas et al. (2015a)
V. faba	*Aspergillus niger* and *Penicillium chrysosporium*	Abscisic acid (ABA) and ethylene	El-Mahdy et al. (2021)
C. sativus	Combinations of different EFs	IAA and GAs	Syamsia et al. (2021)
G. max	*Paecilomyces formosus* and *Penicillium funiculosum*	GAs and IAA	Bilal et al. (2020)
G. max	*Porostereum spadiceum*	GAs	Hamayun et al. (2017)
G. max, *V. faba* and *Z. mays*	*Purpureocillium lilacinum*, *Purpureocillium lavendulum* and *Metarhizium marquandii*	IAA and solubilize P from fluorapatite	Baron et al. (2020)

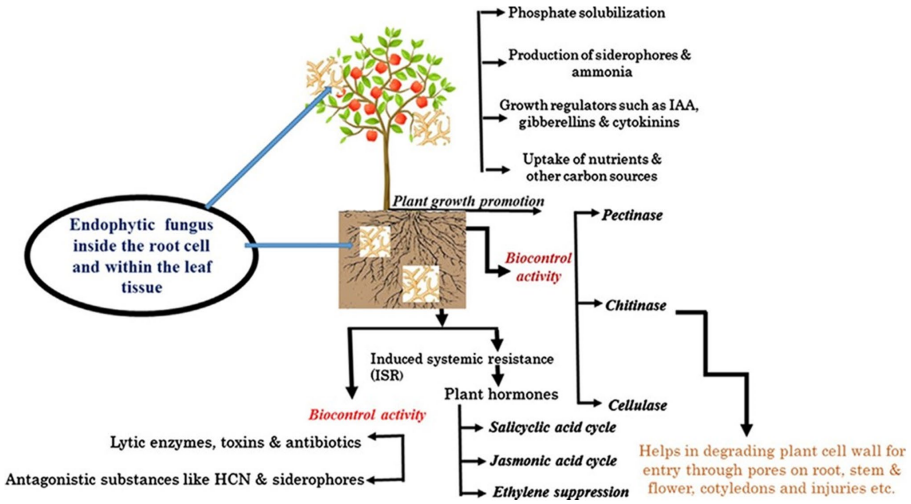

FIGURE 1
The functional dimension of EFs that localize inside living plant root tissues and within root cells and the mechanisms by which they benefit their host plants. Adapted from Aamir et al. (2020).

comprising cellulose, lignin, and hemicelluloses, which enable nutrient cycling (Lata et al., 2018). As reported by Behie and Bidochka (2014), EFs such as *Heteroconium chaetospira* have been shown to transfer N gained from decayed OM in the soil to *Brassica campestris* roots and increase N uptake efficiency. Yang et al. (2014) also indicated that fungal endophyte *Phomopsis liquidambari* colonization increases N, P, S, Zn, and Mg availability and significantly increases plant root length, root number, height, number of buds, chlorophyll levels and biomass in rice. Lugtenberg et al. (2016) revealed that *P. indica* increased shoot dry weight and grain yield and appeared to trigger flowering earlier at low temperatures under the lowest nutrient input, therefore helping to decrease fertilizer inputs while maintaining reasonable yields.

In nutrient acquisition, a process known as the 'rhizophagy cycle' enables EFs to access nutrients in the soil and then transport them back to the plants, where they penetrate the root cells at the tips of the roots closest to the nutrient exudate zone, where the growing root epidermal cells possess thin cell walls (Lata et al., 2018; White et al., 2019; Rigobelo and Baron, 2021; Singh et al., 2021). Rhizophagy symbiosis is a mutualism that involves nutrition exchange between plants and their EF partners (White et al., 2018, 2019; Rigobelo and Baron, 2021), and this process has been hypothesized by White et al. (2018, 2019), as depicted in Figure 2. Nutrient mining by EFs in the rhizophagy cycle, according to Kumar et al. (2020), involves three major steps: (1) Plant roots exudate organic acids, such as malic, citric, and acetic acids, into the soil, which bind with metals found in the soil (Mg^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, etc.); (2) the EFs eventually possess transporters that attach to these organic acid-metal complexes and transfer them into the EF cells; and (3) finally, EFs return to plant roots and enter root cells, where they oxidatively take nitrogen and micronutrients, including Fe, Zn, and Mg, from microbes and eject them back into the soil from root hair tips to acquire more nutrients.

The rhizophagy cycle involves EFs transitioning between the EF/intracellular protoplast phase of the plant root cells and the free-living walled phase of soils, acquiring nutrients in the soil and oxidatively extracting them from the EF/intracellular protoplast phase (White

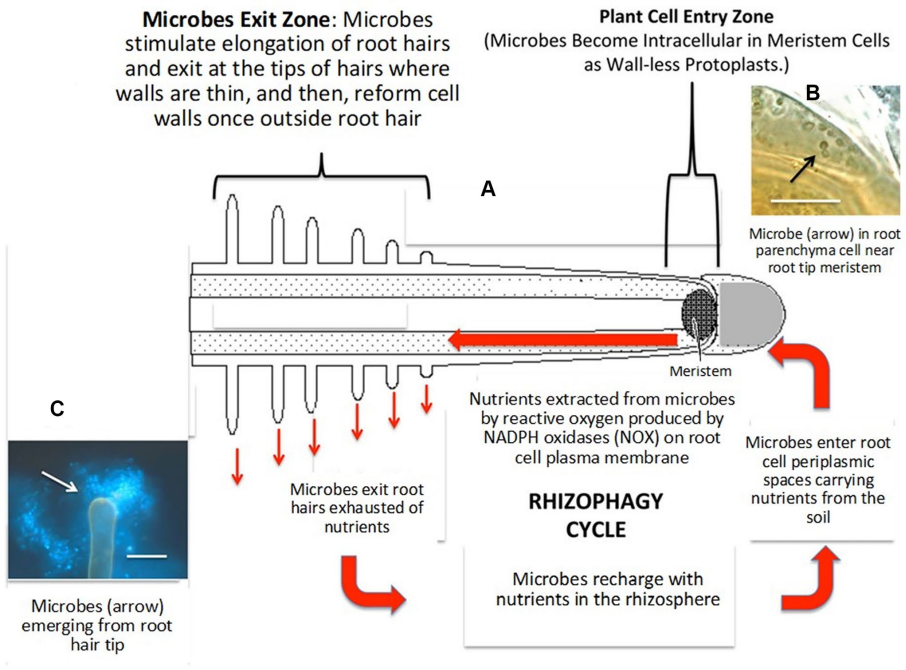

FIGURE 2
Diagram of the rhizophagy cycle showing microbes entering root cells at the plant root tip meristem and exiting root cells at the tips of elongating root hairs **(A)**, microbes (arrow) in the periplasmic space of parenchyma cells near the plant root tip meristem **(B)** and microbes (arrow) emerging from the plant root hair tip **(C)**. Adapted from White et al. (2019).

et al., 2019; Kumar et al., 2020). In this cycle, EFs enhance root exudates and change their composition, which boosts microbial activity and nutrient mining. These root exudates contain proteins, vitamins, enzymes, phenolic acids, flavonoids, fatty acids, tannins, nucleotides, alkaloids, steroids, terpenoids, and polyacetylenes in addition to organic acids (White et al., 2018, 2019). Plants use their symbiotic EF to transport nutrients from the soils into the periplasmic spaces of their root cells, liberate nutrients via oxidation, and deposit used EF back into the rhizosphere by extending root hairs. This process has been found to improve nutrient transport into plants, assisting with nutrient acquisition from symbiotic EFs that sequester soil nutrients utilizing siderophores (White et al., 2018). Yung et al. (2021) further delineated that EFs act as the prey of roots in rhizophagy and serve to nourish plant nutrition and growth by improving the mineral nutrition and root elemental composition of host plants. For instance, Yung et al. (2021) reported that EFs, particularly DBF60 (*Metapochonia rubescens*), DBF79 (*Alternaria thlaspis*), DBF81 (*Trichoderma harzianum*), and DBF107 (*Cladosporium* sp.), isolated from various plant parts improved the root elemental contents of P, K, Mg, Ca, and S by up to five times in some cases.

Furthermore, EFs produce extracellular enzymes such as pectinase, cellulase, lipase, laccase, proteinase, phosphatase, and xylanase, which aid in the breakdown of macromolecules such as sugar-based polymers, lignin, organic phosphate, proteins, and carbohydrates to micromolecules accessible to plants (Kaur, 2020). In particular, EF chitinase enzymes play a vigorous role in the degradation and cycling of C and N from chitin molecules obtained from insect exoskeletons, crustacean shells, and fungal cell walls (White et al., 2019; Kaur, 2020). Chen et al. (2018) showed that *Epichloë festucae* var. *lolii* alleviated nutrient deficiency in ryegrass by promoting root metabolic activity and decreasing the stoichiometric ratios of C:N, C:P, and N:P in leaves and roots and the Cu content of roots. This EF also increased the concentration of C, N, P, K, Ca, Mg, Fe, and Mn, the dry weight of leaves and roots, the Cu content of leaves and root activity in the absence of fertilization. Overall, EFs have been confirmed to be a safe option for achieving sustainable farming due to their ability to increase the transfer of access to nutrients and other essential compounds (Lata et al., 2018; Fadiji and Babalola, 2020; Rigobelo and Baron, 2021; Singh et al., 2021).

5 Endophytic fungi conferring plant resilience under climate change conditions

Sustainable agricultural goals aim to address climate change as a significant impediment to food security for an increasing global population, emphasizing the need for climate-resilient production systems as a key dimension of sustainability. Climate change-driven abiotic stresses, such as climatic variability, drought, salinity, heat, osmotic stress, and nutrient shortages, are increasingly affecting and limiting agricultural productivity in this century (Ferus et al., 2019; Fontana et al., 2021). According to Singh et al. (2021), increasing drought, temperature, and soil salinity and decreasing water availability are growing challenges for improvements in crop production. Endophytic fungi, which inhabit almost all plants in natural Earth's ecosystems, provide different benefits to boost crop performance, yield, and resilience to environmental stresses, hence mitigating the negative effects of climate change (Hereme et al., 2020; Verma et al., 2022). Consequently, the integration of EFs is perhaps a beneficial approach for both mitigating the effects of climate change

on main crops and growing agricultural production on marginal lands. Thus, the use of EFs would be a promising approach for improving agricultural productivity by decreasing the reliance on harmful agrochemicals. For example, Tian et al. (2021) noted that after crossbreeding wild rice (African and Asian wild rice) species with grown rice accessions, wild rice contains more root EFs than the cultivated parent rice used to produce crosses in the first generations (F1 offspring), which opens up new research areas for the impacts of breeding on the inheritance of EFs in successive generations. Similarly, Abdelrazek et al. (2020) reported that carrot genotypes affected EF abundance and suggested the possibility of using EFs in carrot breeding programs for improvements in the health and yield of crops. Wang et al. (2020) also reported that *Fusarium* head blight (FHB) resistance gene Fhb7 (Fhb7) introgressions in wheat confer resistance to both FHB and crown rot in varied wheat backgrounds, offering a solution for *Fusarium* resistance breeding.

Several laboratory and greenhouse experiments have strongly indicated that endophytes could be used to mitigate stresses in agricultural crops to increase productivity (Chitnis et al., 2020; Singh et al., 2021), as demonstrated in Table 6. The colonization of EFs usually triggers physiological changes in plants, imparting tolerance to various abiotic (oxidative, drought, salinity, high temperature, high CO_2, and metal toxicity) stresses (Lata et al., 2018; Morsy et al., 2020; Verma et al., 2022). EFs can enhance plant resilience to climate change by promoting the plant's own molecule production and/or by producing several compounds on their own, which are crucial for adaptation to adverse environmental conditions (Lata et al., 2018; Morsy et al., 2020). In most cases, EFs aid host plants in responding to stresses by regulating plant growth and development using bioactive substances that can work together with hosts for better performance. They secrete phytohormones, saponins, triterpenoids, ginsenosides, proteolytic enzymes, growth hormones, phosphate-solubilizing factors, active volatile and nonvolatile metabolites, IAA, and ACC deaminase during stress to enhance plant growth and enable plants to overcome abiotic stresses (Moraes et al., 2020; Jagannath et al., 2021; Lu et al., 2021). According to Verma et al. (2022), *T. atroviride*, *T. polysporum*, and *T. harzianum*, which inhabit *Phaseolus vulgaris,* phosphate solubilizing factors, exude proteolytic enzymes, and active volatile and nonvolatile compounds that enhance plant growth and improve abiotic stress tolerance. Secondary metabolites such as flavonoids, phenylpropanoids, phytoalexins, and carotenoids are found in stressed plants inoculated with EFs, and they help plants tolerate abiotic stress by acting as antioxidants that scavenge ROS (Kaur, 2020; Jagannath et al., 2021). In a recent study, EFs increased the phenol and proline contents in drought-salinity-exposed plants, where phenols signal symbioses, while proline, a stress-associated amino acid, improves drought tolerance by regulating macromolecule stabilization and redox homeostasis (Ballesteros et al., 2023).

