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Edited by
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Insights in microbe and virus interactions with plants: 2022

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Editorial: Insights in microbe and virus interactions with plants: 2022

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Editorial on the Research Topic
Insights in microbe and virus interactions with plants: 2022

Plants are important mediators of interactions between their associated microbial communities. Plant microbial interaction is empirical research that represents a wide range of molecular interactions between pathogens and plants. Plant associated microbes can enhance stress resistance through the induction of several mechanisms. The interaction between microbiomes and plants is beneficial as well as pathogenic. These microorganisms have a significant impact on plant production and health in natural contexts and are capable of forming intricate co-associations with plants. The development of novel sequencing technology for examining plant microbiomes enables potential opportunities for depth analysis of plant interactions under various management strategies (Islam et al., 2023).

Over the past 450 million years, microbes and plants have associated with each other and formed an assemblage of species known as holobiont. Plants cohabit with different groups of microbes including bacteria, fungi, and archaea, which form complex microbial consortia. Microbe-plant associations in soil occur by the mean of chemical-based interactions (Hassani et al., 2018). Plants influence their interaction with the microbes and soil by releasing rhizodeposits comprised of diverse compounds such as root caps, border cells, volatile organic compounds, soluble exudates, and lost carbon. Among all rhizodeposits, released root exudated modulate the composition of microbes around the plant roots, as they act as chemo-attractants. The plant-microbe interaction varies due to differential gene expression in the microbes (Farrar et al., 2014). Both plants and microbes tend to benefit from each other, for example, plants provide novel metabolites that could be used by the microbes as an energy source. On the other hand, microbes guard the plants from various biotic and abiotic factors of the environment through two different mechanisms i.e., direct, and indirect. Plants' growth and development have been known to be affected by various biotic factors such as pests, insects, and pathogens such as pathogenic bacteria, fungi, and viruses (Yadav et al., 2023b). Through various indirect mechanisms such as the production of antibiotics, lytic enzymes, siderophores, and hydrogen cyanide, microbes lower the concentration of ethylene through the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase shields the plant health and suppresses the growth of pests and pathogens (Yadav et al., 2023a). A lack of nutrient supply and abiotic factors such as drought, salt, and high/low temperature have also been found to deplete the growth of the plants. Microbes

alleviate this stress via direct plant growth mechanisms (Devi et al., 2022). Microbes alleviate the nutrients and abiotic stress by availing the soluble form through mechanisms such as nitrogen fixation, nutrients (zinc, potassium, and phosphorus) solubilization, siderophores production for the chelation of iron, ACC deaminase activity, activation of antioxidants, and the production of various phytohormones such as auxins, cytokinin and gibberellins (Kour et al., 2023; Islam et al., 2023).

This Research Topic includes high-quality research papers and review articles focused on new insights, novel developments, current challenges, latest discoveries, recent advances, and future perspectives in the field. The Research Topic contains six research and three review papers that cover different aspects of viral, bacterial, and fungal pathogens in relation to plant-microbe molecular interaction, plant colonization, detection, and disease control. Canfora et al. evaluated the impact of strains of Beauveria brongniartii and Beauveria bassiana on soil bacterial and fungal communities using an approach based on the terminal restriction fragment polymorphism (T-RFLP) analysis. The findings conclude that the application of the bioinocula induced only a transient and limited effect on the soil microbial community; even though some changes in the structure dynamic and frequency of soil bacterial and fungal OTUs emerged.

Cui et al. isolated and characterized *Priestia megaterium* KD7 for the biological control of pear fire blight. Fire blight, caused by the bacterium *Erwinia amylovora*, is one of the most serious plant diseases that mainly affects Rosaceae, such as apple, pear, medlar, and quince. Biological control with microbial antagonists is considered a powerful and eco-friendly alternative to controlling fire blight. The findings indicate that *P. megaterium* KD7 could be used as a potential source of a new biocontrol agent to control fire blight.

In another article, Xi et al. evaluated the microbiome diversity, composition, and assembly in a *California citrus* orchard. The environmental impact of agrochemical pesticides and fertilizers leads to changes in consumer behavior toward sustainably grown food and food products and as a result, farmers are increasingly relying on biological-based technologies and less on synthetic chemistries. Microbiomes have been shown to provide many benefits to plants by priming the immune system and protecting them from diseases, facilitating nutrient acquisition, and overall enhancing health and increasing yield. Data indicated that compartmentalization of microbiomes with distinct profiles occurs between above and below ground microbial communities. These findings highlight key microbial taxa that could be engineered as biopesticides and biofertilizers for citriculture. Interaction between the flagellum of *Candidatus* Liberibacter asiaticus and the vitellogenin-like protein of *Diaphorina citri* significantly influences CLas titer evaluated by Peng et al.. As a regulatory factor, *Vg_VWD* was upregulated in CLas-infected *D. citri* compared with uninfected ones, and the CLas titer increased significantly after *Vg_VWD* was silenced. The study provides a foundation for studying the roles that flaA and *Vg_VWD* may play together or separately in insect and plant hosts.

Deja-Sikora et al. explored the potential role of *Rhizophagus irregularis* and *Funneliformis mosseae* for the growth of *Solanum tuberosum* L. in a different way. This study indicated that multipartite interactions can take place in plant hosts inhabited by phytopathogens and endophytes. The application of AMF inoculum can reduce the economic losses caused by the virus. In the study of Duduk et al. the correlation between the occurrence of sugar beet RTD and the presence of root rot fungal pathogens in a semi-field "*Ca*. P. solani" transmission experiment with the cixiid vector *Reptalus quinquecostatus* (Dufour), in addition to naturally infected sugar beet in the open field. Zboralski and Filion summarized the effects of climate change-induced stresses on plants and detailed the mechanisms used by plant-beneficial *Pseudomonas* strains to alleviate them. Recommendations are made to promote targeted research on the stress-alleviating potential of these bacteria.

Another review by Kumar et al. on *Stenotrophomonas* in diversified cropping systems. The review discusses various plant growth and biocontrol attributes of the genus *Stenotrophomonas* in various food crops along with knowledge gaps. Additionally, the potential risks and challenges associated with the use of *Stenotrophomonas* in agriculture systems have also been discussed along with a call for further research in this area.

Al-Turki et al. summarize recent advances in PGPR-mediated resilience toward interactive effects of drought and salt stress in plants. The advancements made in the field of PGPR-mediated resilience through multi-omics approaches (*viz.*, genomics, transcriptomics, proteomics, and metabolomics) to unravel the intricate interactions between PGPR and plants have been discussed, including the molecular pathways involved in stress tolerance.

Research on plant microbes has greatly increased in the past few years. Integrated strategies such as multi-omics, engineering, theory, experimental biology, computational biology, and statistics offer quantitative insights into plant microbiome interactions (Islam et al., 2023). The importance of positive interactions between viruses and microbes in the plant has frequently been overlooked in plant breeding programmes, which have traditionally focused on examining the genetic variability of the crop for improved yield and stress tolerance. In the future, these integrated strategies will offer methods for evaluating as well as implementing the use of plant microbiome interaction to raise the productivity and sustainability of global agriculture.

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Conflict of interest

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The impact of *Beauveria* species bioinocula on the soil microbial community structure in organic strawberry plantations

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Introduction: The multifunctionality of microorganisms, including entomopathogenic fungi, represents a feature that could be exploited to support the development, marketing, and application of microbial-based products for plant protection. However, it is likely that this feature could affect the composition and dynamics of the resident soil microorganisms, possibly over a longer period. Therefore, the methodology utilized to evaluate such impact is critical for a reliable assessment. The present study was performed to evaluate the impact of strains of *Beauveria brongniartii* and *Beauveria bassiana* on soil bacterial and fungal communities using an approach based on the terminal restriction fragment polymorphism (T-RFLP) analysis.

Materials and methods: Soil samples in the vicinity of the root system were collected during a 3-year period, before and after the bioinocula application, in two organic strawberry plantations. Specific primers were used for the amplification of the bacterial 16S rRNA gene and the fungal ITS region of the ribosome.

Results and discussion: Data of the profile analysis from T-RFLP analysis were used to compare the operational taxonomic unit (OTU) occurrence and intensity in the inoculated soil with the uninoculated control. With regard to the impact on the bacterial community, both *Beauveria* species were not fully consistently affecting their composition across the seasons and fields tested. Nevertheless, some common patterns were pointed out in each field and, sometimes, also among them when considering the time elapsed from the bioinoculum application. The impact was even more inconsistent when

analyzing the fungal community. It is thus concluded that the application of the bioinocula induced only a transient and limited effect on the soil microbial community, even though some changes in the structure dynamic and frequency of soil bacterial and fungal OTUs emerged.

KEYWORDS

***Beauveria bassiana*, *Beauveria brongniartii*, soil bacteria community, soil fungal community, T-RFLP**

1. Introduction

Biological control is an essential component of arthropod pest management in both organic and conventional cropping systems (Shah and Pell, 2003; Zehnder et al., 2007), and among the several studied organisms, entomopathogenic fungi (EPFs) are frequently considered for the control of soil pests (Butt, 2002). The evolution of policies worldwide aiming at the reduction of the use of synthetic pesticides has thus favored the development of plant protection products based on microorganisms, including EPF (de Faria and Wraight, 2007). However, the introduction of a bioinoculant to agricultural ecosystems can raise questions about its impact on non-target organisms. To address this, the European Union updated the guidelines to assess the risks of potential effects on non-target organisms during the registration process of biological pesticides (European Commission, 2013), being these organisms either closely related to the target species or being especially exposed, as it could be the case for soil native microbial populations.

On the other hand, the multifunctionality of microorganisms, including EPF-based bioinocula, represents an aspect that could further support the development, marketing, and application of microbial-based products (Harman, 2011; Kowalska et al., 2020). This has been recently shown particularly for EPF able to conduct also an endophytic development: acting as a plant-growth promoter (Raya–Díaz et al., 2017; Tall and Meyling, 2018) or supporting the control of soil-borne pathogens (Jaber, 2018). It is thus likely that all these functions could affect the composition and dynamics of the resident soil microorganisms, possibly over a long period. However, the methodology utilized to evaluate the impact of microbial inoculants on soil microbial community composition is a key to a reliable assessment (Canfora et al., 2014, 2016; Manfredini et al., 2021), due to the complexity of the soil environment (Nannipieri et al., 2017).

In the past few years, we have endeavored an effort to address the control of white grubs of *Melolontha* spp. in a region with a high incidence of this pest (Malusá et al., 2020). The application of EPF belonging to the species *Beauveria bassiana* (Balsamo -Crivelli) Vuill. (Ascomycota: Hypocreales) and *Beauveria brongniartii* (Saccardo) Petch is considered a suitable and effective method to achieve this goal (Zimmermann, 2007). Tartanus et al. (2021) reported the results of a study evaluating the behavior of these species in organic strawberry plantations in relation to the environmental conditions, their abundance after soil inoculation, and their impact on soil microbial communities carried out with a classical microbiological method. The present study was performed to evaluate the impact of the two *Beauveria* strains on soil bacterial and fungal communities using an approach based on terminal restriction fragment polymorphism (T-RFLP) analysis.

2. Materials and methods

2.1. Field trials

In total, two trials were carried out on strawberry plantations, managed according to the organic farming practices, located in the territory of Lubartów district (Lublin voivodeship, South-Eastern Poland), namely, at Brzostówka (51.4365°N, 22.7856°E) (following BRZ) and Nowa Wola (51.4177°N, 22.7238°E) (following NOW). Strawberry plants were planted in springtime (NOW, cv. Polka) or late summer (BRZ, cv. Senga Sengana) in 2014 in commercial fields for a typical 3-year crop. Both sites had a similar soil texture (sandy loam), classified as podsolic, but different pH values (5.3 and 6.8, for BRZ and NOW, respectively), salinity (0.7 and 1.0 dS m-1 for BRZ and NOW, respectively), and organic matter content (1.06 and 1.19% on dry soil weight for BRZ and NOW, respectively).

Both fields (about 1 ha) were highly infested by *Melolontha* spp. larvae, as determined by an initial assessment of their presence made by counting live grubs before planting the strawberries (on average, 2 larvae m^{-2} were found, 4-fold the acceptable damage threshold, i.e., 0.5 larvae m^{-2}). The assessment was performed by collecting the soil samples from 25 cm × 25 cm × 30 cm (w:l:d) wells and checking for the presence of the grubs (minimum 8 holes from each repetition).

A randomized block design with four replicates (for a total of about 1,500 m^2 per treatment) was established for the following treatments in both trials:

1) A *Beauveria bassiana* (Balsamo -Crivelli) Vuill. (Ascomycota: Hypocreales) strain (BB59, hereafter Ba) isolated from rhizospheric soil of an apple orchard

located in Valle d'Aosta by the company CCS Aosta, (Aosta, Italy), which genomic sequence of ITS region of the ribosome has been deposited in the GenBank database and can be accessed to ID KT932307 (see below).

2) A *Beauveria brongniartii* (Saccardo) Petch strain (hereafter Br) isolated from the soil of a potato field highly infested by *Melolontha melolontha* in Romanów locality (Lublin voivodeship, Eastern Poland). The strain is deposited in the Fungal Collection of the Department of Horticulture and Plant Protection, Siedlce University of Natural Sciences and Humanities. The sequence of the ITS region of the ribosome has been deposited in the GenBank database and can be accessed to ID KT932309.

Control plots did not receive the bioinocula. The strains have not been tested before in the laboratory to assess their virulence in comparison with other strains. However, pot experiments carried out under controlled conditions confirmed that both were able to infect *M. melolontha* larvae (unpublished data). The *B. bassiana* bioinoculum was prepared by growing the fungus in a liquid medium based on the malt extract and glucose and formulated as a wettable powder into a carrier material made of a mixture of corn fibers and zeolite (1:10 w-w). *B. brongniartii* was grown on a solid substrate (barley kernels). The concentration of each of the two fungi in the inoculum was about $1{\cdot}10^7$ spores$\cdot g^{-1}$. The colonized kernels were used as a carrier for application. *B. bassiana* was applied as an aqueous suspension, and to reduce the risk of damaging the fungal cells, a fan-less sprayer with large diameter nozzles was used to apply the equivalent of about 2,000 l$\cdot$ha^{-1} of the bioinoculum water suspension. The applications were carried out near the plants' rows. After each application, the soil was mixed on the surface with light hand hoeing.

Each treatment consisted of a dose of 45 kg$\cdot$ha^{-1} applied to the soil. In the case of the trial NOW, the dose was split into four applications in the first year (starting at planting on 20 May 2014), with monthly intervals. For the trial BRZ, the dose was split into two applications with a 3-week interval in the first year (starting at planting on 30 July 2014). For both trials, a single application was performed for the following 2 years (mid-July and mid-May in 2015 and 2016, respectively).

2.2. Soil sampling and DNA extraction

Soil samples were collected as follows: mid-September 2014 (i.e., about 16 and 4 weeks after application for NOW and BRZ, respectively), mid-June and mid-September 2015 (i.e., before the second application and about 8 weeks after it), and mid-May and end of July 2016 (i.e., before the third application and about 8 weeks after it). The soil samples were collected from the vicinity of the plant's root system, with an Egner's sampler from a depth of 0–20 cm from about 25 points randomly distributed on each of the four plots for every treatment. These individual samples were merged to compose a laboratory sample (approximate weight of 1–1.5 kg).

DNA was extracted from 0.6 g of soil using the DNeasy PowerSoil® DNA Isolation Kit (Qiagen Inc., Chatsworth, LA, USA) following the manufacturer's instructions. DNA crude extract yields were calculated using Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), following the manufacturer's instructions. DNA extraction was repeated in duplicates, and then, the DNA solutions were pooled. The extracted DNA was diluted to 10 ng μL^{-1} and stored at −20°C for the following analytical steps.

2.3. T-RFLP analysis of soil microbial community

Primers 63f and 1087r with a dye label VIC™ (Victoria, developed after the modifications of Aequorea victoria Green Fluorescent Protein) on the 5′ were used for the amplification of the bacterial 16S rRNA gene (Canfora et al., 2015). The operons ITS1 and ITS4, labeled with 6-FAM™ (6-carboxyfluorescein) dye, were used for the amplification of the fungal ITS region of the ribosome (primers sequence details are provided in **Supplementary Table 1**). PCRs were repeated in triplicate for each sample and were performed in a 30 μL volume with 50 ng of template DNA and 0.2 U of Taq Phusion hot start Taq DNA Polymerase (Platinum, Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR (for bacteria and fungi) was performed under the following conditions: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; the process was completed with a final extension step of 10 min at 72°C. The PCR products were separated on a 1.5% agarose.

The amplified products were purified with a Qiaquick PCR Purification Kit (Qiagen Inc., Chatsworth, LA, USA); after quantification with Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 600 ng of amplified 16S rDNA was digested for 5 h with 20 U of *Taq*I and *Alu*I (Promega, Madisson, WI, USA) at 37°C and 65°C, respectively. For the ITS region of the fungal DNA, 600 ng of the amplified product was digested with 20 U of *Hin*fI and *Hae*III (Promega, Madisson, WI, USA) for 5 h at 37°C. A 50 ng aliquot of the digested products was resolved by the capillary electrophoresis on an ABI3500 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) using LIZ600 as the size standard for GeneScan analysis. Fragment sizes from 55 to 500 bp were considered for profile analysis and determination of the operational taxonomic unit (OTU) numbers.

2.4. Data treatment and statistical analyses

Data obtained from T-RFLP analysis were used to compare the soil inoculated with *B. bassiana* and *B. brongniartii* with the uninoculated control. A derivative profile was created by the comparison of T-RFLP profiles from each restriction enzyme from duplicate DNA samples following the method reported in the study of Canfora et al. (2017). The quality of T-RFLP data was checked by GeneMarker software (SoftGenetics, LLC, State College, PA, USA). Each peak on the T-RFLP profiles is thought to correspond to a certain anonymous taxon referred to as an operational taxonomic unit (OTU), while the area of the peak is thought to correspond to the proportion of this OTU in the microbial community. Only fragments with fluorescence intensity of ≥55 arbitrary fluorescence units were considered. Alignment of the profiles was performed directly on the output table of the software GeneMarker, considering a range of ± 0.5 bp to discriminate peaks of consecutive sizes. The derivatives of terminal restriction fragment (T-RF) profiles of the different enzymes were combined and transformed into a binary vector, in which the intensity area of peaks was scored as strings, to be used for the analysis. The number of peaks was counted in each sample across the treatments. A heatmap was created on the retrieved number of peaks with a frequency of ≥3, using XLSTAT software 2020.2.3 (Addinsoft, 2020). The map included TRFs with at least one fragment present per sample, thus displaying the common fragments and highlighting the differences in terms of frequency (the presence or the absence of TRFs), as well as in terms of "intensities" (area of the peak). Considering that an error of ± 0.5 bp in discriminate peaks could occur when reading the bands, particularly comparing runs from different sampling periods, we assumed that peaks with ± 0.5 bp of difference correspond to the same OTU (i.e., 135.1 = 135.2 = 134.8; 139.1 = 139.2; 71.2 = 71.7).

3. Results

The T-RFLP data were analyzed based on the assumption that the T-RF number (i.e., each peak in the T-RFLP profiles) represents a different species/strain identified as an OTU. Indeed, while the Shannon H index can evaluate the OTU diversity, its identity remains unknown. Therefore, T-RFLP data were analyzed by assessing the presence/absence of each OTU (peak) and its intensity (as semiquantitative data) for both sites, displaying the occurrence of the common OTU (T-RF with at least one peak per treatment in each sample period) on heatmaps. To better appraise the stringency of the approach followed in the data elaboration, the total number of OTUs identified in the samples is also provided in the relevant tables with the number of OTUs utilized for the assessment.

3.1. Impact of inoculated species on soil bacterial community

In the samples collected in 2014 (after application), the number of bacteria OTUs present in ≥1 treatment samples was 22 in BRZ and 25 in NOW (**Figures 1A**, **2A**). In total, 15 of them were common to both sites. The application of the two bioinocula induced a different response in terms of OTU intensity in the two sites: a general reduction of the intensity was observed in the samples from BRZ (for the 62 and 48% of the OTUs in the case of *B. brongniartii* and *B. bassiana*, respectively), while a general increase was observed in NOW (for the 72 and 48% of the OTUs in case of *B. brongniartii* and *B. bassiana*, respectively) (**Table 1**). Among the 15 OTUs common to both sites, only two of them responded similarly to the application of inocula in both sites (OTUs 71.7 and 144, with reduced intensity) while all others responded differently (either being enhanced or reduced after the application compared to the control) (**Figures 1A**, **2A** and **Table 1**).

In 2015, a total of 16 and 12 OTUs were identified during the first and second sampling, respectively, in NOW, with nine of them common to both sampling times (**Figures 1B, C**). Both inocula induced a general reduction in the intensity of OTUs in the first sampling time: in 75% of OTUs in the case of *B. brongniartii* and 44% of OTUs in the case of *B. bassiana* (**Table 1**). The fall samples, different from the previous ones, showed no impact of *B. brongniartii* inoculation on OTU intensity, while *B. bassiana* induced an increase in the intensity of several OTUs compared to the control (**Figure 1C** and **Table 1**). Concerning the BRZ field, 13 and 11 OTUs were identified in the 2015 first and second sampling time, respectively, eight of which were common to both periods (**Figures 2B, C**). In the spring samples, the presence of *B. bassiana* induced a general reduction of the OTU intensity (on 62% of OTUs), while *B. brongniartii* resulted to induce a similar share among the three categories of changes (**Table 1** and **Figure 2B**). In the fall samples, both inocula induced a reduction in the intensity in the majority of OTUs (64 and 46% for *B. brongniartii* and *B. bassiana*, respectively) with only few of them having unchanged or increased intensity (**Figure 2C** and **Table 1**).

In 2016, 19 OTUs were defined in May and 9 in July soil samples from NOW, three of which were common to both periods (**Figures 1D, E**). *B. brongniartii* induced an increased intensity in the majority of them in both sampling times, while *B. bassiana* affected the OTU intensity only to a limited extent, having the different categories of the changes a quite similar share in both sampling periods (**Table 1** and **Figures 1D, E**). The samples from BRZ were characterized by 13 and 6 OTUs, respectively, for the first and second sampling times, none of them common to both periods (**Figures 2D, E**). The presence of *B. brongniartii* induced an increase of more than half or 67% of OTUs in the first and second sampling times,

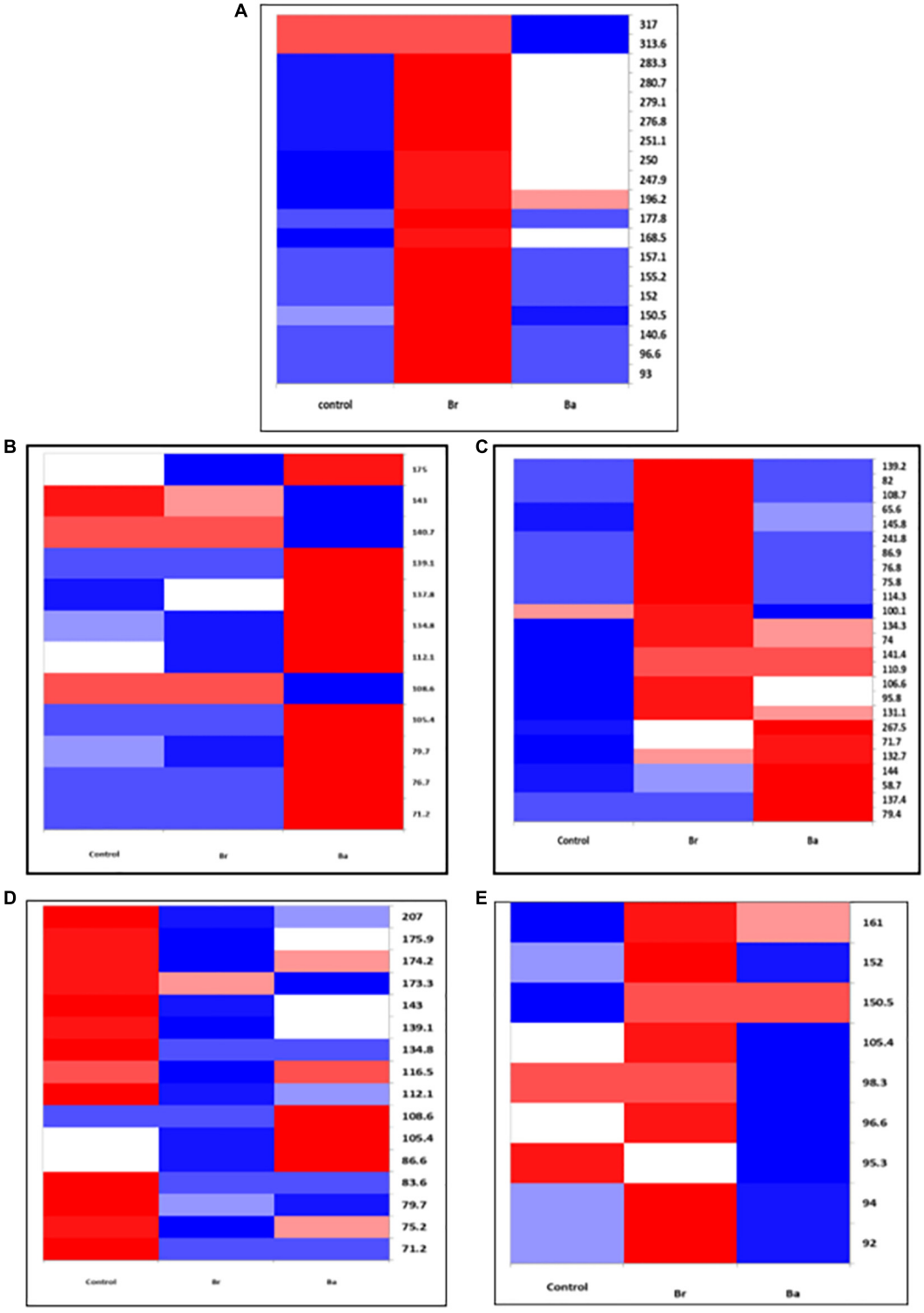

FIGURE 1
Heatmap displaying the occurrences of the common bacterial operational taxonomic units (OTUs) identified, as T-RFs, in the soils treated with *Beauveria brongniartii* (Br) and *Beauveria bassiana* (Ba) bioinocula and untreated (Control) in the field located at Nowa Wola (NOW) as sampled during the 3 years. The color represents the OTU intensity: Blue to red, min to max. The absence of OTU is displayed as white line. The intensity of the color represents the percentage of occurrence in the treatment. Samples were collected in **(A)** September 2014, **(B)** June 2015, **(C)** September 2015, **(D)** May 2016, and **(E)** July 2016.

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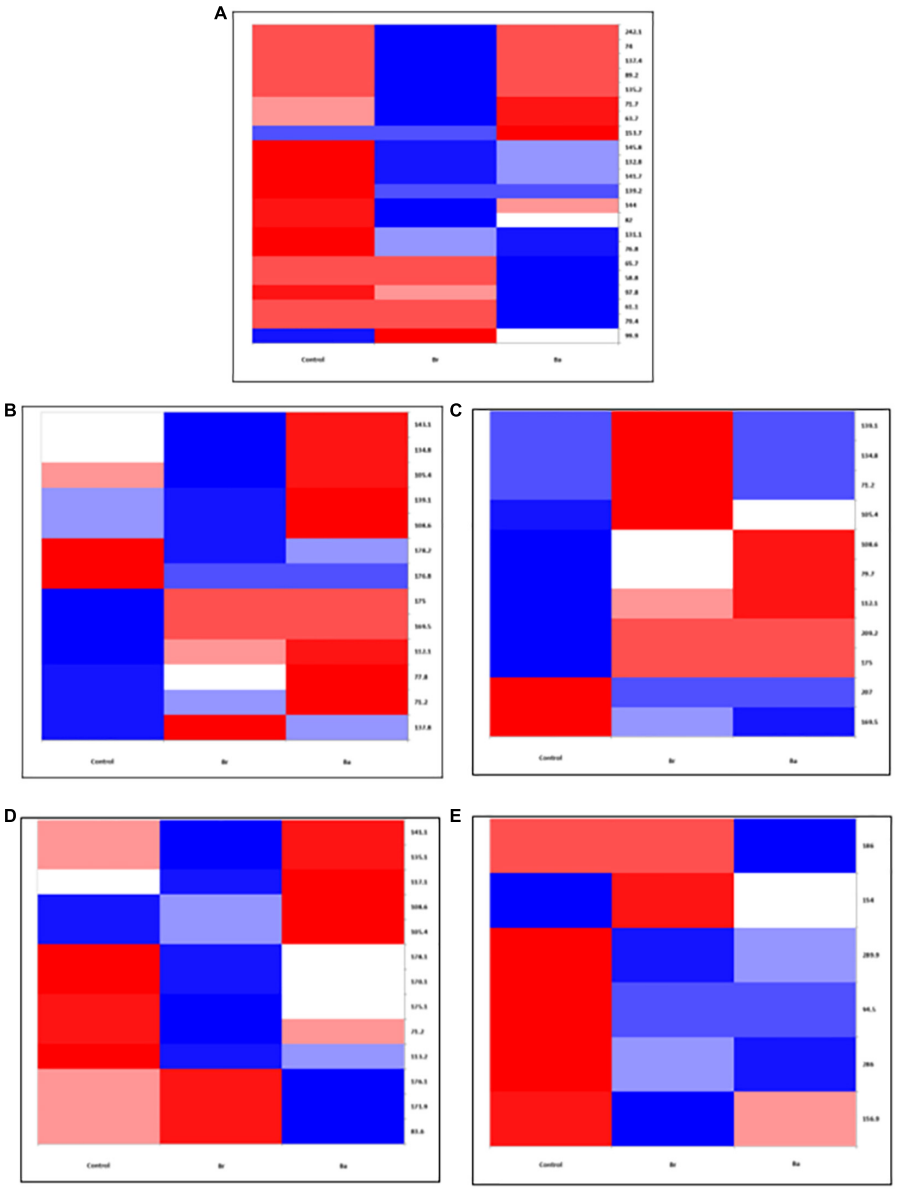

FIGURE 2
Heatmap displaying the occurrences of the common bacterial operational taxonomic units (OTUs) identified, as T-RFs, in the soils treated with *Beauveria brongniartii* (Br) and *Beauveria bassiana* (Ba) bioinocula and untreated (Control) in the field located at Brzostówka (BRZ) as sampled during the 3 years. The color represents the OTU intensity: Blue to red, min to max. The absence of OTU is displayed as white line. The intensity of the color represents the percentage of occurrence in the treatments. Samples were collected in **(A)** September 2014, **(B)** June 2015, **(C)** September 2015, **(D)** May 2016, and **(E)** July 2016.

respectively. *B. bassiana*, instead, induced a relevant increase of OTU intensity only in the second sampling period, while not affecting them during the first sampling (Table 1 and Figures 2D, E).

Analyzing the impact across the seasons, the bioinocula resulted to induce a change in the intensity of the OTUs only few months after the application (i.e., in the T2 samples of 2015 and 2016) (Table 1). This tended to follow an increasing trend across the seasons, particularly in terms of the number of OTUs affected in the last sampling period. However, very few OTUs were consistently observed across the seasons in each field for at least three sampling periods (Table 1). Among them, five were common to both sites (OTUs 71.2, 105.4, 108.6, 139.1, and 175). However, their intensity in relation to the application of the two bioinocula was not consistently affected by them. In general, few OTUs were also not found or additionally present in the treated plots compared to the control in both sites during the different sampling periods.

TABLE 1 Percentage of bacterial operational taxonomic units (OTUs) categorized according to the comparison of intensity with the control.

BRZ																												
	2014						2015												2016									
	T1						T1						T2						T1						T2			
	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total
Br	33	62	5	0	10	21 (47)	23	31	23	8	15	13 (30)	0	64	18	18	0	11 (43)	39	0	54	0	8	13 (91)	17	17	67	0	0	6 (72)
Ba	38	48	5	10	10		8	62	15	0	15		27	46	18	9	0		23	15	31	23	8		17	0	67	17	0	
NOW																												
	2014						2015												2016									
	T1						T1						T2						T1						T2			
	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total
Br	20	0	72	8	0	25 (57)	13	75	0	0	13	16 (48)	75	0	0	8	17	12 (42)	11	0	90	0	0	19 (98)	11	0	56	11	22	9 (90)
Ba	40	4	48	8	0		19	44	6	19	13		0	25	58	0	17		42	11	5	42	0		33	22	22	0	22	

The column "Total" indicates the total number of OTUs selected for the impact assessment according to the criteria specified in the text and, in brackets, the overall number of OTUs identified with terminal restriction fragment polymorphism (T-RFLP) analysis.

3.2. Impact of inoculated species on soil fungal community

The samples collected in 2014 resulted to have 12 OTUs in BRZ and 17 in NOW (Figures 3A, 4A). In total, seven OTUs were common to both sites, and four of them responded similarly in terms of intensity to the application of the two inocula in both sites compared to the control. Considering the impact of each inoculum in comparison with the control, at both sites, the application of *B. brongniartii* induced limited changes, with a similar share among the three possible modifications (Table 2). On the other hand, the application of *B. bassiana* induced a higher intensity on the majority of OTUs in NOW, while in BRZ, the impact was on a lower number of OTUs, balanced among unchanged and changed (either increased or decreased).

In 2015, the samples collected from the NOW site contained 27 and 26 OTUs in the first and second sampling times, respectively (Figures 3B, C), while those from the BRZ site resulted to contain 12 and 22 OTUs (Figures 4B, C). In NOW, twelve OTUs were common in both sampling times, while eight of them were common in the case of BRZ. However, though in BRZ, three of these common OTUs resulted to respond similarly to the application of both inocula in terms of intensity changes compared to the control in both sampling periods, in NOW, any OTU resulted to modify consistently the intensity for the sampling periods concerned (Figures 3, 4). The impact of the inocula on the intensity of the OTUs was different depending on the site and sampling period (Table 2). In NOW, *B. bassiana* induced a reduction to more than half OTUs in the first sampling, while in the second, the modifications were equivalent in share. An opposite pattern was instead induced by *B. brongniartii*, with a common percentage of the different modifications during the first sampling and an enhancement of the increased intensity of OTUs in the second sampling period. In BRZ, *B. bassiana* induced a diverging impact on the OTU intensity when comparing the first (higher share of increased intensity–58%) and second (higher share of decreased intensity–59%) sampling periods (Table 2). Considering *B. brongniartii*, its application did not modify the intensity for the majority of OTUs compared to the control (42%) during the first sampling period, while inducing a decrease in the intensity of the majority of OTUs (59%) in the second sampling period (Table 2). In 2016, we were unable to observe fungi OTUs in the samples collected by applying the criterium of finding at least one T-RF per sample and avoiding rare T-RFs. Only three OTUs were consistently detected across the sampling periods and only in the NOW field: 265.1, 219.2, and 253.4 (Figure 3). However, the impact of the two bioinocula on their intensity was inconsistent.