Fungal endophytes also indirectly promote plant growth by inducing resistance and promoting antibiotic, secondary metabolite, and siderophore production, which protects plants against abiotic stressors (Singh et al., 2021). Thus, root EF can be leveraged as a biotechnological tool to sustain high ecophysiology and productivity in abiotic stressors (Molina-Montenegro et al., 2016; Ferus et al., 2019). In areas under drought conditions, for instance, EFs offer benefits that are related to reduced lipid peroxidation, higher proline levels, and ion homeostasis upregulation or downregulation (Molina-Montenegro et al., 2016; Morsy et al., 2020). Studies have revealed that EFS confers drought tolerance in crops through enhanced photosynthesis, improved water use efficiency (Dastogeer, 2018; Hereme et al., 2020), improved nutrition and root development (Dastogeer, 2018), and induced stress-responsive gene expression (Molina-Montenegro et al., 2016; Hereme et al., 2020). For instance, under water stress conditions, EFs increased chlorophyll content, total biomass, net photosynthesis, relative water contents, and stomatal conductance in plants compared to nonstressed conditions (Dastogeer, 2018; Ismail et al., 2020). Additionally, EFs also aid plant adaptation to drought endurance by secreting phytohormones, exopolysaccharides, ROS, 1-aminocyclopropane-1-carboxylate deaminase, and volatile compounds while altering root morphology and biosynthesizing anti-stress metabolites (Fontana et al., 2021). Ismail et al. (2020) isolated the EF *A. violaceofuscus* from the fern *Dryopteris flix* L. and found that its culture filtrate had a higher concentration of secondary metabolites that improved plant height, chlorophyll contents, soybean seedlings, and sunflower biomass under drought and heat stresses. Drought and heat stresses can also be mediated by

TABLE 6 Endophytic fungi conferring abiotic stress resistance in various plants.

Fungal endophytes	Host plant	Abiotic stress	References
P. indica	*Hordeum vulgare*	Salinity stress	Baltruschat et al. (2008)
Trichoderma sp.	*T. cacao, Brassica rapa* subsp. Pekinensis and *H. vulgare*,	Salinity and Drought stress	Chhipa and Deshmukh (2019)
Curvularia protuberate	*Lycopersicon esculentum*	Temperature stress	Rodriguez et al. (2008)
Paecilomyces formosus LWL1	*Oryza sativa* subsp. Japonica	Temperature stress	Waqas et al. (2015b)
Chaetomium globosum and *Penicillium resedanum*	*Capsicum annum*	Drought stress	Khan et al. (2014)
Penicillium brevicompactum	*Hordeum vulgare*	Drought stress	Chhipa and Deshmukh (2019)
P. indica	*Brassica rapa* subsp. Pekinensis	Drought stress	Sun et al. (2010)
Penicillium roqueforti Thom	*Triticum*	Heavy metal stress	Ikram et al. (2018)
Exophialapisciphila	*Zea Mays*	Heavy metal stress	Wang et al. (2016)
Acrocalymma vagum	*N. tabacum*	Heavy metal stress	Jin et al. (2018)

EFs through mitogen-activated protein kinase (MAPK) and heat shock proteins (HSPs), respectively (Lata et al., 2018).

Fungal endophytes also have the potential to mitigate soil salinity stresses in various crops (Badawy et al., 2021; Moghaddam et al., 2021). Some of the strategies mediated by EFs to mitigate salt (other abiotic) stresses include (1) proline accumulation within cells (Badawy et al., 2021; Gupta et al., 2021); (2) modulation of plant hormones/phytohormones (Gul et al., 2014; Baron et al., 2020; Illescas et al., 2021); (3) maintenance of ionic homeostasis by modulating ion accumulation, ensuring a low cytosolic Na+:K+ ratio and advancing nutrient uptake in plants (Gupta et al., 2021); (4) accumulation of glycine betaine and polyols (Khan et al., 2011); and (5) production of extracellular enzymes (Jagannath et al., 2021). For instance, *Induratia* spp. isolated from coffee plants also produce remarkable amounts of extracellular enzymes, such as protease, cellulase, lipase, phosphates and amylase (Monteiro et al., 2020). A recent study examined 203 EFs from 29 species from *Baliospermum montanum* tissues for extracellular enzymes and showed that 83% of isolates produced amylase, 79% cellulase, 77% phosphatase, 72% protease, and 59% lipase (Jagannath et al., 2021).

Furthermore, EFs can support host plants in responding to various stresses through the involvement and expression of mutualistic genes (Monteiro et al., 2020; Jagannath et al., 2021; Lu et al., 2021). In particular, EFs can upregulate genes involved in secondary metabolite production, osmotic regulation, ion transport, stress signaling pathways, and the synthesis of antistress metabolites and scavenger molecules (Lata et al., 2018; Chitnis et al., 2020; Harman et al., 2021). For instance, Verma et al. (2022) reported that wheat EF altered stress-related gene expression to enhance drought tolerance, while rice EF modified antioxidant defense gene expression to improve oxidative stress tolerance. Several transcriptomic and metabolomic studies have also indicated the involvement of mutualistic gene products in various plant growth and abiotic stressor adaptation pathways. A recent study by Toppo et al. (2023) revealed that *T. longibrachiatum* T6 upregulated the expression of actin, POD, SOD, and CAT biosynthetic genes to reduce salt stress in wheat seedlings, while *T. harzianum* upregulated CsAPX and CsGR genes in cucumber seedlings, mitigating various abiotic stresses. Abdelaziz et al. (2017) also showed that EF can help salt-stressed plants from osmotic stress by regulating the expression of the *P5CS* gene (pyroline-5-carboxylate synthase), which is involved in the biosynthesis of proline, starch-degrading enzyme activation, and glucan-water dikinase. Toppo et al. (2023) further observed that *P. funiculosum* LHL06 upregulates the *GA1, GA3, GA4, GA7*, and *GA9* genes to reduce metal toxicity by modulating hormonal concentration while downregulating the stress mitigation-related genes *G6PDH, GmGST3, GmGST8* and *GmSOD1[Cu–Zn]*. Fungal endophytes such as *Paecilomyces formosus* LHL10 and *P. funiculosum* LHL06 have also shown increased transcript expression of the *GmHsp90A2* and *GmHsp90A1* genes to enhance soybean growth under high temperature and drought stress (Bilal et al., 2020). In addition, MAPK genes are also key players in the MAPK pathway, which is responsible for producing ROS, signaling pathways, and the synthesis of antioxidants in EFs to help their host plants cope with abiotic stresses (Ogbe et al., 2020). In general, EFs demonstrate remarkable promise as a means to safeguard global agriculture and food security amidst climate change through their multifaceted abilities to promote plant growth and strengthen host plant resilience against environmental stresses.

6 Research gaps and future perspectives

Increasing threats to global food security from climate change, population growth, and multiple stress factors emphasize the urgent need to boost crop yields in vulnerable environments. Fungal endophytes show promise through direct and indirect mechanisms that promote plant growth, pest protection, and climate adaptation, offering a cost-effective and environmentally friendly alternative to improving food production and promoting sustainable agriculture practices. However, their agricultural application remains limited due to regulatory standards and difficulties with mass production, inoculant efficacy, and resistance from consumers and farmers (Khiralla et al., 2016; Ballesteros et al., 2023). Secondary metabolite production from EFs is effective at small-scale setts, but large-scale production has been unsuccessful due to poor yields and performance, particularly for functional polysaccharide biosynthesis. A deeper understanding of EF-produced metabolite structures, microbial interactions, and plant support and/or protective mechanisms could aid integrated management approaches, thereby improving production and benefiting the environment. Further research is also needed on EF manipulation of host morphology and physiology to assess the application potential. Fundamentally, elucidating complex relationships between EFs, crops, soil variables, and soil/plant microbiomes and how these influence morphological, physiological and biochemical responses is essential to optimize EF-mediated adaptations and strengthen sustainable agriculture amid climate threats.

There is also a knowledge gap about how biotic and abiotic stressors may alter plant-EF interactions, potentially leading to mutualistic or parasitic partnerships (Kamran et al., 2021), warranting comprehensive analysis of influential factors to determine the possible interactions. The competition between native microbiomes and introduced EFs, particularly in extreme environments, may lead to decreased effectiveness or negative impacts (Aremu et al., 2017), necessitating studies to confirm effects and identify potential shifts in EF efficacy. Comprehensive metabolomics, genomics, proteomics, and transcriptomics analyses could better elucidate these complex interactions, as well as the diversity and beneficial traits of EFs (Kamran et al., 2021; Verma et al., 2022). Furthermore, EF bioactivity, source, kind, and inoculant production need to be considered when determining the optimal quantity of EFs for plant growth, health, and climate change resilience. Overall, the ultimate goal should be translating endophytes into bioinoculants, biofertilizers and other products using interdisciplinary research and microbial modulation. Despite these aims, smallholder farms remain crucial for global food security, accounting for 50%–70% of global food production (Giller et al., 2021); hence, future efforts must integrate beneficial microbes such as EFs into smallholder agriculture to ensure urgent agricultural transformation.

7 Conclusion

Endophytic fungi are promising tools that can be used as biocontrol agents to regulate plant pest populations, enhance plant growth, and promote plant resilience against the challenges imposed by climate change. These microbes are greatly effective against

agriculturally important pests, including insects, plant diseases, nematodes and weeds, while simultaneously having the potential to promote host plant growth and tolerance to various biotic and abiotic stresses. Thus, EFs have enormous potential to be used as effective and eco-friendly replacements for synthetic fertilizers and pesticides in agriculture under rapid climate change scenarios. Overall, EFs can be considered a key device in managing biotic and abiotic stresses due to the production of biologically active substances against both stresses by activating valuable products for agricultural production under rapid climate change scenarios. Generally, species-specific identification of EFs and characterization of their signaling, promotion and stress mitigation mechanisms, development and shelf-life enhancement components as climate-smart biocontrol agents and biofertilizers are highly imperative in agriculture under rapid climate change scenarios.

Author contributions

TF: Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. EK: Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. TT: Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing. ZB: Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Biochar has positive but distinct impacts on root, shoot, and fruit production in beans, tomatoes, and willows

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Positive relationships have been documented between the amount of biochar added to soils and various aspects of plant growth and fertility such as root, shoot, and fruit production. However, these effects depend on biochar source materials, soil characteristics and species of plant examined. This makes it impossible to systematically compare and generalize findings across previous studies that have used different soils and biochar. We conducted a novel investigation to assess the effects of a single source of biochar (hazelnut wood), in a constructed organic soil, on the different plant tissues in three functionally distinct species: tomatoes (*Solanum lycopersicon*), green beans (*Phaseolus vulgaris*), and willow (*Salix* sp.). Five levels of biochar soil amendment were assessed: 0% (control), 3, 9, and 26% by dry weight. We found a highly significant positive relationship between biochar concentration and total plant biomass (roots + shoots + fruits) in all species, with no significant difference in total biomass response among species. Fruit production increased with increased biochar in both beans and tomatoes. However, tomatoes exhibited significant differences in response among plant tissues; fruit production and shoot biomass increased significantly with biochar, but root tissue did not. Bean germination success increased significantly with biochar concentration. Date of first flowering was earlier with increasing soil biochar in beans but not in tomatoes. Control over both sources of biochar and soil composition in this experiment enables us to conclude that biochar addition can have different impacts on different plants and, in some cases, species-specific impacts on different plant tissues and other measures of fertility. Our results are contrary to prior research that found inhibiting effects of biochar at levels comparable to our 26% treatment. Biochar impacts on soil properties such as CEC and percent base cation saturation do not explain our findings, leading us to conclude that microbial interaction with biochar is an important factor that may explain the positive impacts of soil biochar on plant fertility observed. Further research that repeats this experiment in other soil types, with other biochar sources, and with other plant species is necessary to determine the generalizability of these important findings.

KEYWORDS

plant tissue, soil fertility, carbon sequestration, *Salix*, plant growth

1 Introduction

Biochar is promoted as a soil amendment that can concurrently sequester carbon to address climate change and increase soil fertility, thereby addressing the food needs and of a growing population. In regards to soil fertility, biochar has generally been shown to: increase cation exchange capacity (CEC); increase base cation saturation, decrease bulk density,

increase moisture retention, and increase pH (Zhang Y. F. et al., 2021; Kumar et al., 2023). In addition to the direct effects of biochar on the physical and chemical properties of soil, literature suggests that the positive impact of biochar on plant fertility may also result from the habitat and metabolic resources that biochar provides for beneficial microbial communities (Lehmann et al., 2011; Zhu et al., 2017; Tan et al., 2022; Xu et al., 2023) and that microbial inoculation of biochar can positively impact fertility (Castro et al., 2018). A positive relationship has often been observed between the amount of biochar added to soil and plant growth (Graber et al., 2010; Břendová et al., 2015; Haider et al., 2024). However, saturating and even negative effects have also been reported at high levels of biochar (Rondon et al., 2007; Upadhyay et al., 2014; Fornes et al., 2017). An improved understanding of biochar's impact on individual species and on plant tissues within species may be critical to the future of sustainable agriculture and carbon sequestration.

Fortunately, there is a prodigious and growing body of research on the impacts of biochar on different species of plants and different plant tissues. Significant positive effects of soil biochar have been observed for a broad range of different plant types including: woody plants (Lebrun et al., 2017; Lefebvre et al., 2019); broadleaved herbaceous plants (Thomas et al., 2013); grains such as corn (Rajkovich et al., 2012) and wheat (Vaccari et al., 2011); and vegetables ranging from lettuce (Upadhyay et al., 2014) to onions (Khan et al., 2019). However, studies that control for biochar source and soil type have found significant differences in the impact of biochar on different plant species (Hairani et al., 2016; Lefebvre et al., 2019). Prior research likewise reveals that soil biochar can impact different plant tissues differently and that these impacts can differ among species. For example, Li et al. (2022) observed that biochar resulted in an increase in root:shoot ratio in rice (*Oryza sativa*). In contrast, Yang et al. (2015) documented a *decrease* in root:shoot ratio in sugarcane (*Saccharum officinarum*). Graber et al. (2010) found that biochar increased fruit yields in peppers (*Capsicum annuum*) but had no impact on shoot height. In contrast, these same researchers documented that biochar significantly increased tomato (*Lycopersicum esculentum*) stem length but not fruit production. Understanding the potentially distinct impacts of biochar on crops that are economically important for food and fiber is critical to advancing sustainable agricultural practices. However, there is a significant barrier to understanding these differences.