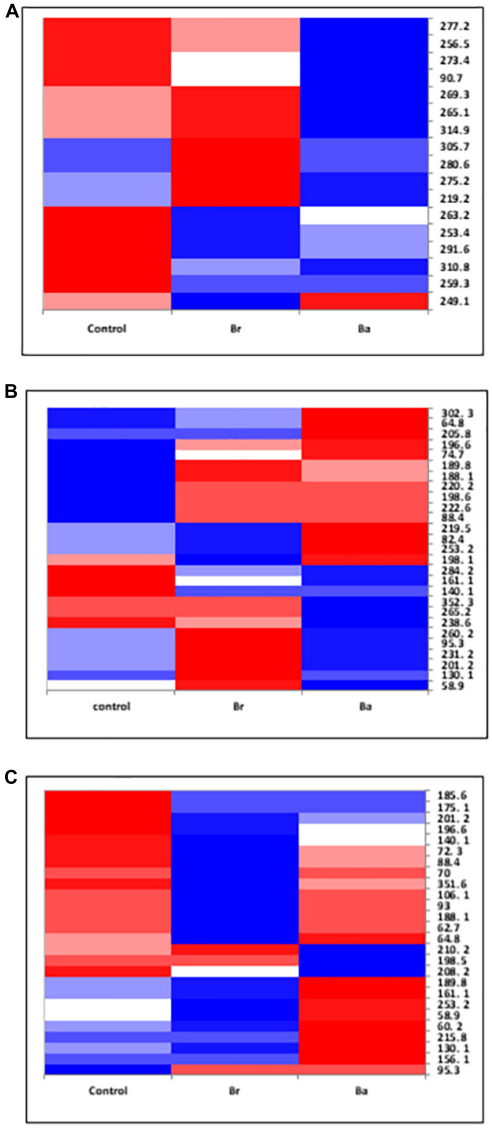

FIGURE 3
Heatmap displaying the occurrences of the common fungal operational taxonomic units (OTUs) identified, as T-RFs, in the soils treated with *Beauveria brongniartii* (Br) and *Beauveria bassiana* (Ba) bioinocula and untreated (Control) in the field located at Nowa Wola (NOW) as sampled during the 3 years. The color represents the OTU intensity: Blue to red, min to max. The absence of OTU is displayed as white line. The intensity of the color represents the percentage of occurrence in the treatments. Samples were collected in **(A)** September 2014, **(B)** June 2015, and **(C)** September 2015.

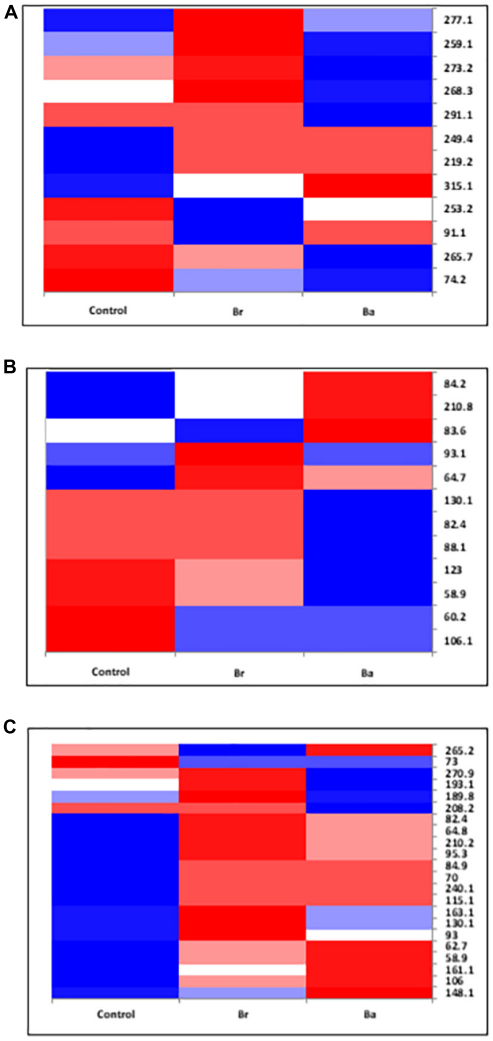

FIGURE 4
Heatmap displaying the occurrences of the common fungal operational taxonomic units (OTUs) identified, as T-RFs, in the soils treated with *Beauveria brongniartii* (Br) and *Beauveria bassiana* (Ba) bioinocula and untreated (Control) in the field located at Brzostówka (BRZ) as sampled during the 3 years. The color represents the OTU intensity: Blue to red, min to max. The absence of OTU is displayed as white line. The intensity of the color represents the percentage of occurrence in the treatments. Samples were collected in **(A)** September 2014, **(B)** June 2015, and **(C)** September 2015.

4. Discussion

4.1. Impact of entomopathogenic fungi inoculants on soil microbial communities

In applying the T-RFLP approach to soil inoculated with *B. bassiana* and *B. brongniartii*, we considered it useful for the analysis when at least one T-RF per sample was observed, indicating the presence of the target gene in the investigated samples, while we did not include rare T-RFs occurring in few specific samples (data not shown). This approach resulted in the lack of fungal OTUs from the samples collected in 2016. However, it is believed that the stringent criterium adopted in the study reduced the risk of a biased analysis and evaluation of the bioinocula impact on the microbial communities, which was possible for the other sampling periods and always for the bacteria community.

TABLE 2 Percentage of fungal operational taxonomic units (OTUs) categorized according to the comparison of intensity with the control.

BRZ																		
	2014						2015											
	T1						T1						T2					
	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total
Br	25	33	25	8	8	12 (50)	42	17	17	17	8	12 (203)	9	73	9	5	5	22 (266)
Ba	25	25	33	8	8		8	25	58	0	8		14	59	18	5	5	
NOW																		
	2014						2015											
	T1						T1						T2					
	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total	Unchanged	Reduced	Increased	Missing	Additional	Total
Br	29	24	35	12	6	17 (60)	33	30	26	7	4	27 (265)	31	4	54	8	4	26 (405)
Ba	29	0	65	6	0		22	52	22	0	4		35	27	23	8	8	

The column "Total" indicates the total number of OTUs selected for the impact assessment according to the criteria specified in the text and, in brackets, the overall number of OTUs identified with terminal restriction fragment polymorphism (T-RFLP) analysis.

The presence of discriminant bands (OTU) could be associated with a putative species that may be characteristic of a certain soil sample. In a previous study (Tartanus et al., 2021), the digestion of the amplicons obtained from the soils treated with *B. bassiana* and *B. brongniartii* inocula resulted in different numbers of T-RFs for bacteria and fungi, ranging from 6 to 80 T-RFs, pointing to a wide genetic diversity of bacteria and fungi in these soils. Overall, the applied strains affected, to a different extent, the soil bacterial and fungal communities of the two investigated sites, which are characterized by different soil physical-chemical conditions, highlighting that the inoculative effect may depend also on the resident microbial community encountered by the inoculant and, consequently, by the interaction between it and the native community (Malusà et al., 2021). We have thus further analyzed the OTU data in an effort of identifying the possible effects of the bioinocula on the soil autochthonous microbiome with an approach that could be useful also to support other methods (e.g., NGS) to define the impact of bioinocula on the environment (Deising et al., 2017; Mawarda et al., 2020). Indeed, studies assessing the effects of applied microorganisms, particularly entomopathogenic fungi, on soil microbial communities have revealed only small or transient effects (Trabelsi and Mhamdi, 2013; Kröber et al., 2014; Zimmermann et al., 2016). Nevertheless, Mawarda et al. (2020) reported that 30% of studies using fingerprinting-based methods (e.g., TRFLP, DGGE, and TGGE) did not show any consistent effect of inoculation, pointing to a likely methodological limitation. In the case of EPF, the potential effects on the soil microbiome after the application were found to vary (Hu and St. Leger, 2002; Schwarzenbach et al., 2009; Hirsch et al., 2013; Mayerhofer et al., 2017, 2019). The results of this study point to similar conclusions, as the modifications observed in the OTU number and intensity were generally of limited effect, not consistent in the two studied fields and across the seasons considered. However, it is noteworthy to consider these results also in relation to the timing of bioinocula application and soil sampling and the soil characteristics of the fields, which are factors that could impact the potential effect of the bioinocula as well as their interactions with the native microbiome.

4.2. Impact of entomopathogenic fungi inoculants on soil bacteria community

The application of the bioinocula modified to a low extent the composition of bacterial communities after each application, as only few new or missing OTUs (about 1/10 of the OTUs identified) were detected in the inoculated soil (i.e., were triggered or not in inoculated soil) compared to the control across the whole sampling period. However, even though probably influenced by other factors, the inoculation affected the intensity of the bacterial OTUs, i.e., their quantitative contribution to the total bacteria community. The impact of

both bioinocula in this respect could not be considered fully consistent across the seasons (sampling periods) and fields. However, considering the time passed since the application, some common patterns were highlighted in each field and sometimes also among them, showing that a certain impact could be attributed to the bioinocula. In 2016, the intensity was increased in the majority of the OTUs by both bioinocula in NOW, but also a significant percentage was not changed. The same amplitude of changes was observed in BRZ, only with the majority of OTUs having a decreased intensity. In the first sampling period of 2015, before the second application of the bioinocula, both sites showed a majority of OTUs with decreased intensity compared to the control and a similar number of unchanged OTU intensity. The impact of both bioinocula on bacteria OTU intensity for the BRZ samples was similar at the end of the 2015 season, 8 weeks after their application, but not for the NOW site, where the two bioinocula affected differently the bacterial community. In 2016, a similar impact on OTU intensity was again observed in both sites and both sampling periods in the plots treated with *B. brongniartii*. This was consistently observed also in *B. bassiana* plots, with the exception of the first sampling in NOW, when, however, an uncommon number of missing OTUs (i.e., not possible to determine considering the identification criteria) was found.

Draganova et al. (2008) reported that strains of *B. bassiana* showed varying degrees of impact on the intensity of significant groups of soil microorganisms (bacteria, actinomycetes, and fungi). Some strains (e.g., *B. bassiana* 224Re) showed no impact; in contrast, the strain *B. bassiana* 412 showed the most substantial stimulation effect on heterotrophic microorganisms, mineral nitrogen utilizing bacteria, free-living nitrogen-fixing microorganisms, and soil fungi, with 14, 15, 7, and 30 times higher density of these microorganisms compared to the control treatments, respectively. However, the same strain caused suppression of the cellulose degrading microorganisms compared to the non-inoculated soil. Isolates of *B. bassiana* had no or little influence on the microbial community composition and function in the rhizosphere up to 30 days after inoculation, as assessed by DGGE and microrespiration analyses (McKinnon et al., 2018). Moreover, the density dynamics of *B. bassiana* introduced into forest soil did not affect the densities of bacteria and actinomycetes (Shimazu et al., 2002). Considering that a significant decline in all detectable *Beauveria* strains was observed in the fields of the study using a PCR-based method (Tartanus et al., 2021), this may indicate that the inocula did not have a lasting effect. Such a hypothesis would be confirmed by the generally consistent similarities in OTU intensity among bioinocula and sites found on the samples collected in spring 2015 and 2016, before the new application, as well as by considering also only the OTUs common to different sampling periods and sites.

Entomopathogenic fungi from the genus *Beauveria* are considered common rhizosphere colonizers in many ecosystems (Jaronski, 2007; Zimmermann, 2007). They are generalist pathogens to various insect species, the cadavers of which may provide a source of nitrogen to plant roots through the fungal mycelial network from an insect cadaver (Barelli et al., 2016), as it has been shown for another entomopathogenic fungal genus–*Metarhizium* (Bruck, 2010; Behie et al., 2012). In this instance, the soil inoculated with EPF could provide more nutrients derived from their metabolism that modify the composition of the bacteria community (González-Guzmán et al., 2020). However, there is limited knowledge of interactions between EPF and other soil microorganisms (Lozano-Tovar et al., 2017). Another mechanism triggered by the EPF affecting the bacterial community could result from the modification of root exudates (composition or quantity). Indeed, the endophytic behavior of *B. bassiana* and its capacity to develop on root exudates, particularly in the absence of the insect host, would likely modify the soil chemical environment, leading to changes in the bacterial community (Vega, 2018; Rolfe et al., 2019).

The nutrient promotion effect may vary under different environmental conditions (Hossain et al., 2017; Yadav, 2020). Therefore, the different impacts observed in the various sampling periods and/or fields could be the expression of the interaction between the EPF activity and the environmental conditions. In some cases (e.g., during the 2015 season), the lower number of common OTUs (T-RFs) and their lowered intensity compared to the control or the previous sampling period could point to a higher genetic diversity promoted by the bioinocula or to the return of the microbial community to a pre-application equilibrium due to bioinoculum level decrease (Scheepmaker and Butt, 2010).

4.3. Impact of entomopathogenic fungi inoculants on soil fungal community

The impact of the two bioinocula on the fungal OTUs was quite limited, as only in BRZ 2015 the samples after the application showed a higher OTU number compared to the sample collected before the application. No pattern of influence emerged in analyzing the effect on OTU intensity. This situation was well highlighted during the last season (2016) when any specific OTU specifically influenced by the bioinocula was detected.

Several studies have detected limited or only transient effects on soil fungi after the application of bioinocula of both *B. bassiana* and *B. brongniartii* (Rai and Singh, 2002; Schwarzenbach et al., 2009; Hirsch et al., 2013). A *Metarhizium brunneum* (Hypocreales: Clavicipitaceae) strain formulated as fungus colonized barley kernels (FCBKs) used in a pot experiment showed to induce some changes in the fungal community structures (Mayerhofer et al., 2017), differently to the field application of the same formulation or previous reports indicating a lack of impact from the application of

Metarhizium anisopliae sensu lato (Kirchmair et al., 2008). However, since the non-formulated fungal spores resulted in not affecting the fungal communities, it was suggested that the effects could mainly derive from the formulation carrier (i.e., the kernels). This could also have occurred in the present study, particularly in the *B. brongniartii* strain, which was also formulated as an FCBK. Moreover, time-related effects on the fungal and prokaryotic soil communities in the pot experiment were similar to or greater than the treatment effects (Mayerhofer et al., 2017). The following study confirmed this hypothesis, showing that neither changes in soil fungal or prokaryotic community structures nor relative sequence abundance of individual OTUs could be detected upon the application of *M. brunneum* formulated as FCBK (Mayerhofer et al., 2019). Seasonal changes in soil microbial composition in relation to the plant developmental stage were also found to exceed the effects of applied fungi and bacteria in bulk soil (Savazzini et al., 2009) as well as in the rhizosphere (Van Dillewijn et al., 2002; Grosch et al., 2006).

Microbial control usually implies the application of large amounts of infective propagules of a biological control agent to soils under treatment. For instance, about 10^{12}–10^{14} propagules of EPF are applied per hectare, translating into 10^5 spores per cm^2 of soil (Jaronski, 2010). Such high loads of propagules may have unintended side effects, leading to changes in soil microbial community structures. The densities of total fungi were significantly increased by *B. bassiana* application in forest soils (Shimazu et al., 2002). A possible effect of the impact of the bioinocula could be the competition with native arbuscular mycorrhizal fungi, which was detected in the case of *B. bassiana* in oak and maize roots (Zitlalpopoca-Hernandez et al., 2017; Matek et al., 2019). A positive outcome of this kind of impact could be the antagonism with soil-borne fungal pathogens (Jaber, 2018; Wiberth et al., 2019) or the synergistic activity with other mycoparasite species (Krauss et al., 2004). Nevertheless, since the host mortality is dose-dependent (Jaronski, 2007; Inglis et al., 2009), it could also be postulated that the impact on the microbiome (or non-target organisms) is transient, as it emerged also from the present study, and decreasing as the inoculum levels decrease.

5. Conclusion

With regard to the impact on the bacterial community, both *Beauveria* species were not fully consistently affecting their composition across the seasons and fields tested. Nevertheless, some common patterns were pointed out in each field and, sometimes, also among them when considering the time elapsed from the bioinoculum application. The impact was even more inconsistent when analyzing the fungal community. It is thus concluded that the analyses performed pointed to a transient and limited effect of both *Beauveria* species on the soil microbial community even though some changes in the structure dynamic and frequency of soil bacterial and fungal OTUs emerged.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Author contributions

LC, EM, and MT: conceptualization and methodology. LC and MT: analysis and investigation. All authors have wrote, reviewed, read, and agreed to the published 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1073386/full#supplementary-material

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Microbiome diversity, composition and assembly in a California citrus orchard

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The citrus root and rhizosphere microbiomes have been relatively well described in the literature, especially in the context of Huanglonbing disease. Yet questions addressing the assembly of root microbial endophytes have remained unanswered. In the above ground tree tissues, leaves and stems have been the research focus point, while flush and flower microbiomes, two important tissues in the vegetative and reproductive cycles of the tree, are not well described. In this study, the fungal and bacterial taxa in five biocompartments (bulk soil, rhizosphere, root endosphere, flower and flush) of citrus trees grown in a single California orchard were profiled using an amplicon-based metagenomic Illumina sequencing approach. Trees with no observable signs of abiotic or biotic stresses were sampled for two consecutive years during the floral development phase. The rhizosphere was the most biodiverse compartment compared to bulk soil, root endosphere, flower and flush microbiomes. In addition, the belowground bacteriome was more diverse than the mycobiome. Microbial richness decreased significantly from the root exosphere to the endosphere and was overall low in the above ground tissues. Root endophytic microbial community composition shared strong similarities to the rhizosphere but also contained few taxa from above ground tissues. Our data indicated compartmentalization of the microbiome with distinct profiles between above and below ground microbial communities. However, several taxa were present across all compartments suggesting the existence of a core citrus microbiota. These findings highlight key microbial taxa that could be engineered as biopesticides and biofertilizers for citriculture.

KEYWORDS

Huanglongbing, biofertilizers, rhizosphere, citrus flush, caulosphere, anthosphere, biopesticides, endophytes

Introduction

Developing integrated agriculture systems has become increasingly needed in the face of mounting global challenges. The environmental impact of agrochemical pesticides and fertilizers is leading to changes in consumer behavior toward sustainably grown food and food products and as a result, farmers are increasingly relying on biological-based technologies and less on synthetic chemistries. Microbiomes have been shown to provide many benefits to plants by priming the immune system and protecting them from diseases, facilitating nutrient acquisition, and overall enhancing health and increasing yield. Taking advantage of the microbiome at work,

i.e., the capitalization on microbial traits that are beneficial to the host or the environment or both, presents a promising avenue for the development of a more sustainable next-generation agriculture (Schlaeppi and Bulgarelli, 2015).

Commonly occurring organisms across similar microbiomes form a core microbial community that is hypothesized to play critical roles in ecosystem functioning within that type of microbial habitat (Shade and Handelsman, 2012; Gopal et al., 2013). While many deep sequencing studies have shown that plant microbiomes are made up of a plethora of microbial taxa, only a few taxa typically predominate in the larger community (Sagaram et al., 2009; Gottel et al., 2011; Weinert et al., 2011; Bodenhausen et al., 2013; Peiffer et al., 2013). Even in a variety of experimental settings, some of the highly abundant taxa in these studies are noticeably conserved across the microbiomes of related plant species. This implies that a core microbial community consistently associates with specific hosts at different spatial and temporal scales. However, it is known that the composition of the plant microbiota is influenced by a number of biotic and abiotic factors (Redford and Fierer, 2009; Bulgarelli et al., 2012; Lundberg et al., 2012; Rastogi et al., 2012). There is still much to learn about the composition of the core microbiome community and its significance for plant health, given that only a few studies have identified the key players in plant-associated microbial communities (Bulgarelli et al., 2012; Lundberg et al., 2012; Rastogi et al., 2012).

Citrus is one of the most important perennial fruit crops in the world. Being a good source of vitamins, fiber, and minerals, it is commended for its nutritional qualities and advantages for human health. Citrus is also a major contributor to the economic value of the agricultural sector. It accounts for 16% of the total value of the United States fruit production (Li et al., 2020) with California representing 80% of the nation's fresh fruit market with an annual value of 2.3 billion dollars (CDFA, 2021). There has been tremendous interest in exploring the structure and function of the citrus phyllosphere and rhizosphere microbiomes and engineering its assembly to address current challenges in citriculture (Zhang et al., 2021; Ginnan et al., 2022). Root microbiome has emerged as a focal point of citrus health especially in the context of Huanglongbing (HLB) disease (Blaustein et al., 2017; Xu et al., 2018; Ginnan et al., 2020; Wu et al., 2020).

Huanglongbing (HLB) or citrus greening is considered the most serious problem of citrus worldwide (National Research Council, 2010). HLB is caused by an uncultivable Gram-negative phloem-limited bacteria belonging to the *Candidatus* Liberibacter species (i.e., *Ca.* L. asiaticus, CLas; *Ca.* L. africanus and *Ca.* L. americanus), which are transmitted from infected to healthy plants by citrus psyllids (Bové, 2006). *C*Las infection causes phloem sieve occlusion and impairs translocation of photo-assimilated carbon to the root zone thereby weakening trees by decreasing the energy pool of non-structural carbohydrates (Etxeberria et al., 2009). Lasting infection leads to root collapse and dysbiosis of root associated microbial communities including depletion of keystone taxa and enrichment of saprobes and parasitic soilborne fungi such as *Fusarium* and *Phytophthora* (Ginnan et al., 2020). However, despite our better understanding of the importance of root health on disease management, tree health and productivity, gaps remain to confidently develop effective guidelines for long-term disease management.

The highest concentrations of *C*Las can be found in midribs of flush (Chiyaka et al., 2012). A flush shoot may be defined as a new shoot growth with immature leaves but can range from as small as newly breaking buds of just feather flush to fully elongated shoots with expanded, tender leaves. In California and Mediterranean climates, flush is produced twice annually in relatively well-defined cycles, one related to plant growth in summer-autumn, and one related to flowering and fruiting in spring. Timing of flush development is genetically and environmentally governed, with temperature, photoperiod, solar radiation and rainfall (Moss, 1969, 1976; Olesen et al., 2013). The most critical of these for fruit production is the spring leaf flush since it coincides with both flowering and early fruit development. However, the microbial composition of the citrus flush has to our knowledge not been elucidated, even though this tissue is at the forefront of the infection in the HLB pathosystem. Profiling the citrus flush microbiome could identify potential beneficial organisms that are inhibitory to *C*Las or provide the host with environmental fitness and horticultural advantage.

Similar to the flush, the study of flower microbiome has surprisingly received little attention despite its direct role in fruit production. In citrus, flowering time and abundance depend largely on the species, the tree age, and the climatic conditions (Lau and Lennon, 2011; Agustí et al., 2020). However, research indicated that rhizosphere microbiome can also drive changes in the host phenological traits including flowering period (Lau and Lennon, 2011; Lu et al., 2018). The host phenological stage appears to be a major driver of the leaf microbiome assemblage indicating that it could also influence flower microbiome composition (Ginnan et al., 2022). Flowering is the most important determinate of yield and quality of citrus fruit production (Stander, 2015). Particularly, flowers provide ephemeral but unique nutrient-rich and protective habitats for microorganisms (Aleklett et al., 2014) and the microbial make-up of flowers may affect disease outcome and in turn fruit yield. For example, fire blight disease severity of apple blossoms caused by *Erwinia amylovora* can be mitigated by treating flowers with endogenous microbial taxa (Cui et al., 2021). The understanding of the reproductive microbiome function on flowering may hold the key to enhance productivity in agroecosystems.

The objective of this study was to fill in the knowledge gap about root microbial assemblage in citrus to better identify key microbes recruited by the host that likely harbor beneficial properties, increase the host environmental fitness, and support tree health. In addition, our goal was to profile the microbiome of the flower and the flush, two young tissues that had not been extensively studied despite their critical importance in the tree vegetative and reproductive cycles. Flush is also critical to the HLB disease epidemiology. Here, we provide a microbial map of five distinct compartments (bulk soil, rhizosphere, root endosphere, flush and flower) of citrus from a single orchard over a two-year period and discuss in what capacity the information acquired with this research may help citrus production.

Materials and methods

Plant sampling and processing

The experimental orchard is located at the Lindcove Research and Extension Center, California (GPS coordinates 36°21′10″N; 119°03′40″W). The plant materials were collected from 11 years-old conventionally farmed citrus cv. 'Tango' on 'Carrizo' rootstock (*Citrus*

sinensis L. Osbeck × *Poncirus trifoliata* L.). All samples were collected at the flower initiation stage on 4/7/2021 and 3/28/2022. Flower, flush (the new foliar growth between bud break and shoot expansion), root and rhizosphere samples were collected each year from 12 random trees. Flush and flower samples were collected from the four quadrants and pooled. Feeder roots were sampled from two sides of the tree approximately 0.3 m away from the base of the trunk. Five bulk soil samples were also collected each year from the four corners and the middle of the citrus grove. Gloves were changed and clippers and shovels were sterilized with 30% household bleach between each sampled tree. All samples were immediately placed on ice in a cooler for transit to the laboratory and were frozen. All samples were processed within 24 h. Root and rhizosphere samples were processed as described by (Lundberg et al., 2012). Briefly, roots were placed in sterile 50-mL conical tube with 25-mL of PBS with 200-μL L^{-1} Silwet® L-77 surfactant. Samples were vortexed at maxim speed for 15 s. Roots were then transferred to a clean 50-mL conical tube with 25 ml of PBS. The first tube was centrifuged at 3200 *g* for 15 min and the aqueous layer was removed. The pellet was retained as the rhizosphere fraction. The roots continued to be vortexed and were moved to a clean PBS tube until PBS remained clear after vortexing. Roots were then sonicated using a Branson Sonifier 450 at a low frequency for 5 min (five 30 s bursts followed by 30 s breaks). Roots were then stored at −70°C for further processing. Flowers, flushes, and roots were then lyophilized in the FreeZone 2.5-L benchtop freeze dry system (Labconco, Kansas City, United States) for 72 h. Specifically, flower and flush samples were not surface sterilized; thus, the aboveground microbial next-generation sequencing datasets included both epiphytes and endophytes. Samples were then ground to a powder using the MM300 grinder (Retsch, Haan, Germany) in a 35-mL stainless steel grinding jar with 20-mm stainless steel balls at 25 oscillations per second in 30-s increments until sample was fully pulverized.

Microbiome library preparation

DNA was extracted from all samples using the ZymoBIOMICS DNA miniprep kit per manufacturer's protocol, using 100 mg of dried tissue or 250 mg of wet rhizosphere (Zymo Research, Irvine, United States). DNA was assessed for quality and quantity using the Qubit 4 Fluorometer with the Qubit dsDNA HS Assay (Thermo Fisher Scientific Inc., MA, United States). Both bacterial 16S–V4 and fungal ITS rRNA regions were amplified using the Earth Microbiome protocol and primers.[1] Briefly, primers 515F(GTGYCAGCMGC CGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) were used for bacterial microbiomes and ITS1f (CTTGGTCATTTAG AGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) for fungal ITS amplification (Caporaso et al., 2010). PCR reactions of 25 μl contained 10 μl of Phusion hot start flex 2 × master mix, 0.5 μl of each primer (10 μm) and 2 μl of DNA. In bacterial above-ground tissue (flower and flush), universal pPNA and mPNA clamps were added at a starting concentration of 1.25 μl (5 μm). These clamps were designed to reduce the amplification of host chloroplasts and mitochondria while having no effect on bacterial amplification (Fitzpatrick et al., 2018b). A negative control was added to each PCR to ensure barcodes and master mix were not contaminated. Successful amplification was verified on a 1% agarose gel and DNA was quantified using the NanoDrop 2000 Spectrophotometers (Thermo Fisher Scientific Inc., MA, United States). A total of 1,200 ng of each sample in a library were combined into an Eppendorf tube and cleaned using the AMPure XP PCR purification system (Beckman Coulter, Brea, United States) per manufacturer's protocol. Final concentration of libraries was determined using both qPCR and bioanalyzer before being sequenced on the MiSeq instrument (Illumina, San Diego, United States) using Miseq run (2 × 300 paired end) for fungal reads and Miseq run (2 × 250 paired end) for bacterial microbiome at the UC Riverside Genomics Core facility. Fungal and bacterial sequences were deposited in NCBI under the accession number SUB12502574 and SUB12495203, respectively.

Computational analysis

The R Core Team v4.1.1 was used to perform all computational analysis. Most processing for the reads were done in DADA2 v 1.16.0 (Callahan et al., 2016) including further quality control sequencing filtering, dereplication, chimera identification, merging paired end reads, and construction of sequence tables. Taxonomy identification was assigned using the SILVA SSU r138.1 reference database for bacterial taxa and Unite database v 10.5.2021 for fungal taxa. Phyloseq v 1.36.0 (McMurdie and Holmes, 2013) and ggplot2 v3.3.5 packages (Wickham, 2016) were used for much of the graphical and statistical analyzes of the data. Unidentified microbes at the kingdom or phylum level, or microbes that occurred less than two times within all 24 trees (12 tree samples per year) were removed from the full dataset. The bacterial dataset totaled 106 samples (24 flower, 24 flush, 24 rhizosphere, 24 root and 5 soil samples) and the fungal dataset totaled 104 samples (23 flower, 24 flush, 24 rhizosphere, 23 root and 5 soil samples) after filtering out poor quality reads, chloroplast, mitochondria, taxa with unidentified phyla. After removal of singletons and doubletons, the total ASVs were of 10,483 (soil = 4,395; rhizosphere = 7,635; root = 1997; flush = 129; flower = 128) and 5,155 (soil = 707; rhizosphere = 2,964; root = 1,333; flush = 860; flower = 905) for the bacterial and fungal datasets, respectively. Shannon diversity index was used as a metric of taxa diversity within the communities. Kruskall–Wallis and pairwise Wilcoxon rank sum tests were run to verify statistical differences among groups. Phylum bar charts and genus bar charts were constructed by aggregating taxa at the phylum level and genus level, respectively. Samples were also constructed by tissue compartments and transforming to relative abundance. Bray–Curtis dissimilarity was used to calculate the compositional similarities between samples and was visualized with NMDS (Non-metric MultiDimenstional Scaling) plots using the Vegan package v 2.5-7. To determine statistical significance of beta diversity, Adonis tests were run. Venn diagrams were created using UpSetR v 1.4.0 by transforming to relative abundance and filtering taxa to those that occur greater than 0.1% and are prevalent in at least two samples of that tissue type. Data was aggregated by genus and transformed to relative abundance for the prevalent Venn diagrams. Taxa were denoted as prevalent in each biocompartment. Graphs were generated using VennDiagram v1.6.20. Data was aggregated to the ASV or genus

1 http://www.earthmicrobiome.org/

level and transformed to relative abundance for the concentric pie charts representing core microbiome. ASVs/genera were filtered based on core microbiome as previously defined. DeSeq2 v 1.30.1 was utilized and visualized using Pheatmap v1.0.12 to find microbes associated with a biocompartment and above-and belowground sections. Genera were filtered by relative abundance, p value and log2 fold change, keeping only genera occurring at $\geq 1\%$ relative abundance of whole dataset with $p < 0.01$ and having a log2 fold change > 5 or < -5. Heat maps represent the relative abundance of the data.

Results

The Shannon index indicated that the rhizosphere had a significantly higher microbial richness among all plant tissue types for both bacteriome and mycobiome ($p < 0.001$ [pairwise Wilcox]; Figure 1). All the below ground bacteriome samples showed a significantly higher Shannon diversity index as compared to the above ground samples ($p < 0.001$ [pairwise Wilcox]). In contrast, the root mycobiome had a significantly lower fungal community richness of all tissue types ($p < 0.001$ [pairwise Wilcox]) and there was no significant difference with the soil fungal diversity ($p = 0.25$ [pairwise Wilcox]). The bacteriome richness was higher than the mycobiome richness in the below ground samples (soil, rhizosphere, and root), while the opposite was true for the above ground tissues (flush/flower). Flower and flush microbiome richness level were similar to each other in both groups. There was a year effect on the Shannon diversity index in the mycobiome communities ($p < 0.05$ [pairwise Wilcox]) but not in bacteriome ($p = 0.23$ [pairwise Wilcox]).

Bray–Curtis beta-diversity metrics with NMDS were used to visualize how biocompartments impacted fungal and bacterial community composition (Figure 2). Our data indicated distinct clustering between above- and below-ground in both bacterial and fungal communities ($p < 0.001$ [Adonis]). Among belowground samples, clear clustering was measured for the soil, rhizosphere, and root in both bacterial and fungal groups. Among aboveground samples, flush and flower showed overlapping patterns in bacterial year-2 data and all fungal data. Year also had a significant effect ($p < 0.001$ [Adonis] for bacteriome; $p < 0.05$ [Adonis] for the mycobiome) in the clustering pattern, particularly for the bacterial flower and flush datasets.

Proteobacteria and Ascomycota were the most abundant phyla within the entire dataset representing on average 47.7 and 81.6% of all taxa, respectively (Figure 3). Phyla Basidiomycota and Actinobacteria were also important phyla as they occurred in greater than 10% on average across the entire datasets. Several phyla with a relatively great abundance (greater than 5%) were unique to belowground or aboveground biocompartments. For example, Glomeromycota, Mortierellomycota, Acidobacteria, Gemmatimonadota, and Verrucomicrobiota were mainly found in soil, root and rhizosphere, whereas Cyanobacteria was only found in flower and flush samples. Although the most abundant phyla were the same in each tissue at the

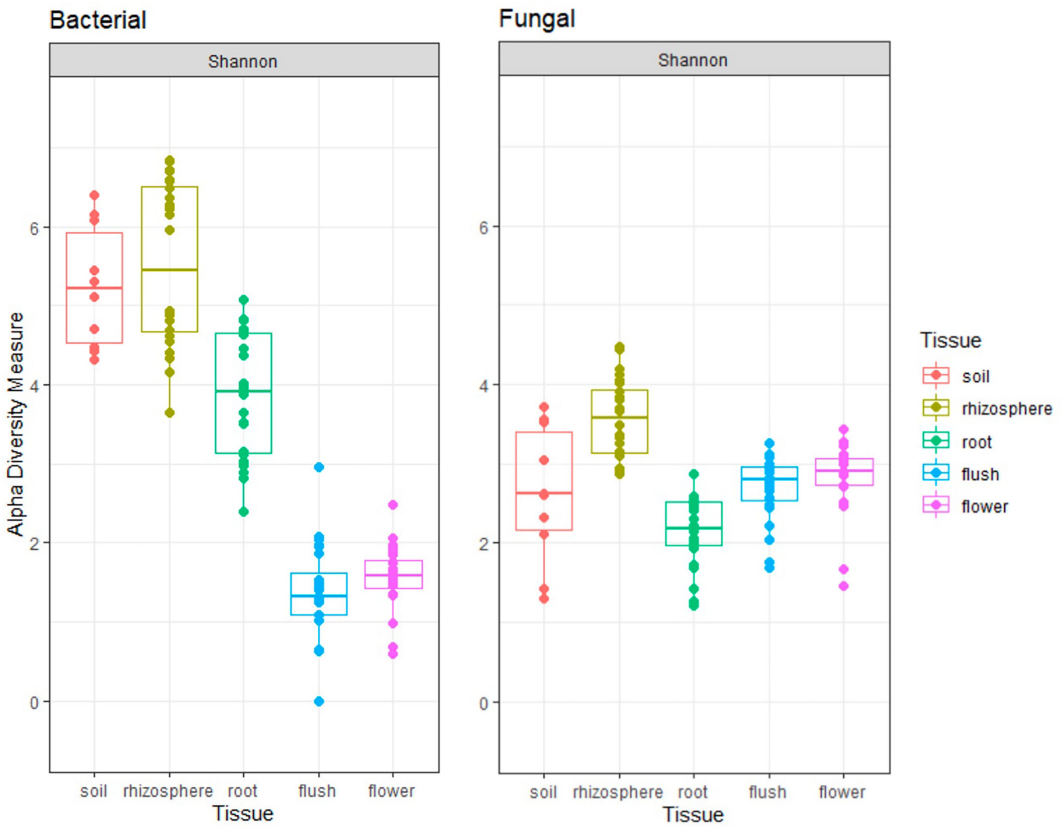

FIGURE 1
Shannon alpha-diversity plots indicate bacterial (left panel) and fungal (right panel) richness across five different citrus biocompartments (soil, rhizosphere, root, flush, and flower).