In spite of clear and compelling evidence that biochar can increase soil fertility, there are at least two important challenges to drawing general inferences from the literature about the differential effects that biochar might have on different plants and different plant tissues. One is that biochar is not a uniform material. Different feedstocks result in biochar with distinct chemical (Haider et al., 2022) and physical (Xu et al., 2023) properties that affect plants differently (Güereña et al., 2015). Likewise, different production processes affect biochar chemistry (Kumar and Bhattacharya, 2021; Bo et al., 2023). For example, increased temperature of biochar production (pyrolysis) has been found to increase: pH, ash content, surface area, and CEC (Elad et al., 2011), H:C ratio (Ronsee et al., 2012) and porosity (Ghorbani et al., 2024). Unsurprisingly, biochar produced with a uniform feedstock but different production processes has been found to result in differences in microbial communities, earthworm preferences, and plant biomass (Chan et al., 2008). These studies indicate the necessity of controlling for biochar source and production process when comparing biochar impacts on plant fertility. A second problem is that the impact of biochar on fertility may be dependent on the soil to which it is applied (Vanapalli et al., 2021; Singh et al., 2022). For example, Manolikaki and Diamadopoulos (2016) found that biochar addition increased ryegrass growth in sandy loam but not in loam soil. Other studies have likewise demonstrated that impacts of biochar vary depending on soil texture (Butnan et al., 2015; Manolikaki and Diamadopoulos, 2018). These studies suggest that it is simply not possible to directly compare the impact of biochar among species or among tissues from reports in the literature if the studies being considered differ in either biochar source or soil type. In this paper we describe what we believe to be a unique controlled experiment to compare the impact of different levels of biochar on different plant tissues in three functionally distinct species of plant.

We compared how different plant tissues in three functionally and economically distinct species of plant responded to a single source of biochar applied to a single soil type. Specifically, we designed an experiment to examine the impact of biochar produced from coppiced hazelnut trees on cherry tomatoes (*Solanum lycopersicum var*), green beans (*Phaseoulous vulagris* var. green bean), and hybrid willows (*Salix* spp.). Beans and tomatoes were selected for two reasons: they are important vegetable crops and they are functionally distinct, as beans are in the legume family and have different nutritional requirements. Hybrid willow was chosen because it is a fast-growing woody plant, often used for remediating damaged land and as a source of biofuel (Karp and Shield, 2008). Each plant type was grown in the same organic planting mix (peat moss and vermiculite) subjected to four distinct levels of biochar (0, 3, 9 and 26% by dry weight). We chose these levels based on reviewing literature on other biochar experiments. The 26% level was chosen because several prior experiments have shown saturating or inhibitory effects at this level (Upadhyay et al., 2014) or other high levels (Rondon et al., 2007; Fornes et al., 2017). We choose these quasi-logarithmic treatment levels with the goal of fitting a hyperbolic saturation curve to define the relationship between biochar concentration and growth parameters. This would have allowed us to assess and compare how different plants and tissues might saturate at different biochar levels. We examined multiple growth parameters within and among the different plant species to assess differences in the impact of a single source of biochar on different species and on different tissues in these species. Different authors refer to plant tissues differently. For the purposes of consistency within this paper we will use: "roots" to refer to all below ground tissue production (biomass); either "aboveground biomass," or "shoots" to refer to both stems and leaves, excluding fruit and seed production; and "fruits" to refer to fruit and seed biomass.

Prior studies have documented a variety of impacts of biochar soil amendment on the three species of plants selected for our experiment. Positive effects of biochar observed for beans include increases in: the biomass of roots and shoots (Güereña et al., 2015; da Silva et al., 2017); total plant biomass (Melo et al., 2018); fruit biomass; and the number of pods and seeds (da Silva et al., 2017). However, some experiments have also documented negative impacts of biochar on bean shoot and fruit biomass (Velez et al., 2018). Saxena et al. (2013) found that biochar significantly increased: percent germination, root length, shoot length, flowers per plant, pods per plant, number of seeds, seed biomass, and tolerance of high temperatures and extreme light conditions. Increases in root:shoot ratio in response to biochar addition have also been observed in beans (Torres et al., 2020).

Conflicting results have been observed regarding the impact of biochar on bean root nodules, with some studies showing a decrease in abundance (Castro et al., 2018) and others showing increases (Güereña et al., 2015). Several studies have observed increases in nutrient levels in bean tissues grown in soils augmented with biochar, including P, Fe, Mg (Gao et al., 2016), N, K, Ca, Zn, Cu, Mn (Inal et al., 2015), and B (Rondon et al., 2007). Several experiments demonstrate that benefits saturate or are even inhibited with increasing soil biochar. For example, Rondon et al. (2007) found bean biomass increased up to 6% soil biochar and decreased after 9%.

A considerable body of literature exists on the effects of biochar on growth and fertility of tomatoes. The addition of soil biochar has been shown to increase many growth parameters including aboveground biomass, total biomass, fruit yield, and plant height (Ronga et al., 2020; Guo et al., 2021). Tartaglia et al. (2020) found that biochar addition resulted in an increase in: early growth, the number of flower buds and the average number and weight of fruit. Akhtar et al. (2014) reported that biochar improved fruit quality as well as yield. Documented impacts are not entirely positive or consistent. Two studies documented a negative impact of biochar on tomato seedling growth (Yu et al., 2019; Vaughn et al., 2021). Two other studies found positive impacts on aboveground and root tissues, but no increase in tomato fruit yield (Vaccari et al., 2015; Velli et al., 2021). Other experiments have reported no significant impact on biomass production (Liao et al., 2021) or fruit yield (Dunlop et al., 2015). Research has also investigated the combined effects of fertilizer, microbial inoculation, and biochar application. Several studies have found that the positive impact of soil biochar on tomato plant height, shoot and root biomass, and fruit production is enhanced when it is augmented with either microbial inoculant (Castro et al., 2018) or compost (Sani et al., 2020). However, Nzanza et al. (2012) observed no effects of the combined addition of biochar and inoculation on tomato root biomass.

Fewer studies have examined biochar impacts on willow and the impacts observed have been more equivocal. Several studies have documented positive impacts on willow growth. Saletnik and Puchalski (2019) found that biochar addition increased growth in *Salix viminalis L*. Similarly, Kuttner and Thomas (2017) documented increased diameter, height, and drought resistance in *S. exigua Nutt*. Seehausen et al. (2017) documented increases in root:shoot ratio in *S. purpurea*. Several studies have documented positive impacts of the addition of soil biochar on willow biomass in contaminated soils (Lebrun et al., 2017, 2019; Mokarram-Kashtiban et al., 2019). Břendová et al. (2015) demonstrated a positive correlation between soil biochar and aboveground biomass of the hybrid *Salix* × *Smithiana*. Lebrun et al. (2017) observed differing effects on different tissues; in *S. alba* and *S. purpurea*, root and leaf biomass increased with soil biochar, while stem dry weight was not affected. Kuttner and Thomas (2017) found that biochar addition did not significantly influence biomass and growth in *Salix exigua Nutt* but did mitigate drought stress. Detrimental effects have also been observed. A mix of biochar and compost treatment caused *Salix triandra x Salix viminalis* to grow more slowly than a control group (von Glisczynski et al., 2016). A study conducted with *S. purpurea* documented negative effects of soil biochar on aboveground biomass, and mean total branch length (Seehausen et al., 2017).

As stated, the challenge of interpreting the literature that we have summarized for beans, tomatoes and willows is that the experiments discussed were conducted using a wide range of biochar in a wide range of soils. This makes it essentially impossible to clearly identify whether the impacts observed in different experiments might have resulted from differences in biochar and soil or represent generalizable results. We designed an experiment to assess the impacts of varying levels of one type of biochar used in one type of soil on different plant species and different tissues within those plant species. Given the important functional and physiological differences between beans, tomatoes, and willows we hypothesized the plants and the individual tissues of these plants would exhibit distinct responses to biochar. Our research objective was to test this hypothesis by addressing several specific questions that could only be assessed through experimental conditions that control for biochar source and soil type. Specifically, we asked:

1. How do three functionally distinct plant species (green beans, tomatoes, and willows) differ in their response to different levels of soil biochar?
   a. Do these species differ in response with respect to: root growth, shoot growth, fruit production, root:shoot ratio, fruit:(root+shoot ratio), percent moisture content of fruit, and individual fruit weight?
   b. Are certain tissues more affected by biochar than others and does this response differ among the species examined?
   c. How does biochar level affect germination rate of beans, and date of first flower for beans and tomatoes?
2. Does the response of willows to biochar change during a second season of growth?
   a. Does additional fertilization of willows impact second year growth?
3. How does biochar affect the properties of an organic-rich soil and how is this mediated by plants and microbial inoculation?
   a. Does biochar addition affect soil properties differently when different species are grown in that soil?
   b. Independent of biochar addition, do tomatoes and bean plants impact soil properties differently?
   c. Does enhanced fungal and bacterial inoculation improve or alter biochar's impact on plant growth?

2 Materials and methods

2.1 Plant preparation and management

Four plant types were used: cherry tomatoes (*Solanum lycopersicum* var. "Supersweet 100"), bush beans (*Phaseoulous vulgrais var*. Burpee's "Stringless Green Pod"), willows (*Salix sp*), and a no plant control. Tomato starts approximately 10 cm in height were acquired from Thome Farms Greenhouse in Elyria, Ohio in early June of 2021. One plant was added to each treatment pot. Cages were later added for support.

Green bean seeds were acquired from Tractor Supply Company. Beans were soaked overnight, drained, rolled in rhizobia bean inoculant, and planted in treatment pots at a depth of ~1.5 cm with 6 seeds per pot. Once bean sprouts reached an average height of 8 cm, they were thinned to 3 sprouts per pot. In late August, beans were thinned to two plants per pot. Green bean plants were attacked by bean leaf beetles (*Cerotoma trifurcata*) throughout the course of the

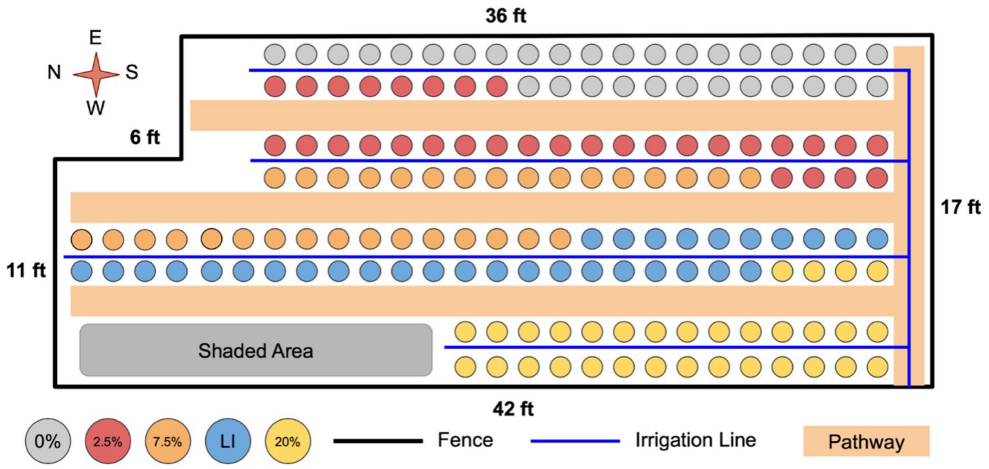

FIGURE 1
Layout of the experimental system. Plants were placed in the order: bean, willow, no plant, tomato. Experimental units were placed in the order of replicate number. Plants were moved regularly as described in text to minimize the impact of variability in shading.

experiment. A natural pesticide consisting of 1 L of water, 8 mL of neem oil, and 3 mL of 1% Liquinox dish soap solution was regularly applied to the beans to control damage through the end of the experiment.

Cuttings of hybrid willows were taken in early June from an existing tree that was originally obtained from Gurney's Seed and Nursery Company.[1] Cuttings were 30 cm length and 1–3 cm in diameter. Three willow cuttings were directly rooted in each treatment pot. Labels were fixed around the base of each cutting to track growth of individual plants.

Each experimental unit consisted of a 2-gallon (7.6 Liters) fabric pot (PHYEX brand, Dongguan, CN) containing the same planting mix with four different levels of biochar: 0, 3, 9 and 26% by biochar and soil dry weight. In addition to the plants, we included a no-plant control. Our 4×4 design thus consisted of 4 plant types (including the no-plant control) and 4 biochar levels. We used 8 replicates per treatment resulting in 128 experimental units for the main experiment. As described below, we also included an additional "low inoculation" treatment for each of the four plant types at the 9% biochar level to which we applied only a single microbial inoculation with 8 replicates resulting in an additional 32 experimental units.

2.2 Site and management

The experiment was conducted at Oberlin College's Adam Joseph Lewis Center for Environmental Studies (41.2959° N, 82.2211° W) from June–October 2021, and extended to November of 2022 for the willow plants. Oberlin is in a temperate climate in USDA hardiness zone 6a. An experimental area of 700 square feet was covered in a black weed block fabric, covered in straw to combat heat island effects, and fenced to prevent unwanted herbivory from humans and other animals. Plants were watered by hand until July 26 when an automatic irrigation system was installed to ensure water was not a limiting factor.

Figure 1 shows how treatments were generally arranged in the experimental area. As depicted, pots within a treatment were not randomized by treatment level, rather treatment levels were kept together to avoid confusion and cross contamination. The main uncontrolled variation in the experimental area was variability in shading by buildings, trees, and other structures. To minimize the impact of this variability, once a week, plants were rotated by 10 units to negate any effects of placement within the experimental area. Plants were moved in an s-shaped rotation so that every plant was periodically in every row and therefore experienced similar variation in shading.