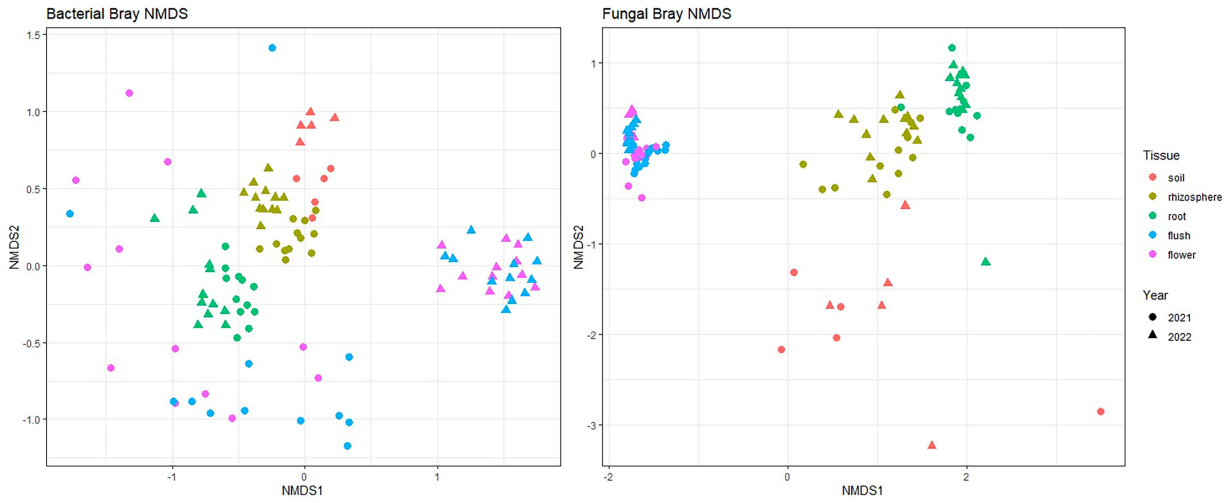

FIGURE 2
Bray–Curtis beta diversity for bacteria (left panel) and fungi (right panel) across five different citrus biocompartments (soil, rhizosphere, root, flush and flower). Points represent individual sample communities for one biocompartment from one citrus tree at 1year. Points are colored by biocompartment and shaped by year collected.

phylum level, differences were observed at the genus level especially between above and belowground. In the mycobiome, the most abundant genus in belowground samples was *Neocosmospora* (36.4%), while aboveground *Cladosporium* was the most dominant (60.4%). In the bacteriome, *Acinetobacter* was the most abundant genus in aboveground samples (38.7%), and the belowground samples were more diverse, with no single dominant genus.

We used DeSeq2 analyzes to indicate enrichment/rarefaction patterns of taxa along the soil, rhizosphere root axis and signature microbial taxa for the three plant biocompartments, root, flush and flower. We focused our analysis on the most prevalent and abundant taxa and applied a filtering metric that consisted of ≥ 50% incidence in roots and > 10% of bacterial flush and flower samples with a relative abundance > 1% (Figure 4). Our results indicated a root enrichment of several bacterial genera in the rhizosphere that were dominant in soil, but only a few of these were found in the root including *Actinoplanes*, *Burkholderia*, *Mucilaginibacter*, and *Rhizobium* and fungi *Glomus*, *Neocosmospora*, *Rhizophagus*, and *Setophaeosphaeria*. Several bacterial and fungal taxa were unique to roots and included the bacterial genera *Bradyrhizobium*, *Cupriavidus* and *Rhizobium* and the fungal genera *Glomus*, *Neocosmospora*, *Rhizophagus* and *Setophaeosphaeria*. In contrast, the bacterial genera *Acinetobacter*, *Aquabacterium*, *Gilliamella*, *Romboutsia* and fungal genera, *Alternaria*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, *Mycospherella*, *Sigarispora*, and *Symmetrospora* were signature above ground taxa because only found in those compartments. Bacteria *Burkholderia* and *Streptomyces* were present in both below and aboveground tissue.

The identity of the most prevalent ASVs that were unique to each biocompartment or shared across biocompartments were determined using Venn diagrams with a filtering consisting of ≥ 50% incidence for the belowground compartments and > 10% of bacterial flush and flower samples, with a relative abundance > 0.1% (Figure 5). This filtering narrowed the dataset to a total of 794 ASVs (491 bacterial and 303 fungal ASVs). The rhizosphere was the biocompartment with the highest number of unique filtered ASVs for both bacteria and fungi (429 ASVs total = 54%), whereas root, flower and flush only had 14, 6.5 and 5.8% of unique ASVs for the combined fungi and bacteria datasets, respectively. The fungal *Epicoccum* and bacterial *Acinetobacter*, *Aquabacterium*, *Gilliamella*, *Kocuria*, *Romboutsia*, *Snodgrassella*, *Tychonema* ASVs were biomarkers of the above ground tissues because they were only found in the flush, flower or both. The fungal *Beauveria*, *Fusarium/Giberella/Fusicola*, *Mortierella*, *Setophaeosphaeria*, *Solicoccozyma* and bacterial *Bradyrhizobium*, *Cupriavidus*, *Mucilagnibacter*, *Pseudathrobacter*, *Steroidobacter* ASVs were biomarkers of the belowground citrus because they were only found in the root, rhizosphere, or both. The majority (84%) of total number of fungal and bacterial ASVs inhabiting the roots (111 ASVs) were also found in the soil/rhizosphere suggesting they entered the root from the soil/rhizosphere. Only 3.4% of the total number bacterial and fungal ASVs (27 ASVs total) were capable of colonizing at least one of the below and above ground compartments highlighting their ubiquitous nature and included ASVs belonging to the fungal genera *Alternaria*, *Cladosporium*, *Mycosphaerella*, *Neocosmospora*, *Sigarispora*, and *Symmetrospora*, and the bacterial genera *Actinoplanes*, *Bacillus*, *Burkholderia*, *Firmicutes*, *Mesorhizobium*, *Pseudomonas*, *Massilia*, *Sphingomonas*, and *Streptomyces*.

Discussion

The aim of this study was to characterize the citrus microbiome across five biocompartments (flush, flower, root, rhizosphere and bulk soil) during the floral development phase. At this stage, trees undergo drastic physiological shifts with respect to carbon reallocation, water dynamics and phytohormone production (Goldschmidt and Koch, 2017; Agustí et al., 2022) that are linked to significant shifts in microbial community assemblage (Ginnan et al., 2022). Both flower and flush are short-lived organs. Flowers host a unique set of microbes that may act as mediators of host reproduction and disease control (Burgess and Schaeffer, 2022). The flush is also a tender tissue fed on

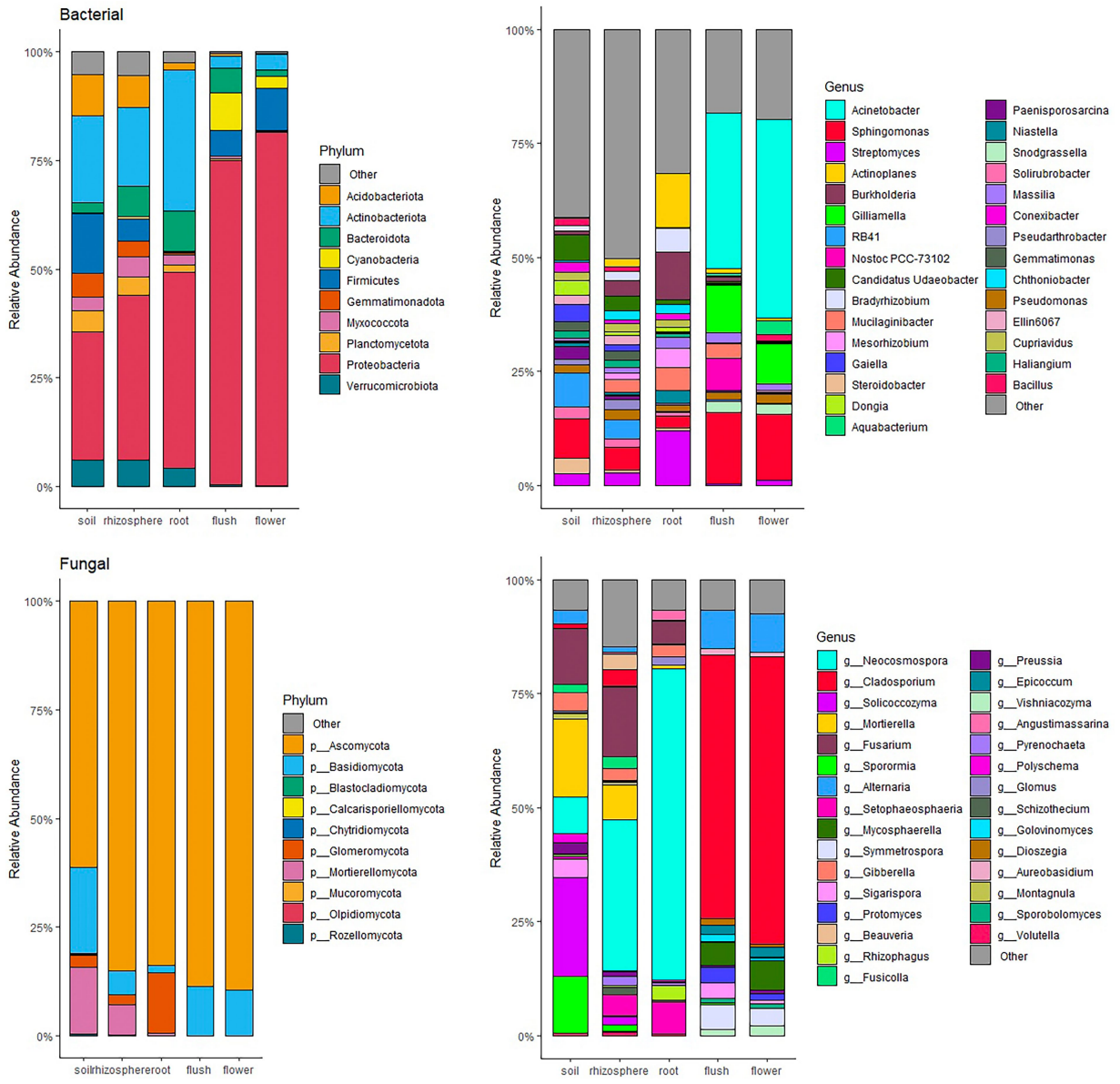

FIGURE 3
Relative abundant bar chat of bacteria (top panels) and fungi (bottom panels) community at phylum (left panels) and genus (right panels) level within individual citrus biocompartment (soil, rhizosphere, root, flush and flower). Only top 10 phyla and top 30 genera occurring at ≥1% relative abundance are displayed.

by several insect pests, including the Asian citrus psyllid (*Diaphorina citri*) vector of CLas, the causal bacterial agent of HLB (Hall and Albrigo, 2007), and profiling its microbiome could reveal potential biocontrol agents for HLB management. The citrus rhizosphere microbiome has also been a research focus because of its role in nutrient fixation, absorption and cycling as well as defense against pathogens (Xu et al., 2018; Ginnan et al., 2020; Zhang et al., 2021). Here we provide a better understanding of the acquisition of specific microbes along the soil-rhizosphere-root axis as plant endophytes may have bioactive functions relative to bioinoculant development (Lugtenberg et al., 2016; Santoyo et al., 2016).

The microbial biodiversity of citrus trees was primarily located in the plant rhizosphere, with the bacteriome showing higher taxonomic richness than the mycobiome (Blaustein et al., 2017; Ginnan et al., 2020). Trees were predominantly colonized across all compartments by Ascomycota fungi and Proteobacteria (Trivedi et al., 2010; Passera et al., 2018; Xu et al., 2018; Bai et al., 2019) but microbial composition within those groups was vastly different between the above and below ground compartments as indicated by beta diversity plots. Microbial diversity in the flush and flower was low and Ascomycota and Proteobacteria represented overwhelmingly 80% of the microbial relative abundance in those tissues. In contrast, belowground microbial assemblage was more complex especially for bacteria and included a wide range of taxonomic groups spanning across several bacterial phyla. Our data indicated that a minority of taxa (3.4%) were capable of colonizing both below and above ground habitats. We defined the core taxa of the citrus holobiont as genera prevalent in at least 50% of our samples and with a relative abundance of at least

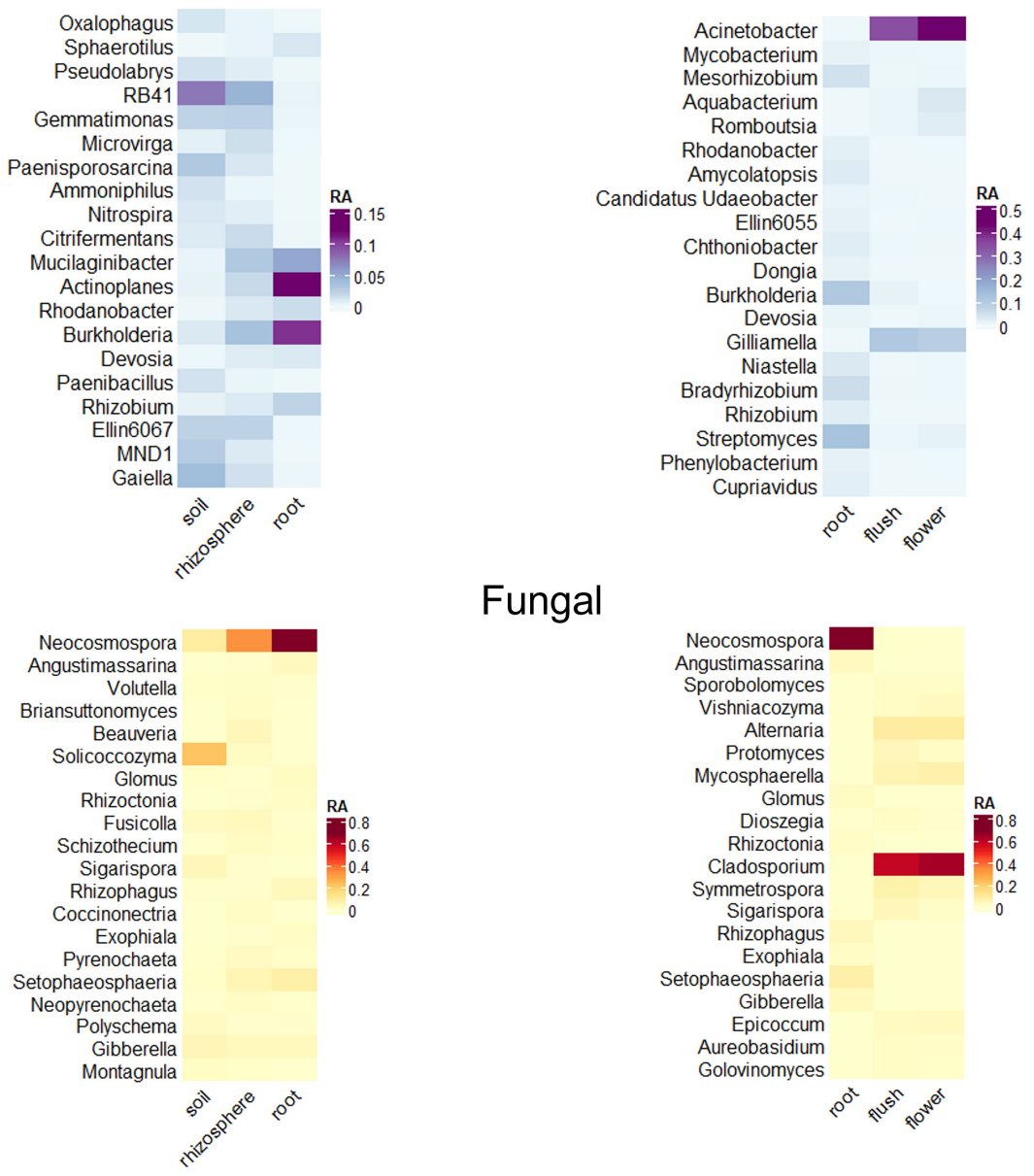

FIGURE 4
Relative abundance heat maps of significant taxa as determined through DESeq2 analyzes indicate recruitment of bacteria (top panels) and fungi (bottom panels) along the soil/rhizosphere/root endosphere axis (left panels) and signature taxa for the root/flush/flower (right panels) compartments. Data was filtered to a $p<0.01$ cutoff and log2 fold change of >5 or <−5. Only genera occurring at ≥1% relative abundance with the top 20 highest log2 fold changes are displayed.