2.3 Biochar source and preparation

Biochar was created from local coppiced hazelnut wood. Harvested branches, ranging in diameter from approximately 1–10 cm were combusted in a retort oven that was fabricated for this experiment. Specifically, a 120-gallon (450 L) propane tank was converted into a rocket stove; the top was cut off; air holes were cut into the bottom and a 3-m chimney was added to the top. A standard 50-gallon (190 L) steel drum was used as the inner chamber. The overall size and design of the inner chamber was similar to the biochar retort furnace used by Wijitkosum (2021). The hazelwood was packed into this chamber and capped with a lid that did not form a complete airtight seal and placed upside down into the rocket stove. The space between the two containers was then filled with scrap wood, the top and chimney were placed back on, and a fire was lit from below. The outer fire then heated the hazelnut wood in the absence of an oxygen source to the point that pyrolysis released combustible gasses that heated the unit until the run was completed. A complete run from ignition through termination of combustion was typically approximately 4 h. Although we did not directly measure internal temperature, given similarities in design to the apparatus described by Wijitkosum (2021), we estimate combustion at 450–600°C. The product of pyrolysis in the retort oven contained charcoal with no evident ash production of any kind. Material from multiple combustion runs was processed through a woodchipper and then

1 www.gurneys.com

homogenized resulting in biochar that ranged in size from fine powder to 5 mm in diameter.

2.4 Soil preparation

In order to achieve a uniform and controlled soil for the experiment, a soil mixture was created from a 1:1 volumetric ratio of perlite and shredded peat moss. To create the mix, 1 gallon (3.8 L) of perlite and 1 gallon (3.8 L) of peat moss were added to a hand concrete mixer and homogenized. The wet weight of 1 gallon (3.8 L) of perlite was 370 grams (g) and the wet weight of 1 gallon (3.8 L) of peat moss 500 g. The moisture content of both assessed and determined to be low (<8%). Biochar was then added in addition to the 870 g mass of soil per pot. For the four treatment levels, 0 g, 22.3 g, 70.5 g, and 217.5 g of dry biochar were added, respectively, to achieve the 0, 3, 9, and 26% by dry weight. The soil mix was added to the fabric pots. The chipped biochar was heterogeneous in particle size. In order to control for this and ensure uniformity in biochar treatments, we passed the chipped biochar through a 2 mm soil sieve to separate fine and coarse particle sizes. The same ratio of course and fine particles was added to every experimental unit that received biochar.

All experimental units were inoculated with two separate commercial sources of microbial inoculant. Manufacturers of the "Mikro-Myco" (4655 Waterford Dr., Suwanee, GA) state that it contains a combination of plant growth promoting rhizobacteria (PGPR) and phosphate solubilizing fungi (PSF) with 4 species of Endo Mycorrhizae and 7 species of Ecto Mycorrhizae. We also used "Wildroot Organic" (PO Box 4800, Horseshoe Bay, TX). An inoculant solution including both products was applied three times over the course of the summer: July 1st, July 22nd, and August 8th. The inoculant was prepared according to instructions, and a total of 100 mL of both inoculants were applied to each experimental unit at each inoculation. As mentioned, a side experiment was conducted to assess the impact of inoculation. One set of plants of each species grown at the 9% biochar level were inoculated like all other treatments. A second set of low inoculant (LI) plants were also grown at the 9% biochar level. The LI group was only inoculated once near the start of the experiment.

Every experimental unit was fertilized once, shortly after planting, using Osmocote 14–14-14 N:P:K time release fertilizer granules.[2] Based on the manufacturer's recommended application rate; 15 mL of this fertilizer was sprinkled on the soil surface in each pot.

2.5 Measurements

A variety of different growth parameters were measured for different plants at different times in the experiment. For example, we measured the germination rate for green beans and the flowering timing for both beans and tomatoes. Ripe tomato and bean fruits were harvested and immediately frozen throughout the growing season. Frozen fruits were cleaned of condensate, weighed, oven-dried and then reweighed. As common comparison measurements for all plants, we assessed the final root and shoot biomass for tomatoes, green beans, and willows, as well as the total fruit biomass for tomatoes and green beans. All reported weights for plant tissues are oven-dry weights unless otherwise specified. We also assessed total biomass, the sum of root, shoot, and fruit biomass for the species that produce fruit. Total biomass for willows was the sum of root and shoot biomass as there was no flower or seed production in these young plants. Root:shoot ratio was calculated by dividing the root biomass by the shoot biomass. The fruit:(root+shoot) ratio was calculated by dividing the fruit biomass by the sum of the root and shoot biomass. Fruit percent moisture content was measured after collecting all fruit produced from a plant throughout the season and measuring both wet and oven-dried weight (Equation 1). The equation used to determine percent moisture content was:

$$percent\ moisture\ content = \frac{wet\ weight - dry\ weight}{wet\ weight} * 100 \quad (1)$$

The average dry weight of the fruit was calculated by dividing total fruit biomass dry weight by the total number of fruits produced.

We measured the germination rate in beans by simply counting how many of the six bean seeds planted in each pot produced green shoots that broke the soil. In green beans and tomatoes, we also measured the day of first flowering for each plant (there were two plants per pot for the green beans and each plant was measured). Date of the first flower was recorded beginning on July 29. On this date, several plants had already had multiple flowers for several days, but we counted this as the first flowering date for these.

At the end of the 2021 growing season in October, any remaining fruit on the green bean and tomato plants was harvested and the remaining shoot tissue was cut where it met the soil. Aboveground shoot biomass was placed in a paper bag, oven dried and then weighed. No attempt was made to separate leaf from stem tissue in either beans or tomatoes; these were collectively treated as "aboveground biomass" or "shoots." Tomato and bean roots were carefully separated from the soil by hand. Root material greater than 1 cm in length was captured, smaller root fragments were not. The aboveground tissues from the two individual bean plants in each experimental unit for beans were processed and weighed and analyzed as a single unit. The root tissue of the two bean plants was inseparable and also treated as a single unit.

Root material was rinsed with water, to further remove soil particles, and placed in paper bags for drying. Both root and shoot material were dried in a drying oven at 100°C until they no longer lost weight. A subset of root samples was incinerated in a muffle furnace for 24 h at 400°C. On average, mineral content of roots was less than 20% (Table 1). Mineral content of roots was not significantly larger than mineral content of shoots and fruits. We therefore concluded that we had effectively removed the vast majority of the soil particles from

TABLE 1 Mineral content of plant tissue.

Plant & Biochar level	Root	Fruit	Shoot
Beans 0%	12.9% a	5.4% c	13.3% d
Beans 20%	20.7% b	6.9% c	10.5% e
Tomatoes 0%	21.8% b	18.3% b	13.7% f
Tomatoes 20%	20.3% b	17.4% b	14.2% f

Values with different letters differ significantly in percent mineral content at the alpha = 0.05 level.

2 www.growwithosmocote.com

the roots. Final dry weights of roots, fruits, and aboveground biomass that we report in the results section include the mineral content.

Since our intention was to continue growing a subset of the willows for a second season, experimental units in the willow treatment were processed differently from the beans and tomato units. Each experimental willow unit contained three plants. Following the first season, the roots of these three plants in each unit were carefully separated from each other. Of these three plants in each unit, the one of intermediate height was processed to measure above and belowground biomass. The other two plants were retained for a second growing season. The intermediate height plant was cut at soil level, and root and shoot material was processed and dried using the same procedures described for tomato and beans. However, in contrast to beans and tomatoes, the dry weight reported for aboveground plant tissue in willows includes only stem tissue and not leaf tissue.

The willows that were retained for a second growing season were transplanted from 2-gallon (7.6 L) to 4-gallon (15.1 L) pots. We planted each of the two remaining willow plants from each experimental unit in its own individual pot, effectively doubling the number of experimental units from 8 to 16 at each biochar level. We reused the soil retained from the beans and tomato treatments for repotting the willows. Soil from each biochar level for the beans and willows was homogenized prior to using it for this purpose. During the second year, the willows were grown in the same plot (Figure 1) as the previous year and rotated in the same manner as described earlier. Additional microbial inoculant was not applied to the willows during the second season.

Willows were not fertilized at the start of the 2nd growing season. However, in response to leaf yellowing, we decided to fertilize half the willows at the end of July of the 2nd season. The decision to fertilize only half of the plants was made so that we could assess whether biochar level influenced the willows response to the fertilization. Each of the willows fertilized received 30 mL of the Osmocote sprinkled on this soil surface as in the first year (doubled from the first year to account for the doubling of pot volume).

In 2021 an attempt was made to capture willow leaves with netting prior to leaf fall. Unfortunately, this proved ineffective and willow leaves were not captured. We successfully captured leaves in the fall of 2022. We determined that leaves accounted for only a small fraction of total aboveground biomass in willows. In the results section, aboveground biomass for willows therefore includes only the woody tissue, not leaves.

Since all the willows were fertilized in 2021, the results presented in section 3.7 compares fertilized 2022 willows with 2021 willows. In section 3.8 we assess differences between fertilized and unfertilized willows in 2022.

2.6 Soil analysis

Our experiment focused on the impact of biochar soil amendment on plant fertility. Nevertheless, we thought it important to assess differences in soil chemistry resulting from biochar addition. Due to constrained resources, we were unable to conduct soil analysis for all biochar levels for all species. Soil taken from beans, tomatoes, and no plant pots were analyzed from the 0% biochar level and 26% biochar level. Soil from the willow plants was not analyzed. After all biomass was removed from tomato and green bean pots, the soil from each was re-homogenized and samples were sent for soil analysis. Soil was analyzed by Logan Labs (620 North Main Street, Lakeview, OH 43331) using standard methods of soil analysis (NCR-13 Soil Testing and Plant Analysis Committee, 1998). Analyses included: pH, organic matter, cation exchange capacity (CEC), S, P, Ca, Mg, K, Na, B, Fe, Mn, Cu, Zn, Al, and percent base saturation.

2.7 General statistical model

As stated, we selected quasi-logarithmic treatment levels, with our highest biochar level to be within the range in which prior experiments have shown saturating or inhibitory impacts on plant growth. Our intent was to fit a hyperbolic saturation curve to data on biochar level vs. growth parameter. However, initial examination of our data provided no evidence for this expected non-linear response. We therefore applied linear statistical models to quantify the impact of biochar on the various growth parameters examined. In particular, in order to answer our research questions, we assessed whether the slope of the relationship between each growth parameter measured and biochar was greater than zero. In other words we assessed whether biochar concentration had a significant positive relationship on each growth parameter.

As indicated in the introduction, a key goal of our analysis was to be able to compare the overall effects of biochar among species and among tissues within and between species. In order to be able to do this, we standardized data for each growth parameter by calculating z-scores for that variable (Equation 2). The magnitude of the slope calculated by regressing z-scored response variables against biochar then provides a non-dimensional measure of the strength of the impact of biochar on each growth parameter examined. This standardization allows slopes (essentially effect size) to be directly compared both among tissues within a plant species and among species. Specifically, a significantly larger slope indicates a stronger response to biochar application. We assessed whether slopes for different growth parameters differed within each species (for example, are the roots of tomato plants more affected by biochar than shoots?). We also assessed whether slopes differed among species (for example, is the response of root tissue to biochar greater in tomatoes than beans?). Unless otherwise noted, all p-values reported in the results section are the p-values assessing whether slopes are different from zero (i.e., testing for a positive or negative impact of biochar) and whether slopes differ from each other (more impact on one growth parameter and or species than another).

We used Analysis of Variance (ANOVA) as an additional model for assessing whether responses differed among individual treatment levels for a given species. Although we compared all the individual treatment levels, for the sake of simplicity, our results section (Table 2) only reports on ANOVA comparisons between the 0 and 26% treatment levels. The comparisons we examined among other treatment levels did not lead to conclusions that differ from simply comparing 0 and 26% levels.

RStudio was used to conduct both linear regressions, and ANOVA using the mosaic package. When only comparing two conditions, ANOVAs were run using RStudio to test for significant differences between the two conditions.

2.8 Standardizing data for comparison among tissues and among species

Different tissues within a plant can be expected to accumulate different amounts of biomass. Standardization of data was therefore necessary to be able to directly compare the impact of biochar among plant tissues within each plant species (for example, are the roots of tomato plants more affected by biochar than shoots?). Similarly, different species of plants also accumulate different amounts of biomass from each other. Standardization of the data was therefore also necessary to compare impacts of biochar among plant species (for example, is the response of root tissue to biochar greater in tomatoes than beans?). Z-scores were used for standardizing data for each variable that was compared among tissues and among species (Equation 2). The equation used for calculating the z-score was:

$$z = \frac{x - \mu}{\sigma} \quad (2)$$

where z is the z-score, x is the observed value for one growth parameter for one experimental unit, μ is the mean of all values for that growth parameter for that plant species across all treatments within the biochar treatment experiment, and σ is the standard deviation of all values for that growth parameter for that plant species. All reported data on plant growth and biomass is reported as z-scores. z-scores are not used to analyze seed germination rate, number of flowers, timing of flowering and soil variables.

We conducted a concurrent experiment to assess the impact of low versus higher levels of biochar inoculation. Calculations of statistics, including z-scores for the main experiment (the effect of biochar on growth parameters in three species) completely exclude experimental units in the low inoculant group. Statistical analysis conducted on the high versus low inoculation assessment include only experimental units in the 9% biochar level (the level at which this assessment was conducted).

3 Results

3.1 How does biochar affect plant growth in different species?

3.1.1 Total biomass

Biochar had a highly significant impact on total biomass (for beans and tomatoes: root + shoot + fruit and for willows just root + shoot) for all three species considered ($p = <10E-15$, Table 2 and Figure 2A). The impact of biochar on total biomass was similar among species, with no significant differences in impact at the alpha = 0.05 level. Beans exhibited a marginally stronger response to increasing biochar than tomatoes ($p = 0.08$, Table 2) and were not different from willows ($p = 0.1$).

3.1.2 Root and shoot biomass

Biochar had a highly significant positive impact on root biomass in all three species ($p = <10E-4$, Table 2 and Figure 2B). However, the strength of this impact differed among species. Bean roots exhibited a significantly stronger response than the roots of tomatoes ($p = 0.001$, Table 2). Though not as significant as beans, willows also exhibited a stronger response than tomatoes ($p = 0.05$, Table 2). Bean and willow roots did not differ from each other in their response to biochar treatment ($p = 0.2$).

Similar to roots, Biochar had a highly significant positive effect on shoot production for all three species ($p = <10E-9$, Table 2 and Figure 2C). The strength of this relationship did not, however, significantly differ among species ($p = 0.2$).

3.1.3 Root:shoot ratio

In contrast to the significant positive impact of biochar on roots and shoots in all three species, biochar did not have a significant and definitive overall impact on root:shoot ratios when the three species were considered together ($p = 0.9$, Table 2 and Figure 2D). Considered individually, the root:shoot ratio was not significantly impacted by biochar in either willow or bean plants. Tomatoes, however, exhibited a marginally significant decrease in root:shoot ratio with increasing biochar ($p = 0.07$, Table 2). To investigate this effect further, we conducted a pairwise comparison of the root:shoot ratio in tomatoes between the 0 and 26% treatment groups and found that the 26% groups had a significantly lower root: shoot ratio than tomatoes in the 0% treatment group ($p = 0.04$).

3.1.4 Fruit biomass

Biochar had a highly significant positive overall impact on total fruit production (dry weight of total harvest) in beans and tomatoes when considered collectively ($p = <10E-6$, Table 2 and Figure 3A) and individually ($p = 0.001$, Table 2). The strength of this relationship did not, however, differ significantly between these two species ($p = 0.4$).

3.1.5 Fruit:(root+shoot) ratio

In contrast to the significant impacts of biochar on fruit, roots and shoots, biochar did not have a significant overall impact on the ratio of fruit:(root+shoot) when bean and tomatoes were considered together ($p = 0.5$, Table 2 and Figure 3B). Likewise, considered individually neither species showed a significant response in this ratio in response to biochar addition. Not surprisingly, there was no significant difference in this ratio between tomatoes and beans ($p = 0.3$). Nevertheless, a pairwise comparison to test if there was a difference in ratio of beans between the 0 and 26% biochar levels indicated that the 26% groups exhibited a lower fruit:(root+shoot) ratio than beans in the 0% treatment group ($p = 0.04$, Table 2).

3.1.6 Fruit percent moisture content

Biochar did not have a significant overall impact on the percent moisture content of bean and tomato fruits considered together ($p = 0.6$, Table 2 and Figure 3C). However, this lack of general pattern is attributable to the fact that the two species exhibited distinct responses. The moisture content of beans increased significantly with biochar addition ($p = 0.04$, Table 2). In contrast, the moisture content in tomato fruits was not significantly impacted by biochar addition ($p = 0.2$). Not surprisingly, the strength of the relationship between percent fruit moisture and biochar addition (Table 2 and Figure 3C) differed significantly for the two species ($p < 10E-3$).

TABLE 2 Effect of biochar on different growth parameters considered within and among species.

Growth parameter	Variable	Slope	*p* value slope	*p* value ANOVA 0 vs. 26%
Total Biomass (root + shoot + fruit)	Three species combined	0.0810	<10E-15	<10E-15
	Tomatoes	5.5816	<10E-5	<10E-4
	Green beans	8.4015	<10E-13	0.008
	Willows	5.9498	<10E-4	0.002
	Beans > Tomatoes	n/a	0.08	n/a
Root biomass	Three species combined	0.0782	<10E-4	<10E-7
	Tomatoes	1.2001	0.05	0.95
	Green beans	8.1128	<10E-5	<10E-4
	Willows	5.4963	0.008	0.004
	Beans > Tomatoes	n/a	0.001	n/a
	Willows > Tomatoes	n/a	0.05	n/a
Shoot biomass	Three species combined	0.0848	<10E-9	<10E-15
	Tomatoes	6.8173	0.05	0.004
	Green beans	8.2459	<10E-4	<10E-4
	Willows	6.0189	0.008	0.002
Root:shoot ratio	Three species combined	−0.0174	0.9	0.6
	Tomatoes	−4.2592	0.07	0.04
	Green beans	0.2205	0.7	0.3
	Willows	2.2177	0.2	0.3
Fruit biomass	Two species combined	0.0701	<10E-6	0.002
	Tomatoes	0.0457	0.001	0.002
	Green beans	0.0335	0.001	0.001
Fruit:(root+shoot) ratio	Two species combined	−0.0165	0.5	0.06
	Tomatoes	0.0657	0.9	0.07
	Green beans	−2.5676	0.3	0.04
Fruit percent moisture content	Two species combined	0.0017	0.6	0.7
	Tomatoes	−0.0250	0.2	0.04
	Green beans	0.0332	0.04	<10E-8
	Beans > Tomatoes	n/a	<10E-3	n/a
Average fruit biomass per fruit	Two species combined	0.0512	0.001	0.02
	Tomatoes	0.0599	0.007	0.05
	Green beans	0.0425	0.05	<10E-17
Combining all plant types	Root	0.3055	<10E-4	0.3
	Fruit	0.0984	0.004	0.002
	Shoot	0.1096	< 10E-9	0.002
	Shoot > Fruit	n/a	0.01	n/a
Tomatoes	Root	1.2001	0.06	0.95
	Fruit	5.7198	0.001	0.002
	Shoot	6.8173	0.001	0.004
	Shoot > Fruit and Root	n/a	0.01	n/a
	Fruit > Root	n/a	0.08	n/a
Beans	Root	8.1128	<10E-4	<10E-4
	Fruit	7.4421	<10E-4	0.001
	Shoot	8.2459	<10E-4	<10E-4
Willows	Root	5.4963	0.008	0.004
	Shoot	6.0189	0.008	0.002

The analyses of fruit consider only tomatoes and green beans. Since slopes were calculated from z-scores and as a function of percent biochar, they are dimensionless and can be directly compared among species. The *p* values reported for each tissue indicate whether the slope of a z-score regressed against biochar level is significantly greater than zero. *P* values reported for comparisons among tissues (i.e., shoot>fruit) assess whether the slopes for these two tissues differ from each other. *P* values for these comparisons among tissues are only reported for cases in which significant relationships were found. The final column in the table reports *p*-values for ANOVA conducted to assess differences between 0 and 26% biochar levels.

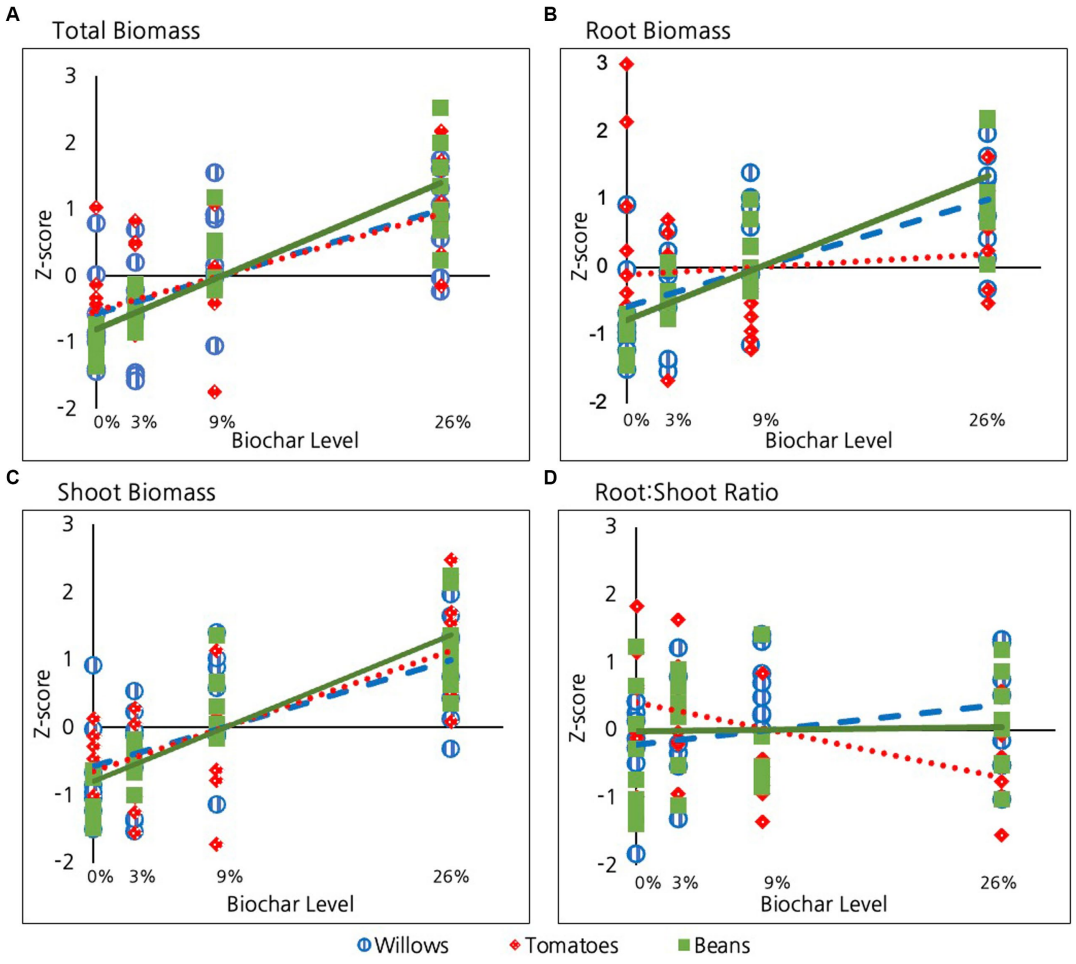

FIGURE 2
Effect of biochar on: **(A)** Total plant biomass, **(B)** Root biomass, **(C)** Shoot biomass and **(D)** Root:shoot ratio in the three species examined.

3.1.7 Individual fruit biomass

When considered together, biochar had a significant positive impact on the average dry weight of individual fruit ($p = 0.02$, Table 2 and Figure 3D). There was, however, no significant difference in the strength of response between tomatoes and beans ($p = 0.4$).

3.2 How does biochar affect plant growth in different tissues?

3.2.1 Across species

As discussed, when data are combined for all species, biochar has a highly significant positive impact on root, on fruit, and on shoot production ($p < 0.01$ in all cases, Table 2 and Figure 4A). When we combine root and shoot biomass together, we observe a significant increase in total plant biomass with increasing biochar ($p < 10E\text{-}15$). As reported, we saw small differences in the strength of the relationships among species, with total bean biomass showing a marginally stronger response than tomato biomass. No other differences were evident.

3.2.2 Within species

Of the three species examined, tomatoes were the only ones to exhibit significant difference in response among root, shoot and fruit tissues (Figure 4B); Specifically, shoot tissue in tomatoes exhibited a significantly stronger response than fruit and root tissues ($p = 0.01$, Table 2). Fruit tissues in tomatoes also exhibited a marginally greater response than root tissues ($p = 0.08$, Table 2). However, no difference was evident between the response of fruit and shoot to biochar addition in tomatoes ($p = 0.6$). No differences were evident in the response of different plant tissues to biochar in beans or willows (Figures 4C,D respectively, $p > 0.6$ in all cases).

3.3 Does biochar affect fertilized willow growth over a two-year period?

As mentioned before, only half of the willows were fertilized in 2022. Since all the willows were fertilized in 2021 the analysis presented in this section compares fertilized 2022 willows with 2021 willows. In a subsequent section we assess differences

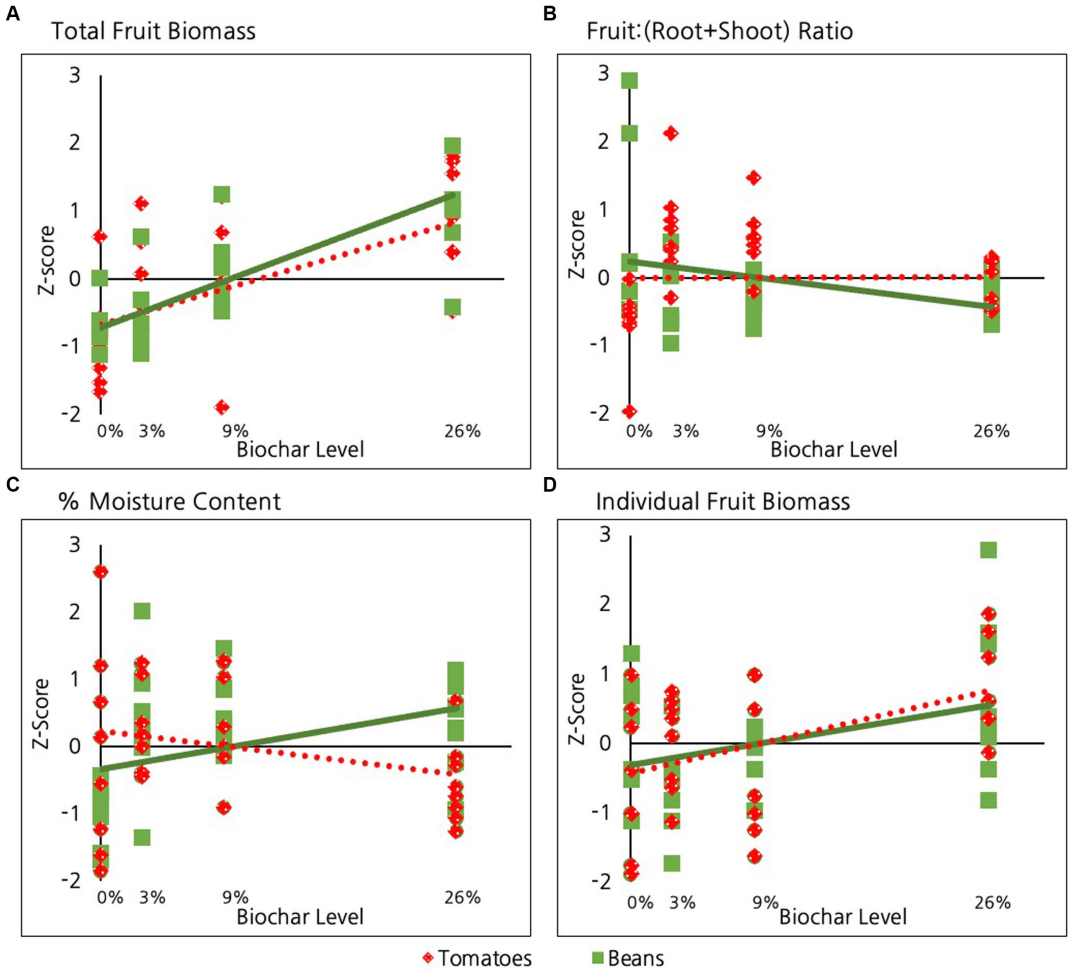

FIGURE 3
Effect of biochar on different aspects of fruit production in tomatoes and beans including: **(A)** Total fruit biomass, **(B)** Fruit:(root+shoot) ratio **(C)** Percent moisture content and **(D)** Individual fruit biomass.