1% and with ASVs within those groups capable of colonizing at least one below and above ground biocompartment. Based on these criteria we found that the fungi *Alternaria*, *Cladosporium*, *Fusarium* (syn. *Fusicolla, Gibberella*, *Neocosmospora*,), *Mycosphaerella*, *Sigarispora*, and *Symmetrospora*, and bacteria *Actinoplanes*, *Bacillus*, *Burkholderia*, *Firmicutes*, *Massilia*, *Mesorhizobium*, *Pseudomonas*, *Sphingomonas*, and *Streptomyces* represented core members of the citrus holobiont. Although additional sampling from orchards located in different citriculture areas may narrow that list. Many of these bacterial taxa are known plant growth promoters and biocontrol agents and can provide fitness advantage to the host (Lemanceau et al., 2017; Xu et al., 2018).

However, the role of fungal taxa remains elusive. Several genera within the *Fusarium* species complex were found in our dataset (*Fusarium*, *Giberella*, *Fusicolla*, and *Neocosmospora*) and members within this group have a broad range of lifestyles including commensalism, mutualism, and parasitism (Crous et al., 2021). For example, *Fusarium solani* is a known pathogen of citrus causing wood dry rot (Sandoval-Denis et al., 2018) but the lifestyle of other species belonging to the *Fusarium* complex is unclear. Other fungal taxa within the citrus holobiont such as *Alternaria alternata*, *A. arborescens*, and *Mycosphaerella citri* were also reported to blemish fruits (Mondal et al., 2003; Wang et al., 2021). On the other hand, *Sigarispora* and

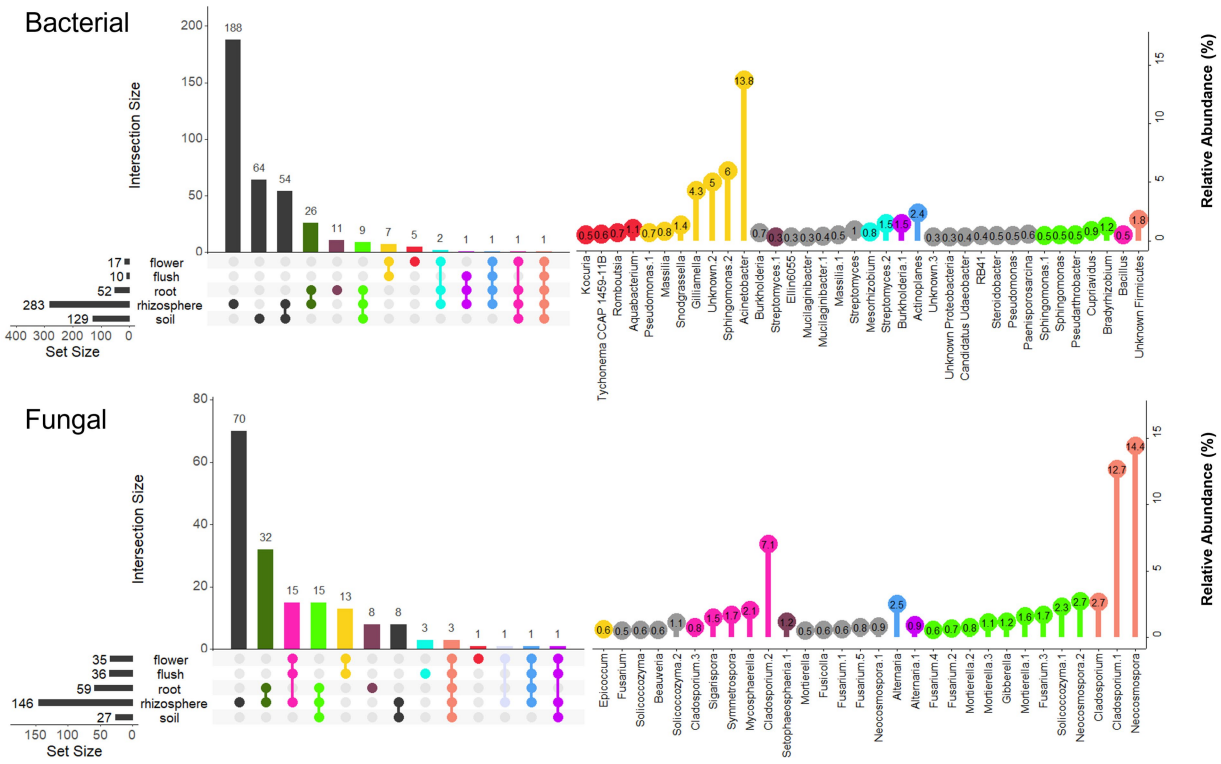

FIGURE 5
Prevalence Venn diagrams at the Amplicon Sequence Variant (ASV) level showing overlapping bacterial (top panel) and fungal (bottom panel) taxa that occur in ≥50% of the soil/rhizosphere/root samples and >10% of the flush/flower samples within and across biocompartment. Only ASVs occurring at ≥0.5% relative abundance are displayed. Left panel represents the total number of ASVs for each biocompartment and intersection size for all biocomparment combinations. The right panel represents the ASV relative abundance (%) and color refers to the left panel and the biocompartment(s) where the ASVs were found.

Symmetrospora have been found in several habitats but with no known functions in citrus. Only *Cladosporium cladosporioides* was shown to inhibit *Liberibacter crescens*, a culturable surrogate of CLas (Blacutt et al., 2020), and could provide some benefits to the host. Deeper amplicon-based sequencing will help naming the fungal species associated with citrus which may provide some information about their lifestyle. Large scale sampling coupled with -omics technologies will shed light on the geographical distribution and functional attributes of the core fungal taxa within the citrus holobiont, although this approach remains limited by the availability of reference genomes (Xu et al., 2018).

The root-associated microbiome of healthy plants is a relatively stable ecosystem because roots are immersed in a buffered environment (the soil) that is not in under the direct constraints of extreme weather conditions and agricultural practices that above ground plant compartments experience. Roots are also less affected by the host phenological changes unlike flower and flush tissues (Ginnan et al., 2022). Root microbial assembly has been described as a two-step process, involving acquisition of specific microbes from the soil to the rhizosphere and a host-driven sorting step mechanism that subsets specific microbes into the root (Bulgarelli et al., 2013). Our DeSeq2 data clearly supported this mechanism in citrus, with enrichment of several organisms from the bulk soil to the rhizosphere, but with only few of these further capable of entering the citrus root endosphere (e.g., *Actinoplanes*, and *Burkholderia*). The microbiome of the rhizosphere was composed of the aforementioned core members of the citrus holobiont, plus signature underground taxa that included the bacterial genera *Bradirhizobium*, *Cupriavidus*, *Mucilaginibacter*, *Rhizobium* and *Steroidobater*, and fungal genera *Glomus*, and *Rhizophagus*. Comparative profiling of bulk soil and rhizosphere samples collected across distinct biogeographical regions from six continents also supported that these bacterial taxa were enriched in the rhizosphere (Xu et al., 2018). Root exudates act as signal molecules and food sources for the selective recruitment of microbes from bulk soil in exchange for increased nutrients assimilation and improved tolerance against abiotic and biotic stresses. Metagenomic sequencing of citrus soil and rhizosphere communities clearly showed that the functional traits enriched in the rhizosphere influenced microbial assembly and plant health (Xu et al., 2018). Specifically, enriched functional attributes affecting microbial assembly were involved in plant-microbe and microbe-microbe interactions (e.g., antimicrobial synthesis, biofilm formation), nutrient acquisition of microbes, and bioremediation of aromatic compounds. In addition, enriched functional traits that benefit the host were involved in nutrient acquisition, hormone balance, and pathogen inhibition (Xu et al., 2018).

Our data indicated that the microbial communities inhabiting the citrus root endosphere most likely originated from the rhizosphere (84% of ASVs) but with a threefold and fivefold decrease for both fungal and bacterial richness, respectively, which support previous findings (Reinhold-Hurek et al., 2015; Wang et al., 2020). The selective forces imposed by the plant host in the endorhiza are

a bottleneck to biodiversity as observed in several plant systems (Fitzpatrick et al., 2018a; Deyett and Rolshausen, 2020; Zhang et al., 2021). Interestingly, the backbone of the root endospheric communities was comprised of taxa from the core rhizophere microbiome, suggesting that similar functional microbial traits overlap between the rhizosphere and root endosphere. We measured a strong enrichment pattern for some taxa including the bacteria *Actinoplanes*, *Burkholderia*, *Mucilaginibacter*, *Rhizobium*, *Rhodobacter*, and fungi *Glomus*, and *Rhizophagus*. All five bacteria can promote plant growth by either fixing nitrogen, solubilizing phosphorus, producing phytohormone production, and increasing abiotic stress tolerance as well as and protect against pathogens by producing antimicrobial compounds or priming plant defense (Santi et al., 2013; Zhang et al., 2017; Orsi et al., 2021; Boukhatem et al., 2022; Fan and Smith, 2022). *Glomus* and *Rhizophagus* are arbuscular mycorrhizal fungi (AMF) that commonly form symbiotic associations with the plant host, including citrus. AMF can facilitate water and nutrient acquisition (phosphorus and nitrogen) and support host defenses against pathogen attack (Hohmann and Messmer, 2017; Chen et al., 2018; Xi et al., 2022).

In contrast to rhizocompartments, above ground microorganisms associated with plants are under strong selective pressure because they are continually exposed to changing environmental conditions (rainfall, heat, and UV radiation) and agricultural practices (agrochemical sprays) but are also influenced by the host phenology (Vorholt, 2012; Burgess and Schaeffer, 2022; Ginnan et al., 2022). The strong year effect measured on bacteriome and mycobiome beta-diversity for above ground tissues clearly support the evidence that microbiome composition in flower and flush is volatile and under environmental constraints. The citrus flower and flush microbiome composition was very similar to the leaf and included both core taxa (*Acinetobacter*, *Romboutsia*, and *Sphingomonas*,) fulfilling community-stabilizing function and transient taxa (*Gilliamella* and *Snodgrassella*) with likely specialized function in the community (Ginnan et al., 2022). Interestingly, the fungus *Epicoccum* surfaced as a signature fungus capable of colonizing flush and flower. It was previously reported as inhibitory to *Liberibacter crescens* (Blacutt et al., 2020) and given those characteristics should be further explored as a potential biocontrol for HLB management. Other signature and ecologically important bacteria within the flush and flower microbiome included *Acinetobacter*, *Gilliamella*, *Snodgrassella*, and *Sphingomonas*. *Acinetobacter* is highly abundant in the floral nectar microbiome of *Citrus paradisi* and other plant species (Fridman et al., 2012; Alvarez-Perez and Herrera, 2013) and *Sphingomonas* has been reported as a frequent member of the citrus rhizosphere (Xu et al., 2018). Both bacteria have also been identified in the sap of other perennial hosts (Deyett and Rolshausen, 2020). *Snodgrassella* and *Gilliamella* are important members of the honeybee gut microbiome and have been speculated to be immigrant taxa introduced to the phyllosphere by pollinators during dispersal event (Powell et al., 2014; Ginnan et al., 2022). Bacteria can be introduced to plants by bees and potentially migrate from the flower to the vascular bundles resulting in systemic movement within the plant (Cellini et al., 2019; Kim et al., 2019). Together, this supports that members of the citrus microbiome can move acropetally and basipetally through the xylem and phloem (Compant et al., 2010; Deyett and Rolshausen, 2019, 2020). Abundance of these bacteria has been shown to peak at the flowering stage in citrus and grapevine (Deyett and Rolshausen, 2019; Ginnan et al., 2022). These bacteria have well known plant growth-promoting capabilities through phytohormone production, phosphate solubilization, and degradation of organometallic compounds and are also antagonistic toward pathogens (Liu et al., 2007; Kang et al., 2009, 2012; Asaf et al., 2020). It is tempting to speculate that similar the rhizosphere, microbial recruitment mechanisms of beneficial bacteria also occur in the phyllosphere to provide the host with exogenous services and promote reproductive and vegetative cycles in sync with the host phenology.

Conclusion

This study provides new information about assemblage of microbial communities in citrus. Our results from a single orchard support that the citrus microbiome is composed of core taxonomic groups that are mainly of soil origin and that can systemically colonize trees. There is also evidence of a microbial niche compartmentalization with specialized taxa capable of colonizing either the above or the below ground biocompartments. Our findings support that transient taxa, whose colonization patterns are in sync with the host phenology, are abundant during flowering and tree flushing. We identified putative plant growth promoting bacteria (e.g., *Burkholderia*, *Sphingomonas*, and *Streptomyces*) enriched in all biocomparments that could be harnessed for bioproduct commercialization to improve tree health. We also identify tissue specific microbes (e.g., *Acinetobacter* and *Epicoccum*) that could colonize the citrus flush and flower and could enhance tree productivity or management against pests and diseases and notably HLB. Broad biogeographical sampling and shotgun metagenomic approach have greatly helped comprehend the structural and functional composition of the citrus rhizosphere microbiome. The next frontier is to expand this approach to the plant endosphere because it could harbor host-selected microbes with bioactive functions. Understanding in what capacity beneficial microbes respond to citricultural practices will help developing recommendations to improve fertilization and pest and disease management programs. These research efforts will narrow the search for active biofertilizers and biopesticides that could be commercialized by agrochemical companies into new green technologies.

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/, SUB12502574 and SUB12495203.

Author contributions

MX: methodology, data collection and analysis, writing original manuscript draft, review and editing. ED: data analysis, scientific guidance and manuscript review. JS: scientific guidance and

manuscript review. AK: project logistics and data collection. MR: secure funding, manuscript review and editing. PR: project conceptualization, secure funding, methodology, data collection and analysis, writing original manuscript draft, and 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

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Isolation and characterization of *Priestia megaterium* KD7 for the biological control of pear fire blight

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Erwinia amylovora is a plant pathogen that causes fire blight disease in Rosaceous plants, such as pear and apple. To develop an effective biocontrol method to suppress *E. amylovora*, a total of 16 bacteria were isolated from pear orchard soil in China and screened for antagonistic activity *in vitro*. Among them, 9 isolates that exhibited antagonistic activity against *E. amylovora* were identified, including *Bacillus atrophaeus*, *Priestia megaterium* (previously known as *Bacillus megaterium*) and *Serratia marcescens* based on the partial 16S rDNA sequence analysis and similarity search. The plate confrontation experiments showed that strain 8 (*P. megaterium* strain KD7) had strong antagonistic activity against *E. amylovora*. The methanolic extract from cell-free supernatant of strain KD7 displayed high antibacterial activities against *E. amylovora*. Furthermore, the active compounds of strain KD7 were separated by thin layer chromatography (TLC) and the amino acids were detected by the presence of a spot with retention factor (Rf) of 0.71. Next, three lipopeptides were identified with high-resolution mass spectrometry (HRMS), including C13-surfactin $[M+H]^+$ at m/z 1008.14, C15-surfactin $[M+H]^+$ at m/z 1036.50, and C14-iturin A $[M+H]^+$ at m/z 1043.17. Strain KD7 showed multiple antibiotic resistance, such as ampicillin, erythromycin, penicillin and tetracycline. The detached pear leaves, twigs and fruits assay showed that both protective and curative action with strain KD7 had the ability to decrease the development of fire blight. Taken together, *P. megaterium* strain KD7 is a potential effective biocontrol agent against fire blight.

KEYWORDS

biocontrol, antibacterial activity, *Priestia megaterium*, extracellular metabolites, fire blight

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora*, is one of the most serious plant diseases that mainly affects Rosaceae, such as apple, pear, medlar and quince. The disease spreads easily and rapidly during warm and moist, especially during the bloom stages. All parts of the plant can be affected by fire blight, including leaves, shoots, flowers and fruits. The characteristic symptoms include wilt and death of flower clusters, wilting and dieback of shoots, and the affected shoots often bent into a 'shepherd's crook' shape (Eastgate, 2000). Since fire blight was first reported in the United States in the 1870s, this disease has spread to more than 50 countries around the world, including countries in North America, Europe, Africa and Asia, causing enormous economic losses to fruit production (Zhao et al., 2019).

Due to the strong ability of survival and migration of *E. amylovora*, as well as high diversity of susceptible hosts, it is difficult to find a unified and effective target for eradication of *E. amylovora*, which makes fire blight difficult to control (Klee et al., 2019). Until now, the common strategy to prevention and control fire blight including pruning of infected twigs and branches, copper compounds (copper hydroxide, tribasic copper sulfate, copper sulfate basic, and copper oxychloride), and antibiotics (streptomycin, oxytetracycline, validamycin, and oxolinic acid) (Joos et al., 2014; Park et al., 2017). However, the role of copper compounds is limit because they only have protective but not curative effect against *E. amylovora*, and can cause side effects in the form of fruit russets, seriously affecting the appearance quality and commercial value (Mikiciński et al., 2019). The use of antibiotics has possible increase the risk of pathogen resistance development and cause environmental problems (McManus et al., 2002; Mikiciński et al., 2019), which has been either not permitted or prohibited in many countries.

Biological control with microbial antagonists is considered as a powerful and eco-friendly alternative in controlling fire blight (Vanneste et al., 2004). Multiple bacterium-based biological control agents have been used to control fire blight, such as *Pantoea agglomerans, Pseudomonas fluorescens*, *Rhanella aquatilis* and *Bacillus subtilis* (Chatterjee, 2001; Stockwell et al., 2010; Sharifazizi et al., 2016). Some bacteria strains have been registered or commercially available and others are in the process of registration. The most famous are *P. fluorescens* A506 (Wilson and Lindow, 1993), *P. agglomerans* P10c (Vanneste et al., 2002), *P. agglomerans* E325 (Pusey et al., 2008), *Pantoea vagans* C9-1 (Ngugi et al., 2010), *B. subtilis* QST713 (Aldwinckle et al., 2002; Bahadou et al., 2017) and BD170 (Broggini et al., 2005). However, the effectiveness of biopesticides for fire blight control was reported generally low and variable because their effects were highly influenced by environmental conditions and disease severities in each orchard, and requires combination with antibiotics (Ngugi et al., 2011). Therefore, new antagonistic bacterial strains with potential characterization and various modes of action are still need to be explored for fire blight disease control.