between fertilized and unfertilized willows in 2022. As described in the methods section, because we were unsuccessful in our attempt to collect willow leaves in 2021, we operationally defined willow "shoots" to include only stem material. We were somewhat successful in our efforts to isolate willow leaves in the 2022 growing season. Some leaf tissue may have been lost due to strong winds, but most of the leaf tissue was captured. However, in order to systematically compare data among years, we continued to define willow "shoots" as stem material and total biomass as the sum of this stem material and root biomass (Figure 5). We nevertheless assessed willow leaf biomass in 2022. A negative trend is evident between leaf biomass and biochar addition (Figure 5D), however, the strength of this relationship is only very marginally different from zero ($p = 0.1$).

As reported previously, during the first growing season (2021) willows exhibited a significant positive response to biochar with respect to shoot growth (stems only), root growth, and the combined total biomass of these two tissues (total biomass). Although the trend of positive response of roots, shoots, and total biomass remained in the second year of growth (Figure 5), the strength of this relationship decreased and was no longer significantly different from zero for any of the variables examined ($p > 0.2$ for biomass, root, shoot and leaves). A different way of assessing the changing impact of biomass in year two is by considering changes in the strength of the relationship between biochar and willow growth parameters (i.e., slope of the lines). This slope decreased significantly between 2022 and 2021 for root tissue ($p = 0.03$), shoot tissue (marginal decrease $p = 0.07$), and biomass ($p = 0.03$).

3.4 Does fertilization affect biochar's impact on willow growth during a second growing season?

As discussed, in the 2nd year we divided our experimental units in half to examine whether the level of biochar addition mediated how willow plants responded to a late season fertilization treatment. We found that the shoots (stem material) in fertilized willow plants exhibited a non-significant positive response to biochar while the unfertilized willows actually exhibited a negative response, with the slopes of these lines differing significantly (Figure 6A, $p = 0.03$). A similar pattern of response was evident for roots, but differences between slopes

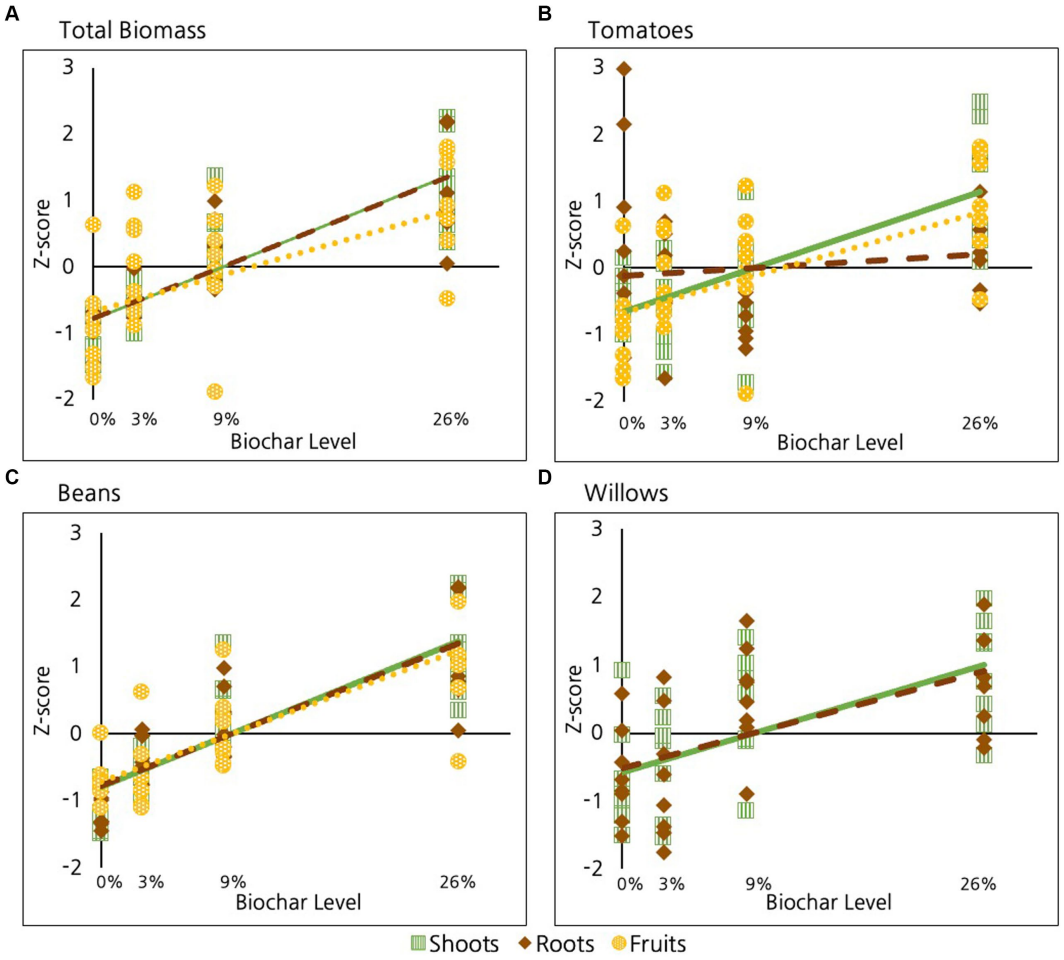

FIGURE 4
Effect of biochar on different plant tissues compared for: **(A)** All species, **(B)** Tomatoes, **(C)** Beans, and **(D)** Willows.

were only marginally significant (Figure 6B, $p = 0.1$). The pattern of a marginally significant decrease in growth with increasing biochar in unfertilized shoots, roots, leaf tissue, and total biomass was further supported when we used ANOVA to directly compare the 0% treatment with the 26% treatment for the unfertilized root tissue ($p = 0.06$), shoot tissue ($p = 0.06$), leaf tissue (Figure 6C, $p = 0.3$), and total biomass (Figure 6D, $p = 0.05$).

3.5 How does biochar affect seed development and first flowering?

As described in methods, we assessed germination rates for green bean seeds in the different treatments. We found increases in germination with increasing biochar with rates of 79, 88, 94 and 100% for the 0, 3, 9, and 26% treatments, respectively. More specifically, we found a significant linear relationship between biochar level and bean seed germination ($p = 0.003$), with an enhancement of 21% for the 26% biochar amendment level germination rate compared to the rate at the 0% amendment.

We also observed significantly earlier first flowering in bean plants with increasing soil biochar ($p < 10E\text{-}6$), with first flowering occurring 8 days earlier in the 26% level than the 0% level. In contrast to beans, in tomato plants we observed no significant impact of biochar addition on the date of first flowering ($p = 0.6$).

3.6 Does biochar affect soil properties?

As discussed, our primary goal was to assess the impact of biochar on plant fertility. A secondary goal was to assess whether biochar addition had a detectable impact on soil parameters and whether these impacts were mediated by the plants grown in that soil. Because this was a secondary goal, in this case we considered only two of the plant species and only the 0 and 26% biochar soils. More specifically, select properties of soil were compared in experimental units that included beans, tomatoes, and no plant treatment at the 0 and 26% biochar levels.

Table 3 reports on the percent difference in soil parameters to document increases in measured soil parameters that might be attributable to biochar addition. *P*-values resulting from pairwise comparisons among treatments are also included to indicate the significance of differences between 0 and 26% biochar level across soils used to grow tomatoes and beans at both 0 and 26% biochar levels. A dash stands for not significant in the table when *p*-values were greater than 0.1.

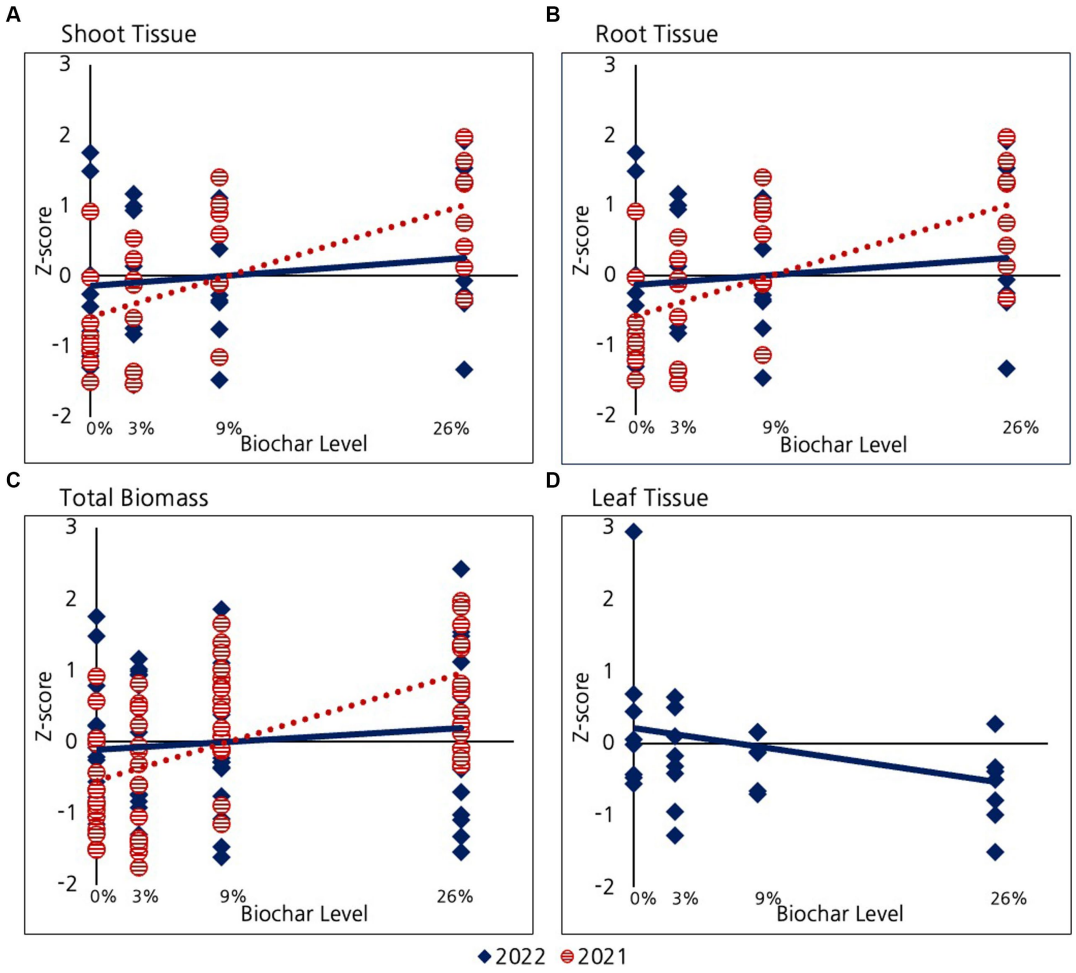

FIGURE 5
Comparison of the effect of biochar on Willows in their first and second growing season for: **(A)** Shoot tissue including only stems, **(B)** Root tissue, **(C)** Total biomass (shoot+root), and **(D)** Leaf tissue in 2022. Leaves were only successfully collected and measured in 2022.

The percent difference used to compare impacts on different soils was calculated as indicated below (Equation 3). Here we use cation exchange capacity (CEC) as an example:

$$\%\ difference = \frac{CEC\ at\ 26\% - CEC\ at\ 0\%}{CEC\ at\ 26\%} * 100 \qquad (3)$$

In Table 3, positive values in rows 1–4 indicates that the soil property was higher at 26% biochar compared to 0%. Negative values in these rows indicate that the soil property was lower at 26% biochar compared to 0% biochar. For rows 5–7, negative values indicate that the soil property was lower for tomatoes than beans. Values were only reported if significant. Although soils were analyzed for P, Ca, and Mg, no significant differences attributable to either biochar or plants were evident for these elements and so they are not reported in Table 3.

When the soil results were combined for beans, tomatoes, and the no plant controls to examine if there was a general trend in soil properties with the addition of biochar, it was found that CEC, percent base saturation, and Fe all exhibited significant differences between the two biochar levels. As is evident in the table, CEC, percent base saturation, and Fe all decreased between 0 and 26% biochar, while pH increased.