In recent years, several mechanisms of action have been reported to explain the suppression of *E. amylovora* by antagonistic bacteria, such as the production of secondary metabolites, nutrient competition and colonization. Various antibiotics produced by *P. agglomerans* strains including phenazine, pantocin A and B were confirmed to be effective in suppressing the fire blight pathogen (Wright et al., 2001; Giddens et al., 2002). Lipopeptides produced by *Bacillus* species have been shown to be an efficient agent against *E. amylovora* (Mora et al., 2015). *Pseudomonas* species are reported to compete with *E. amylovora* for space and nutrients (Vanneste et al., 2004; Cabrefiga et al., 2007).

In this study, bacteria from the soil of pear orchard in China were isolated, strains with antagonistic activities against *E. amylovora in vitro* were identified. Among those isolates, strain KD7 which identified as *Priestia megaterium* (formerly known as *Bacillus megaterium*) (Gupta et al., 2020) showed higher antagonist activity against *E. amylovora*, and the antibiotic substances were determined by HRMS techniques. Furthermore, the efficacy of strain KD7 for control of fire blight was evaluated on detached pear tissues. Our results will help for the effective control of fire blight.

Materials and methods

Bacterial strains, culture conditions, and plant material

E. amylovora strain 0017 which was originally isolated from pear in Kyrgyzstan was used as a reference strain. This strain was identity by polymerase chain reaction (PCR) using the primers AMS3/AMS4c, based on chromosomal *ams* gene (Kim et al., 2001). *E. amylovora* cultures were maintained and grown on nutrient agar (NA) (3.0 g/l beef extract, 5.0 g/l peptone, 15.0 g/l agar, and 5.0 g/l NaCl at pH 7.2–7.4) (Schaad et al., 2001).

Two pear varieties, 'duli' pear (*Pyrus betulifolia* Bunge) and Korla fragrant pear, were used to evaluate the biocontrol activity of antagonistic bacteria against *E. amylovora*. The detached leaves and twigs were selected from 'duli' pear, a wildtype pear widely used rootstock for grafting in China due to its resistance to biotic and abiotic stress (Song et al., 2022). The fruits were collected from Korla fragrant pear (*Pyrus sinkiangensis* Yü), a popular cultivated pear variety in China.

Bacteria isolation and identification

The potential bacterial antagonists were isolated from the pear orchard soil in Korla of Xinjiang, China. The surface debris were removed from the soil, and soil samples were collected (5–15-cm depth) in plastic bags and stored at 4°C. Ten grams of soil sample was suspended in 90 ml of sterile distilled water (SDW), under shaken (200 rpm) for 30 min, and the 1.0×10^{-1} dilution was obtained. Then, the supernatant was serially diluted (1:10) in SDW, and 200 µl of the dilutions (1.0×10^{-5} and 1.0×10^{-6}) were plated onto NA medium at 28°C for 24–48 h. The colonies were picked up and streaked on nutrient broth (NB) (3.0 g/l beef extract, 5.0 g/l peptone, and 5.0 g/l NaCl at pH 7.2–7.4) (Schaad et al., 2001) separately, and pure cultures were stored at 4°C for further use.

Phenotypic and biochemical traits of antagonistic bacterial isolates were tested by conventional bacteriological methods. Morphological traits such as colony color and cell motility as well as physiological fingerprints were performed on NA medium. Gram staining was performed as previously described (Ait Bahadou et al., 2018).

Genomic DNA was extracted using CTAB/NaCl method (Ashmawy et al., 2015). The bacterial isolates were identified by PCR of the 16S rDNA gene sequence using fD2 (5′- AGAGTTTGA TCCTGGCTCAG-3′) and rP1 (5'-ACGGTTACCTTGTTACGACTT-3′) primers (Weisburg et al., 1991). The PCR product was purified, sequenced, and submitted to NCBI database[1] to identify matches with existing characterized reference sequences. A phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA 4.0 software with 1000 bootstrap replicates (Tamura et al., 2007).

1 https://blast.ncbi.nlm.nih.gov/

Antimicrobial activity assays

The pathogenic bacterial cells of *E. amylovora* 0017 and antagonistic bacteria were inoculated on NB medium, respectively, and cultured at 28°C with shaking at 200 rpm. After 24 h, the bacterial suspensions were diluted to an OD_{620} of 1.0 (about 1.0×10^8 to 1.0×10^9 /ml) (EPPO, 2013).

The plate confrontation method was used to verify the functional activity of these isolated bacteria. 200 µl of diluted *E. amylovora* suspension was poured into an NA plate (12 cm in diameter), spread and dried at room temperature for 5 min. A single colony of potential antagonistic bacteria was picked up and spotted on the NA plate containing *E. amylovora*. After incubation (28°C, 48 h), the diameter of the inhibition zone was measured, and the antagonistic activity was determined by measuring the diameter of the inhibition zone (mm) around the isolated bacteria.

Antibiotic resistance of *Priestia megaterium* strain KD7

The antibiotic susceptibility of strain 8, namely *P. megaterium* strain KD7, was investigated by the disk-diffusion method as described previously (Jorgensen and Ferraro, 2009). Eight commercial antimicrobial susceptibility disks (Hangzhou Microbial Reagent Co., Ltd., Hangzhou, China), each containing a defined antibiotic concentration were used for this analysis: streptomycin, kanamycin, tetracycline, erythromycin, penicillin, cefotaxime, ampicillin and ciprofloxacin. First, *P. megaterium* strain KD7 inoculum was serially diluted, and 200 µl of 1.0×10^{-5} dilution was spread onto 90 mm diameter NA plates. When the surface of the plate dried, each antibiotic disk was placed on the inoculated plates. Afterwards, the plates with antibiotic disks were incubated at 28°C for 24 h. The appearance of bacterial growth around the disk indicates resistant bacteria, while the inhibitory diameters around the disk indicates sensitive bacteria (Slama et al., 2019).

Antibacterial activity of *Priestia megaterium* strain KD7 against *Erwinia amylovora in vitro*

P. megaterium strain KD7 was grown on NB medium in a shaking incubator at 28°C for 24 h and 200 rpm. The cultures were then centrifuged (6,000 rpm, 10 min), and the supernatant was sterilized through a 0.22 µm filter. The bacterial cells were disrupted by an ultrasonic cell breaker and filter sterilized to obtain sterile liquid inclusion.

The antibacterial activity of these two filtrates against *E. amylovora* was measured by disk-diffusion method (Zaidan et al., 2005). 10 µl of each filtrate was loaded on a sterile filter paper disk (about 6 mm diameter) and placed onto NA plate containing *E. amylovora*. As a negative control, disk was impregnated with 10 µl of SDW. After incubation (48 h, 28°C), the diameter of the inhibition zone around the disk was measured. All experiments were repeated three times independently.

Extraction of active molecules from *Priestia megaterium* strain KD7

P. megaterium strain KD7 was cultured in 100 ml of NB medium for 24 h (180 rpm, 28°C). Bacterial culture was centrifuged for 10 min (6,000 rpm, 4°C), then the supernatant was collected and filtered through a 0.22 µm sterile filter.

Liquid–liquid extraction method was used to isolate antibacterial bioactive compounds from *P. megaterium* strain KD7. This method is a process of transferring compounds from one liquid phase into another based on their relative solubility in two different immiscible liquids (Huddleston et al., 1998). In this experiment, we used three different solvents (n-hexane, ethyl acetate and n-butanol) to obtain the fraction of n-hexane, ethyl acetate and n-butanol, respectively. The above cell-free supernatant was partitioned with n-hexane, ethyl acetate, and n-butanol in sequence, and the ratio of the volume of solvent and initial supernatant was 3:1. The mixture was rotated for 10 min, shaking for 30 min, and then centrifuged for 10 min at 4°C. The solvent layers were collected and dried by an evaporator.

The lipopeptide crude extracts from strain KD7 were obtained according to method previously described (Vater et al., 2002). The cell-free supernatant was adjusted to pH 2.0 with 6 M HCl and stored at 4°C overnight to precipitate the lipopeptides. After centrifugation (6,000 rpm, 10 min), the extracted lipopeptides were dissolved in methanol for *in vitro* evaluating antimicrobial activity.

The antibacterial activity of extracts was evaluated using the disk-diffusion method. 10 µl of each extract was loaded onto a sterile filter paper disk (6 mm diameter), then placed on NA plate containing *E. amylovora* and incubated at 28°C for 24–48 h. Antibacterial activity was determined by measuring the zone of inhibition around the disks.

Thin layer chromatography

The antibacterial components of the extracted supernatant were detected by thin layer chromatography (TLC) (Batrakov et al., 2003). A chloroform-methanol–water mixture (65: 25: 4, v/v/v) was used for the separation, and the spot was visualized by spraying TLC plate with ninhydrin solution. The properties of the separated molecules were determined by calculating the retention factor (Rf) values. The experiments were repeated three times.

Mass spectrometry analysis of antibacterial components

To identify the substances produced by strain KD7, the crude methanol extracts were analyzed by High-resolution mass spectral (HRMS) on an Orbitrap Exploris 480 mass spectrometer and EASY-nLC liquid chromatography system (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) in positive mode. The ion source was set up as follows: capillary voltage: 2300 V, ion transfer tube temperature: 320°C. Detection was performed with resolution of 120,000, scan range from 200 to 1,200 m/z, 1.6 m/z isolation window. Separation of compounds was performed on ChromXP C_{18} column (3 µm, 250 mm × 75 µm, Eksigent) using H_2O (A)/acetonitrile (B) both

containing 0.1% HCOOH as a mobile phase. The injection volume was 2 µl and the flow rate was 0.3 ml min^{-1}.

Evaluation of antagonistic activity of *Priestia megaterium* strain KD7 in detached pear tissues

Under laboratory conditions, the potential protective and curative effect of *P. megaterium* strain KD7 against *E. amylovora* was investigated on detached pear tissues: leaves and twigs of 'duli' pear, and fruits of Korla fragrant pear. The streptomycin was used to evaluate the efficiency of the treatment.

To evaluate the antimicrobial effects of strain KD7 on leaves, the detached leaves of 'duli' pear were washed with tap water, disinfected by immersion in 3% bleach for 10 min, and then washed with SDW three times. For negative control, leaves were injected with SDW (100 µl) into the intercellular spaces on the hollow petiole of the mature leaves using a syringe and then sprayed with SDW to keep all the leaves in the same conditions (Vanneste et al., 2004; Medhioub et al., 2022). The same thing was repeated for positive control by injecting 100 µl suspensions of pathogenic bacteria *E. amylovora* 0017 (1.0×10^8 CFU/ml) instead of SDW. After 48 h of incubation, leaves were sprayed with suspensions of antagonistic bacteria strain KD7 (1.0×10^8 CFU/ml) and streptomycin solution, separately. For protective tests, leaves were sprayed with strain KD7 or streptomycin, separately. After 48 h, leaves were injected with 100 µl pathogenic bacterial suspensions. For curative tests, leaves were treated inversely to the protective treatments. To observe the effect of strain KD7 in the leaves, a treatment control was designed as follows: leaves were sprayed with suspensions of strain KD7. After 48 h, the suspensions of strain KD7 were sprayed on the leaves again. The treated leaves were incubated in a climate chamber (28°C, 12:12 L:D) and symptoms were assessed from 3 to 15 days of post-inoculation (dpi). Three leaves were used for each treatment and all experiments were repeated three times. Disease severity was assessed by evaluating the percentage of leaf area affected (Vincelli and Hershman, 2011). The scale from level 1–13 was used to evaluate foliar necrosis index.

To investigate the antimicrobial effects of strain KD7 on twigs, healthy detached twigs of 'duli' pear were cut into equal-sized pieces (1.5 cm diameter and 20 cm long) and surface sterilized as described above. Each twig was artificial injured before treatment. For negative control, twigs were sprayed with SDW. For positive control, twigs were sprayed with suspensions of pathogenic bacterial (1.0×10^8 CFU/ml). For protective tests, twigs were sprayed with suspensions of antagonistic bacteria strain KD7 (1.0×10^8 CFU/ml) and streptomycin, separately. After 48 h, twigs were sprayed with suspensions of pathogenic bacterial (1.0×10^8 CFU/ml). For curative tests, twigs treated reversely to the protective tests. Three twigs were used for each treatment with three independent experiments. All twigs were incubated at 28°C in darkness for a period of 7 to 15 days. After 15 dpi, disease severity was assessed by measuring the necrosis at the inoculation site based on the vascular browning index of twigs (0–4 scale): 0 = absence of browning, 1 = 1–10% browning, 2 = 11–25% browning, 3 = 26–75% browning, and 4 = 76–100% browning (Medhioub et al., 2022).

To evaluate the antimicrobial effects of strain KD7 on fruits, Korla fragrant pear fruits were surface sterilized and air dried under filter-sterilized air flow. For negative control, three small holes (2 mm wide, 5 mm depth) were made on each fruit and 100 µl of SDW was injected on each hole. For positive control, 100 µl pathogenic bacterial suspension (1.0×10^8 CFU/ml) was injected instead of SDW. After 48 h, wounded fruits were sprayed with strain KD7 (1.0×10^8 CFU/ml) and streptomycin solution, separately. For protective tests, fruits were sprayed with antagonistic bacteria strain KD7 (1.0×10^8 CFU/ml) and streptomycin, separately. After 48 h, wounded fruits were sprayed with suspensions of pathogenic bacterial (1.0×10^8 CFU/ml). For curative tests, fruits treated reversely to the protective treatments. Symptoms were recorded from 6 dpi to 15 dpi. Disease severity was assessed based on a fruit infection index (0–4 scale): 0 = no necrotic spots, 1 = necrotic spots of 1–5 mm in diameter, 2 = necrotic spots of 6–10 mm in diameter, 3 = necrotic spots of 11–20 mm in diameter, and 4 = necrotic spots over 21–30 mm in diameter (Medhioub et al., 2022).

Statistical analysis

SPSS 17.0 and GraphPad Prism 7 were used for statistical analysis and mapping of experimental data, and variance analysis was performed by LSD multiple comparison method ($p \leq 0.05$).

Results

Isolation and identification of antagonistic bacteria strains

A total of 16 bacterial strains were isolated from pear orchard soil of infected pear trees, among which nine strains showed effective antagonistic activity against *E. amylovora* using the plate confrontation method (Figure 1A). The diameter of the inhibition zones of the antagonistic bacteria against *E. amylovora* varied from 11.17 ± 0.60 to 19.57 ± 0.23 mm. Among them, strain 8 exhibited stronger inhibition activities against *E. amylovora*, with the diameter of the inhibition zone reaching 19.57 ± 0.23 mm (Figure 1B), which was larger than that of other antagonistic bacteria. Thus, strain 8 was selected for further characterization and identification.

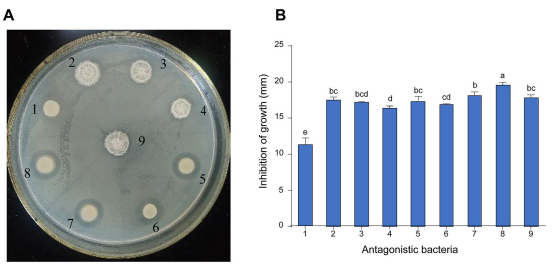

FIGURE 1
Screening and inhibition of *E. amylovora* by antagonistic bacteria. **(A)** The effect of antagonistic bacteria against *E. amylovora*. (1) *Serratia marcescens* D6; (2) *Bacillus atrophaeus* B4; (3) *B. atrophaeus* KB16; (4) *B. atrophaeus* KB14; (5) *Priestia megaterium* KB3; (6) *S. marcescens* D5; (7) *P. megaterium* KB17; (8) *P. megaterium* KD7; (9) *B. atrophaeus* B8. **(B)** Diameter of antagonistic inhibition zone of nine strains. Data with the same letters are not significantly different at $p \leq 0.05$.

Table 1 summarizes morphological, physiological and biochemical traits of nine antagonistic bacteria. Results of Gram staining showed that seven antagonistic bacteria (2, 3, 4, 5, 7, 8 and 9) were Gram-positive, rod-shaped producing white colonies. Furthermore, all bacterial isolates were motile, and showed positive to glucose, saccharose, catalase, gelatin liquefaction and starch hydrolysis. All isolates showed MR reaction negative, while three isolates (5, 7 and 8) showed V-P test negative. According to these phenotypic fingerprints, the selected putative antagonistic bacteria against *E. amylovora* were grouped into the following genera: *Bacillus* (2, 3, 4, and 9), *Priestia* (5, 7, and 8) and *Serratia* (1 and 6).

A phylogenetic tree based on partial 16S rDNA sequence of isolated strains and other bacterial species from the GenBank database was constructed. As shown in Figure 2, strain 2, 3, 4 and 9 shared the highest sequence similarity (>99%) with *Bacillus atrophaeus* (AB680855.1, AB681057.1 and AB363731.1), which was identified as *B. atrophaeus*. Strain 5, 7 and 8 shared the highest sequence similarity (>99%) with *B. megaterium* (KJ155812.1 and MT626661.1), which was identified as *P. megaterium*. Strain 1 and 6 shared the highest sequence similarity (>99%) with *Serratia marcescens* (AJ233431.1 and AB594756.1), which was identified as *S. marcescens*. The 16S rDNA sequences of the 9 isolated strains were submitted to the NCBI for GenBank accession numbers as follows: strain 1 (*S. marcescens* D6, OP565006.1), strain 2 (*B. atrophaeus* B4, ON878208.1), strain 3 (*B. atrophaeus* KB16, OP565005.1), strain 4 (*B. atrophaeus* KB14, OP565018.1), strain 5 (*P. megaterium* KB3, OP565019.1), strain 6 (*S. marcescens* D5, ON878364.1), strain 7 (*P. megaterium* KB17, OP565009.1), strain 8 (*P. megaterium* KD7, OP565020.1), and strain 9 (*B. atrophaeus* B8, OP565021.1).