Next, we examined how plant species mediated the effects of biochar on soil properties. The no plant treatment was designed to serve as something of a control. In this control five soil properties, pH, percent base saturation, K, Na and B were significantly different between 0 and 26% biochar treatments (Table 3). Tomato plants appeared to slightly enhance the degree of difference in soil properties between the two biochar levels; when tomatoes were grown in the soil, significant differences between the two soil biochar levels were evident in seven soil properties including percent base saturation, organic matter, S, K, Na, B, and Mn. In contrast, beans appear to have decreased differences in soil properties between the two biochar levels; only B was different between 0 and 26% biochar. We compared the properties of the combined values for the 0 and 26% biochar amended soil grown with tomatoes against that grown with beans and found that there were differences between pH and percent base saturation (Table 3). We compared the tomato and bean soils at the 26% level to examine if biochar addition has different

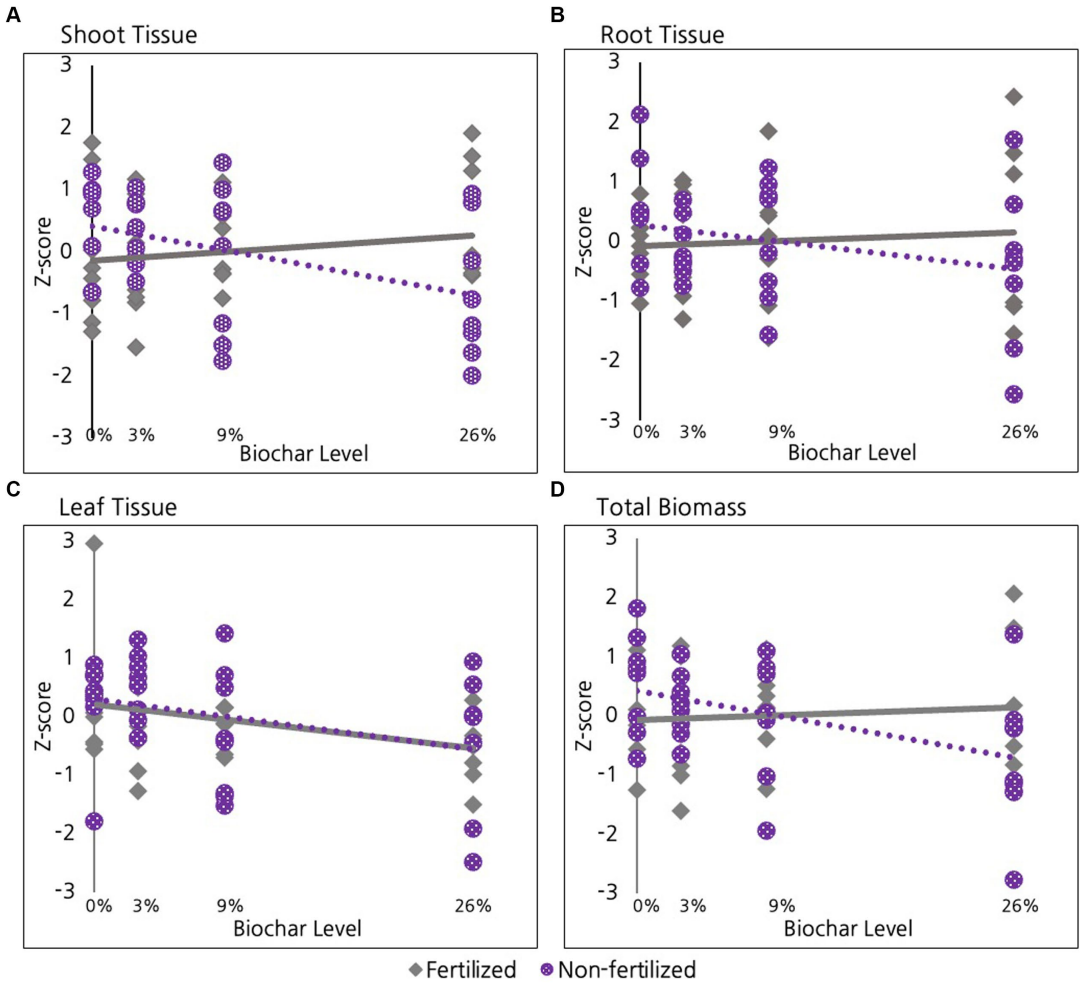

FIGURE 6
Effect of fertilization on Willows in their second growing season for **(A)** Shoot tissue and **(B)** Root tissue **(C)** Leaf tissue and **(D)** Total Biomass.

effects on soils grown with different species and observed a significant difference in S (Table 3). We then compared the two species' soils at the 0% level to examine if the species have an impact on soil properties independent of biochar addition and observed a significant difference in S (Table 3).

3.7 Is there a difference between low inoculant and high inoculant treatments?

As discussed, a concurrent experiment was conducted to assess whether multiple microbial inoculations mediated the impact of biochar on plant growth in the 9% biochar treatment. Repeated inoculation had a positive impact on beans. Compared to the low inoculation treatment, the high inoculation treatment yielded increases in bean biomass ($p = 0.008$), bean shoots ($p = 0.003$), and fruit production (Figure 7, $p = 0.07$). In tomatoes the impacts were less pronounced and in the reverse direction. Compared to the low inoculation treatment, the high inoculation treatment yielded marginally significant decreases in total biomass ($p = 0.09$) and root biomass ($p = 0.05$), with no differences evident in tomato production (Figure 7). No significant differences were found for root:shoot ratio or any willow biomass measures between the low and high inoculant treatments.

We also assessed whether different plants responded differently from each other to inoculation. We compared the results at the low inoculant level between species to examine if some species were affected more than others. We found that beans and tomatoes demonstrated significantly different responses in their biomass production under low inoculation in total biomass, root, shoot, and fruit biomass but in opposite directions. Beans had a moderately significant increase in the high inoculant treatment in fruit ($p = 0.07$), a significant increase in shoot ($p = 0.003$) and total biomass ($p = 0.02$) production. In contrast, the high inoculant tomatoes exhibited a marginally significant decrease in root production and in total biomass ($p = 0.09$). Among the root, fruit, and shoot tissues there were significantly different responses in root biomass ($p = 0.04$), moderately significant differences in fruit biomass ($p = 0.07$), and moderately significant differences in shoot biomass ($p = 0.06$). Comparisons between tomato and willows and willows and beans were not significantly different ($p > 0.1$).

TABLE 3 Effect of biochar addition on soil properties as well as effect of plant type on soil properties.

p values	CEC	pH	% base saturation	Org. Matter	S	K	Na	B	Fe	Mn
All plants (0% vs. 26% BC addition)	−23.5% ($p = 0.05$)	1.49% ($p < 10E-6$)	−13.0% ($p < 10E-6$)	–	−2.0% ($p = 0.09$)	–	–	−5.80% ($p = 0.1$)	−24.5% ($p = 0.01$)	–
Tomatoes (0% vs. 26% BC addition)	−42.7% ($p = 0.1$)	11.6% ($p = 0.03$)	−18.7% ($p = 0.04$)	7.14% ($p = 0.04$)	2.17% ($p = 0.002$)	23.7% ($p = 0.008$)	4.98% ($p = 0.003$)	28.3% ($p < 10E-7$)	−37.5% ($p = 0.08$)	−25.0% ($p = 0.01$)
Beans (0% vs. 26% BC addition)	–	–	–	–	–	–	–	−36.5% ($p = 0.04$)	–	–
No plant (0% vs. 26% BC addition)	–	8.49% ($p = 0.06$)	−8.13% ($p = 0.03$)	–	–	12.8% ($p = 0.03$)	20.6% ($p < 10E-4$)	10.7% ($p < 10E-6$)	–	–
Beans vs. Tomatoes (all BC levels)	–	−0.80% ($p = 0.01$)	1.57% ($p = 0.01$)	–	–	–	−18.9% ($p = 0.08$)	–	–	–
Beans vs. Tomatoes (26% BC)	–	–	–	–	−17.4% ($p = 0.005$)	–	–	–	–	–
Beans vs. Tomatoes (0% BC)	–	−5.31% ($p = 0.005$)	6.89% ($p = 0.05$)	−15.4% ($p = 0.06$)	−26.7% ($p = 0.003$)	−72.4% ($p = 0.07$)	−37.7% ($p = 0.04$)	−112% ($p < 10E-4$)	–	–

% difference is reported along with *p* values when there is a significant difference between biochar levels or plant type.

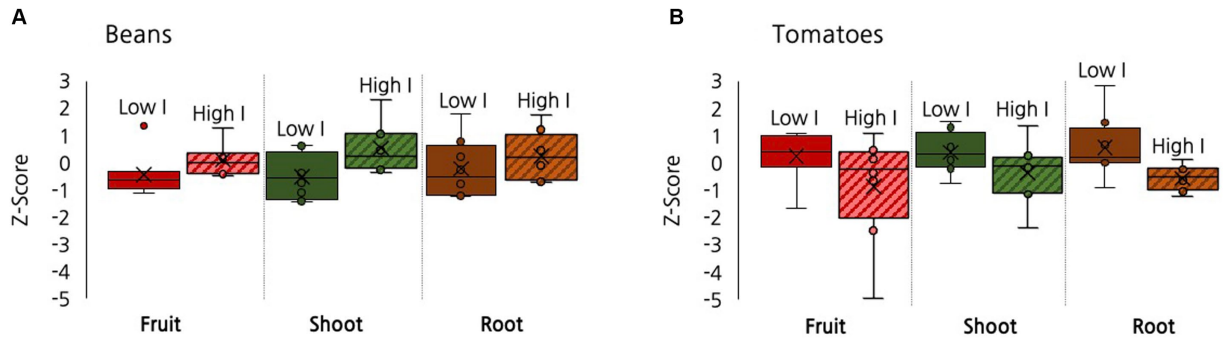

FIGURE 7
A box and whisker plot showing the effect of inoculation, low versus high, on root, shoot, and fruit production when comparing **(A)** Beans and **(B)** Tomatoes. All data is graphed using z-scores as points with an interval between −5 and 3. 'Low I' stands for low inoculation and 'High I' stands for high inoculation.

4 Discussion

Our experiment examined and compared the impact of adding different amounts of biochar to the soil for three functionally distinct plants. By using the same soil and biochar from a single source processed in the same manner, we were able to assess the impacts of biochar on different attributes of plant growth among plant types. Our results showed that biochar had a significant positive effect on root, shoot, and fruit biomass in all three species examined. We also documented improved bean germination and earlier date of first flower in green beans. Although all three species responded positively to biochar, we found important and significant differences in response among the growth parameters examined and among the species. This discussion is organized around addressing the questions posed in the introduction of this manuscript.

4.1 How do three functionally distinct plant species differ in their response to different levels of soil biochar?

Our results are consistent with numerous prior studies that that have documented a positive impact of biochar on total biomass in green beans (Melo et al., 2018), tomatoes (Ronga et al., 2020), and

willows (Saletnik and Puchalski, 2019), and inconsistent with the smaller number of studies documenting negative or null impacts (Velez et al., 2018). As with total biomass, we observed a significant positive effect of biochar on shoot growth in all three species. These results agree with past studies where others also found increases in shoot biomass in beans and tomatoes (Güereña et al., 2015; Ronga et al., 2020) and showed increases in other aboveground growth parameters, such as stem diameter and plant height in willows (Kuttner and Thomas, 2017).

Our experiment was distinct from prior studies in that it was explicitly designed to compare the impact of biochar among species and among plant tissues. In spite of the fact that we chose three functionally distinct species, we did not find a significant difference among these species in the strength of response of total biomass or stem biomass to biochar additions. Furthermore, in contrast to prior experiments (Rondon et al., 2007; Upadhyay et al., 2014; Fornes et al., 2017), we saw no evidence of a saturating impact of biochar, even at our highest treatment level of 26% biochar. These results suggest that, at least for the particular biochar, soil, and plant species examined, the benefits of biochar are robust.

While the pattern and strength of relationships between biochar addition and total plant biomass and stem biomass was similar among species, important differences were evident when we considered root tissues. Although null impacts of biochar on tomato root growth have been observed (Nzanza et al., 2012), the significant increase in root growth with biochar we observed in all three species is generally consistent with the findings of prior studies that we identified in our review of the literature (Güereña et al., 2015; Vaccari et al., 2015; Lebrun et al., 2017; Velli et al., 2021; Wan et al., 2023). What is distinct about our experimental design is that we were able to document differences in the strength of this relationship among the species examined. Specifically, we found that root tissue in beans and willows exhibited a significantly stronger positive response to biochar addition than did root tissue in tomatoes. Although prior studies have documented increases in root:shoot ratio with biochar application in green beans (Torres et al., 2020) and willows (Seehausen et al., 2017), we observed no significant impact of biochar on root:shoot ratio in beans and willows, and a significant decrease in root:shoot ratio for tomatoes with increasing levels of biochar.

Root production is critical to plants' capacity to access nutrients and water. One might therefore expect that differences in the root response among the species examined might lead to parallel differences in shoot and fruit production (Wan et al., 2023). This was not, however, the case in our experiment; as already stated, no differences were evident in biochar impacts on shoot production among the three species. Furthermore, no differences were evident in the impact of biochar on total harvested fruit biomass between beans and tomatoes. This lack of difference in shoot and fruit response occurred in spite of the significant decreases in root:shoot ratio of tomatoes with increasing biochar. This is interesting because it indicates that similar benefits of biochar addition on shoot and fruit production in tomatoes versus beans occur in spite of differences in the response of tomato root tissue. The design of our study does not allow us to directly assess physiological mechanisms. Nevertheless, our results suggest that biochar may enhance the capacity of tomato plants to access water and/or nutrients such that at high biochar levels tomato plants are able to access resources necessary to support elevated stem growth and fruit production with a relatively smaller root system. The fact that this same pattern of reduced relative root growth is not evident in beans and willows indicates that the physiological mechanisms associated with the benefits of biochar differ for these different species.

The goal of annual crop production is obviously to produce as much fruit as possible. Although null effects have been documented for soil biochar impact on tomatoes (Dunlop et al., 2015), most prior research reveals beneficial effects of soil biochar on fruit production in beans (Saxena et al., 2013; da Silva et al., 2017) and tomatoes (Ronga et al., 2020; Guo et al., 2021). Our results show that biochar addition had a significant positive impact on fruit production in both beans and tomatoes, but no significant difference in the magnitude of effect between these two plants. At least under our experimental conditions, biochar addition improves fruit yield, but does not affect total fruit production differently in these two species.

One possible explanation for enhanced fruit production in response to biochar would be if biochar resulted in increases in the ratio of fruit:(root+shoot). For example, one study demonstrated an increase in reproductive tissue of oak trees with biochar addition (Ohtsuka et al., 2021). However, we found no overall enhancement of fruit:(root+shoot) ratio with biochar addition in either beans or tomatoes. It appears that the positive impact of biochar on fruit production scales with the increases in overall plant biomass rather than resulting in disproportionate carbon allocation to fruit tissue.

In addition to documenting impacts of biochar on root, shoot and fruit production, we considered biochar impacts on germination in beans and flowering in both beans and tomatoes. Previous research has demonstrated an increased germination rate in green beans with biochar application (Saxena et al., 2013; Velez et al., 2018). Our results are consistent with these findings; we documented a linear increase in bean germination from 79% in the 0% biochar treatment up to a 100% germination rate in our 26% biochar treatment. These results are inconsistent with the findings of Murtaza et al. (2023) that higher rates of biochar application could restrict germination in a variety of plants. We did not identify prior research on the impacts of biochar on the timing of flowering in beans or tomatoes. We observed significantly earlier first flowering in bean plants with increasing soil biochar, with first flowering occurring 8 days earlier in the 26% level than the 0% level. In contrast to beans, in tomato plants we observed no significant impact of biochar addition on the date of first flowering. These results further contribute to our conclusion that while biochar exhibited similar overall positive impacts on the species examined in terms of total biomass and fruit biomass, there were also species-specific differences in the impact of biochar.

4.2 Does the response of willows to biochar change during a second season of growth?

A touted feature of biochar is that it does not decompose as rapidly as other organic soil amendments such as compost and thus could provide a longer term benefit to soil fertility as well as carbon sequestration potential (Kumar et al., 2022). There are two important considerations related to the long term impacts of biochar on fertility. One is the shifting impact of biochar as the material ages and changes in chemical, physical and ecological composition (for example through microbial colonization). The second is how plants rooted in

soil containing this biochar change in their response to biochar as the plants themselves mature through various growth stages.

With respect to the aging of the biochar itself, previous studies have shown that positive effects of biochar on soil fertility can either increase over time (Kätterer et al., 2019; Jiang et al., 2022; Wali et al., 2022) or decrease over time (Olszyk et al., 2020). We were not able to identify literature that documented changes in how plants respond to biochar as the plants themselves mature. With annual plants, such as the beans and tomatoes we examined, a full evaluation of the impact of biochar on all life phases can take place over a single growing season. Repeating an experiment such as ours in the same soil with multiple crops of the same species over multiple years would provide a valuable direct assessment of the impact of biochar aging on plant growth. Separating the impacts of biochar aging and shifts in response resulting from changes in plant life stage is more challenging for perennial plants. It is clear that for woody plants such as willows, the longer term impacts are an important consideration that requires multiple seasons to assess.

We conducted a two-year study of willows specifically because we wanted to assess whether response during a second year might differ from response during the first year. During the first year of growth, willows exhibited a highly significant positive response to biochar with respect to shoot growth (stems only), root growth and the combined total biomass of these two tissues (total biomass). Although the trend of positive response of roots, shoots, and total biomass to biochar remained in the second year of growth, the strength of this relationship decreased significantly from year one to year two and was no longer significantly different from zero for any of the variables examined in year two. Thus we observed a significant decrease in the positive impact of biochar between the first and second growth season in willows. Because of the design of our experiment, we are unable to determine whether this decrease in response might be appropriately attributed to aging of the biochar or changes in how the more mature willows were responding to this biochar.

While the decreased response of willows to biochar in year two is an evocative and potentially important finding, we note that three experimental conditions beyond the biochar treatment itself may also play a role in explaining this reduced response. First, it is possible that the stress of having their roots separated as they were transplanted into larger pots for the second year may have reduced the willow's response. Second, we did not apply microbial inoculation during the second growing season as we had in the first. Our inoculation experiment in the first growing season demonstrated that repeated inoculations had a significant impact on growth in biochar enhanced soil; it may be that additional inoculation in the second year would have enhanced the response to biochar. Third, during the second growing season we fertilized the willows late in July (i.e., middle to late in our growing season) when we noticed that willow leaves were yellowing. In spite of this late fertilization, we documented that willow shoots exhibited significant differences in their response to biochar addition in fertilized vs. fertilized treatments. Specifically, willow shoots in the fertilized group exhibited a significantly more positive response to biochar addition than those in the unfertilized group. This suggests that ample fertilization of all treatments in year one may have been an important factor determining the strong overall response that first year. So, it is possible that the reduction in response to biochar in year two is a result of nutrient limitation and not a change in response to biochar. Further research is definitely warranted to confirm whether the reduced impact of biochar on willow growth during a second season is reproducible as well as the role of microbial inoculation and fertilization in mediating plant response to biochar.

4.3 How does biochar affect the properties of an organic-rich soil and how is this mediated by plants and microbes?

Since our experiment focused on adding different amounts of biochar to soil, the dramatic and consistent positive relationships between biochar addition and plant growth and fertility parameters must be attributed to interactions that occurred within the soil itself. As discussed in our introduction, physicochemical benefits of soil biochar generally include enhanced CEC; increased base cation saturation; decreased bulk density; increased moisture retention; and increased pH (Zhang Y. F. et al., 2021). These direct effects are mediated and potentially augmented by enhanced habitat and resources for a beneficial soil microbial community (Zhu et al., 2017; Tan et al., 2022).

It is important to note that some of the general physicochemical benefits of biochar may have been less important in the soil we constructed for this experiment. For example, the shredded peat moss which made up 50% of our mix already resulted in a soil with a low bulk density, high water retention capacity, and high total cation exchange capacity, even in the absence of biochar. The 50% perlite in our mix ensured high porosity, good drainage and high moisture retention. Furthermore, the application of a time release fertilizer at the start of the experiment was intended to provide soil nutrients throughout the first growing season. We also applied two different sources of microbial inoculants three times over the course of the first growing season. The inoculant suppliers indicated that these contained multiple species of endo- and ecto-mycorrhizae designed to promote plant growth and phosphate solubilization. Thus we generated what we hoped would be a highly fertile organic-rich soil even in the absence of biochar addition.

The soil analysis we conducted must therefore be considered in light of the particular nature of our constructed soil. Some of the changes in soil chemistry resulting from the addition of biochar are as expected. For example, the fact that pH increased in our 26% biochar soils is consistent with the alkaline nature of biochar and most prior literature (Zhang Y. F. et al., 2021; Zhang M. et al., 2021). We were, however, surprised to find a decrease in cation exchange capacity CEC and reduction in percent base saturation in the 26% versus 0% biochar treatments. A partial explanation is that with the very high background CEC provided by the peat moss and high cation concentrations provided by fertilization, the addition of biochar may have made a relatively minor overall contribution to these soil attributes.

Since biochar is a type of organic matter, most studies find that additions of biochar result in an increase in soil carbon (Juriga and Šimanský, 2018). In our experiment we only documented a significant increase in organic matter with biochar addition in the tomato

treatment. This finding is likely also attributable to the fact that our experiment was conducted in a soil that was already dominated by organic matter in the form of the peat. We found no overall difference in the plant macronutrients P, Ca, Mg or K in 0 and 26% biochar levels. While significant differences were evident in certain elements among treatments, we believe that the use of a time release fertilizer generally overwhelmed most direct impacts of biochar on soil nutrients.

Of particular note in our soil analyses are the ways in which the different plant treatments affected the soils differently and mediated the impacts of biochar on the soil. For example, in the no plant control treatment five soil properties (pH, percent base saturation, K, Na and B) were significantly different between 0 and 26% biochar treatments. Tomato plants enhanced the degree of difference in soil properties between the two biochar levels; in soils supporting tomatoes, seven soil properties exhibited significant differences. In contrast, beans reduced differences in soil properties between 0 and 26% biochar with only a single soil variable showing differences at the two levels. Furthermore we identified significant differences in soil chemistry among plant treatments in both 0 and 26% biochar treatments. These findings are important because they indicate that the plants (or perhaps symbiotic microbes associated with these plant species) are strongly mediating soil chemistry in general as well as mediating the impact of biochar on the chemical properties of the soil.

Perhaps the most important finding related to analysis of soils in this experiment is that the significant beneficial impacts of biochar on plant growth and production can not be easily attributed to the differences in soil chemistry that we quantified. Indeed, one might expect that the decrease in CEC and percent base cation concentration we observed between the 0 and 26% biochar treatments would be associated with a decrease rather than the observed increase in plant fertility. In short, there is very little evidence that the overwhelmingly positive impacts of biochar on plant fertility observed in our experiment can be directly attributed to measured changes in soil chemistry. While it is possible that we did not measure some important direct physicochemical benefit of biochar, we think it is more reasonable to conclude that the benefits of biochar are attributable to their benefits on the microbial community.

The important role that microbes must have played in enhancing fertility is supported by the positive impact we observed in our side experiment on the impact of microbial inoculation. Previous studies have likewise found that inoculating biochar with microbes or compost improves fertility (Castro et al., 2018; Sani et al., 2020). We found that multiple inoculations enhanced shoot, fruit, and total biomass production in beans and root biomass in tomatoes. Taken together, our soil analysis and inoculation experiment both point toward the importance of microbial communities in mediating the impacts of biochar on plant fertility.

5 Conclusion and suggestions for future research

We documented significant positive impacts of increasing levels of soil biochar on root, fruit and shoot production in three distinct plant species. We further documented enhanced germination and earlier flowering in beans with increasing biochar. These findings contribute to, but are also largely consistent with prior research. The novel contribution of this research is an experimental design that allowed us to directly compare the response of plant tissues in three economically and functionally distinct plants by controlling for both soil type and biochar source. Using this approach we found that while overall impacts and effects sizes of biochar addition were generally similar among these three species, biochar addition had significantly different impacts on the different species and, in some cases, species-specific impacts on different plant tissues and other measures of fertility. The physiological and/or ecological mechanisms responsible for these differences warrant further study.

Some of our results are inconsistent with prior research. Differences may stem, in part, from the fact that our study was conducted in a highly organic soil (composed of 50% peat). For example, while numerous studies have documented inhibiting effects of biochar at high levels comparable to our 26% treatment, we documented positive effects even at this highest level. It may be that inhibitory effects are a function of the mineral content in soils. We also did not reproduce many of the enhanced physicochemical properties typically attributed to biochar such as enhanced CEC and enhanced base cation saturation. This again may be attributable to overlapping physicochemical benefits of biochar and fertilized soil that is already rich in organic matter.

In some ways, the lack of substantial differences in measured chemical properties in control soil, which contained 0% biochar, and our highest biochar treatment (26%) may serve to highlight other mechanisms by which biochar may enhance soil fertility. Specifically, the highly significant impacts of biochar observed in the absence of substantial differences in soil chemistry lead us to conclude that microbial interaction with biochar is the critical factor explaining the positive impacts of soil biochar on plant fertility in our experiment.

Our study was conducted in a highly organic, constructed soil with hazelnut wood biochar; further research that controls for soil type and biochar source is necessary to determine the extent to which our findings apply for other biochar sources in other soil types and for other species of plants. Based on our findings we suggest that future work should examine the impact of biochar on several variables that we did not measure including: stem versus leaf growth, nutritional value of fruit (sugars, nutrient concentration, antioxidant properties); microbial dynamics in the soil, changes in the chemistry of biochar and the microbial community that occur as biochar ages; changes in perennial species response to biochar at different life stages; interactions between fertilization and biochar. Future studies should also strive to better characterize the chemical properties of the biochar used as well as the microbial community present in the soil.

Data availability statement

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

Author contributions

SS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. TH: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JP: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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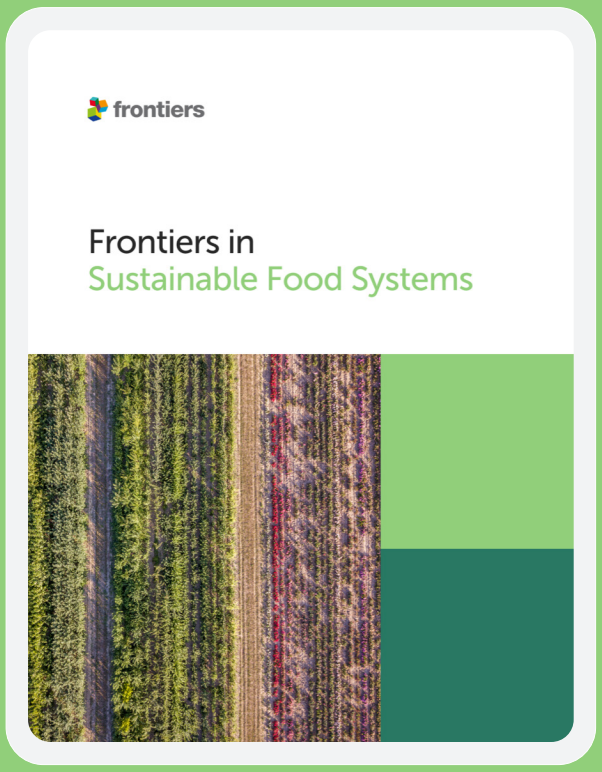

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