Antibacterial activity of *Priestia megaterium* strain KD7

Antibacterial activity of strain 8 (*P. megaterium* KD7) against *E. amylovora* was checked by disk-diffusion method. The cell-free supernatant (CS) of strain KD7 exhibited antimicrobial activity compared to the bacterial cell disruption (BD) (Figure 3A), indicating that the extracellular secondary metabolites secreted by strain KD7 contribute to the antibacterial activity. The growth inhibition activity against *E. amylovora* was detected by the four extracts from strain KD7, which were the extract with methanol (EM), n-hexane (EH), ethyl acetate (EEA) and n-butanol (EB). After incubation, only the bacteria culture broth (BC) and methanol extracts (EM) exhibited an antibacterial activity (Figures 3A,B). The methanol extract was further detected by TLC analysis, which showed a spot with Rf values 0.71 after ninhydrin staining (Figure 3C), suggesting the presence of amino acids in the methanol extract.

HRMS (ESI) analysis of lipopeptides

Using the HRMS(ESI) method, two types of lipopeptides were identified from the crude methanol extracts of strain KD7, including surfactin and iturin A (Table 2; Supplementary Figure S1). From the HRMS results, the major products of m/z 1008.14, and 1036.50 were identified as C13-surfactin $[M+H]^+$ and C15-surfactin $[M+H]^+$, respectively, and the major product of m/z 1043.17 assigned to C14-iturin A $[M+H]^+$ (Kalai-Grami et al., 2016; Wang et al., 2022). The results revealed that surfactin and iturin A played important roles in the antibacterial activities of strain KD7.

Antibiotic resistance of *Priestia megaterium* strain KD7

Antibiotic resistance of *P. megaterium* strain KD7 was tested using disk-diffusion method. As shown in Figure 4, strain KD7 was resistant to ampicillin, erythromycin, penicillin and tetracycline, and susceptible to ciprofloxacin, cefotaxim, kanamycin and streptomycin.

Effectiveness on detached pear tissues

Detached pear leaves, twigs and fruits were used to assay the biocontrol efficacy of strain KD7 against *E. amylovora*. In detached

TABLE 1 Morphological, physiological and biochemical characters of nine antagonistic bacteria.

Bacterial isolates	1	2	3	4	5	6	7	8	9
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+	+	+
Color of colony	White	White	White	White	White	White	White	White	White
Gram Staining	–	+	+	+	+	–	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
MR reaction	–	–	–	–	–	–	–	–	–
V-P test	+	+	+	+	–	+	–	–	+
Catalase	+	+	+	+	+	+	+	+	+
Fermentation of glucose	+	+	+	+	+	+	+	+	+
Fermentation of sucrose	+	+	+	+	+	+	+	+	+

"+" and "–" represent positive and negative reactions, respectively.

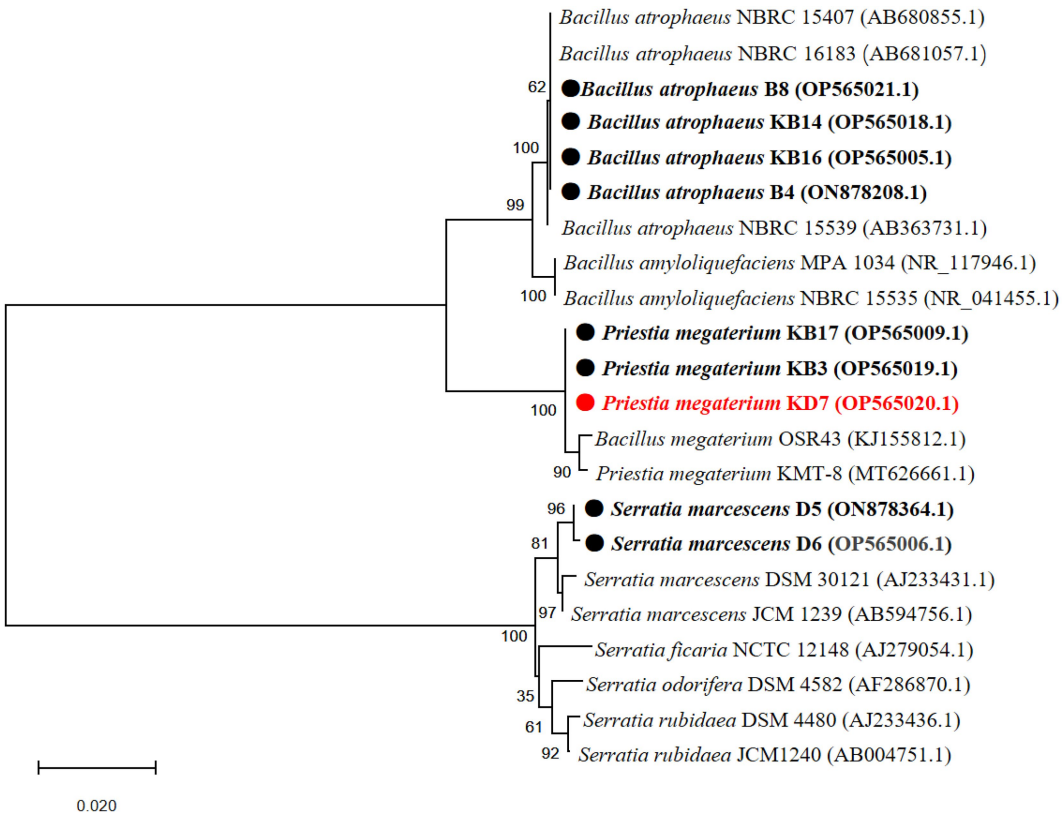

FIGURE 2
Phylogenetic tree of nine antagonistic bacteria isolates with related species on the basis of partial 16S rDNA sequences. The analysis was conducted with MEGA 4.0 using neighbor-joining method with 1,000 bootstrap replicates.

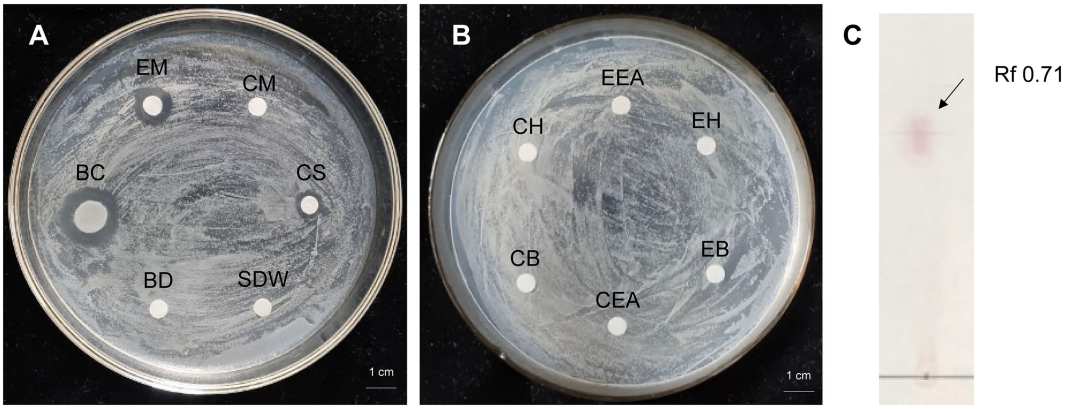

FIGURE 3
The antibacterial activity analysis of organic extracts from *P. megaterium* strain KD7 against *Erwinia amylovora* on NA medium **(A,B)**, and separation of active compounds by thin layer chromatography (TLC) **(C)**. EM, extracted with methanol; CM, methanol control; BC, bacteria culture broth (1.0×10^8 CFU/ml); CS, filtrate from the bacterial cell-free supernatant; BD, filtrate from the bacterial cell disruption; EH, extracted with n-hexane; CH, n-hexane control; EB, extracted with n-butanol; CB, n-butanol control; EEA, extracted with ethyl acetate; CEA, ethyl acetate control; SDW, sterile distilled water (control).

'duli' pear leaves assay (Figures 5A, 6A), curative treatments with strain KD7 reduced the disease severity significantly compared to the control. In the positive control group, necrosis occurred by 3 dpi, exudates released and necrotic spots appeared from the wound site of leaf on the 6th dpi. From 9 to 15 dpi, the remaining leaves were affected rapidly with a foliar necrosis index of 13.00 ± 0.00. By contrast, a small partial of strain KD7-treated leaves appeared disease symptoms, with the foliar necrosis index of 4.72 ± 0.53 and 7.77 ± 0.30 for protective and curative treatment, respectively. Comparatively, foliar necrosis index was 4.74 ± 0.44 and 6.99 ± 0.41 for protective and

TABLE 2 HRMS (ESI) analysis of antagonistic lipopeptides from the crude extracts of strain KD7.

m/z	Lipopeptide assignment
1008.14	C13 surfactin $[M+H]^+$
1036.50	C15 surfactin $[M+H]^+$
1043.17	C14 iturin A $[M+H]^+$

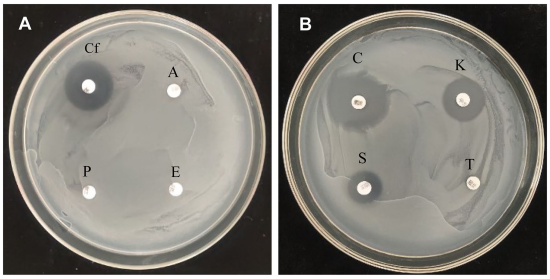

FIGURE 4
Antibiotic sensitivity test of *P. megaterium* KD7. **(A)** *Cf*, ciprofloxacin; A, ampicillin; E, erythromycin; P, penicillin. **(B)** C, cefotaxim; K, kanamycin; T, tetracycline; S, streptomycin.

curative treatment with streptomycin, respectively. The results indicated that strain KD7 exhibited good biological control potential against *E. amylovora*.

In the second experiment on 'duli' pear twigs (Figures 5B, 6B), no necrosis was observed in water-treated twigs (negative control) at 15 dpi. In contrast, the first symptoms began to appear at 3 dpi on *E. amylovora* treated twigs (positive control), and reached the disease incidence by 100% at 15 dpi. Compared with positive control (index = 4.00), vascular browning index was reduced significantly in strain KD7-treated twigs in protective and curative treatments (index = 1.62 ± 0.27 and 2.32 ± 0.28 respectively), which was similar as streptomycin-treated twigs in protective and curative treatments (index = 1.71 ± 0.40 and 2.69 ± 0.27 respectively).

The third experiment on the fruits of Korla fragrant pear (Figures 5C, 6C), positive control inoculated with *E. amylovora* appeared necrosis after 6 dpi, and reached the disease incidence of 100% (index = 4.00) at 15 dpi. However, the infection index was significantly reduced both in both protective and curative treatments with strain KD7 (index = 1.78 ± 0.26 and 2.27 ± 0.37, respectively), indicating that strain KD7 decreased the pathogen development compared with positive control.

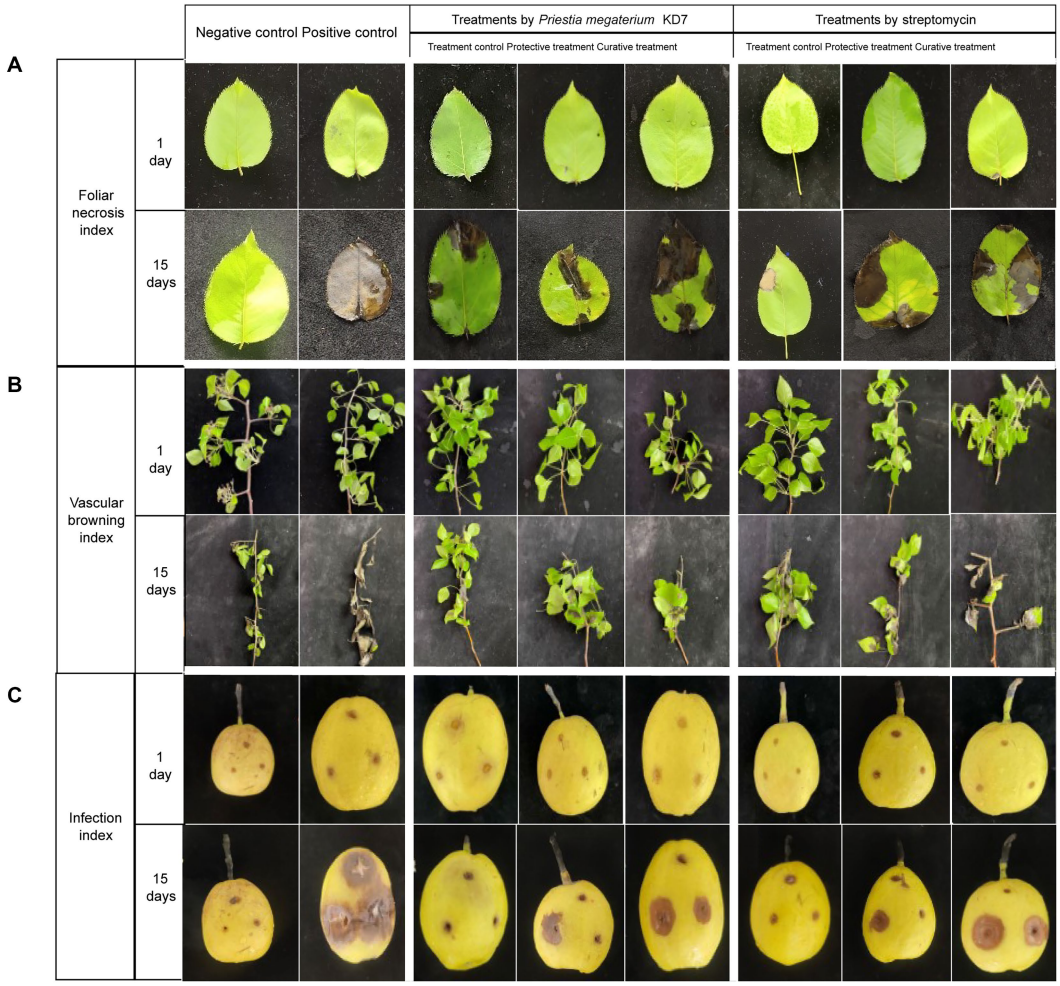

FIGURE 5
Protective and curative effect of *P. megaterium* strain KD7 against *Erwinia amylovora* on detached pear leaves **(A)**, twigs **(B)** and fruits **(C)** after 15days post-inoculation (dpi). The leaves and twigs were collected from wildtype 'duli' pear, and fruits from Korla fragrant pear.

In all *in vivo* assays, disease symptoms and pathogen development in pear tissues treated with strain KD7 were significantly lower than positive control ($p < 0.05$). Furthermore, the effectiveness of protective and curative treatments of strain KD7 against *E. amylovora* were similar to those of streptomycin, indicating its potential biological control agent for controlling fire blight.

Discussion

In recent years, biological control of plant diseases has received increasing attention due to the urgent need to find the eco-friendly alternatives to chemical pesticides (Ongena and Jacques, 2008). In this study, we isolated bacterial antagonists and investigated the inhibition effects against *E. amylovora in vitro* and *in vivo*. A total of 9 bacteria with stable antagonistic against *E. amylovora* were isolated from pear orchard soil. These isolates were identified as *B. atrophaeus*, *P. megaterium* and *S. marcescens*. Among the isolated bacteria, strain 8 (*P. megaterium* strain KD7) showed the highest inhibition effect against *E. amylovora in vitro* experiments. *P. megaterium* is a key microorganism for the biological control of plant diseases which can enhance host plants defenses against diverse pathogens, such as *Septoria tritici* blotch in wheat (Kildea et al., 2008), *Aspergillus flavus* in rice (Mannaa and Kim, 2018). However, little is known about the role of *P. megaterium* in inhibiting fire blight pathogen. Our results showed that the strain KD7 of *P. megaterium* was effective against *E. amylovora* in detached pear tissues, indicating it as a potential biological control agent to control fire blight disease.

It has been shown that *Bacillus* genus against plant pathogens due to the production of antimicrobial compounds (Ariza and Sánchez, 2012). Most important bioactive molecules produced by *Bacillus* genus are non-ribosomal peptides and lipopeptides, polyketides, siderophore and bacteriocins (Fira et al., 2018). *Bacillus* lipopeptides include three families: fengycin, surfactin and iturin (Ongena and Jacques, 2008; Dimkić et al., 2017). The iturins have shown strong antifungal activities against a wide variety of yeast and filamentous fungi but have limited antibacterial activities; The fengycins have strong antifungal activities, especially on filamentous fungi, and their antibacterial activities have been reported recently (Villegas-Escobar et al., 2018); Surfactins have significant bactericidal activities (Zhou et al., 2012; Chen et al., 2020). The molecular mechanisms of disease control for lipopeptides include direct *via* interact with the biological membrane of bacterial and fungal pathogens (Patel et al., 2011), and indirect *via* induction of systemic resistance in plant (Jourdan et al., 2009; Falardeau et al., 2013). *P. megaterium* strains produce a broad spectrum of bioactive lipopeptides, including surfactins, lichenysin, iturin A, fengycins A and B (Pueyo et al., 2009). In this study, both the cell-free supernatant and the methanol extracts from *P. megaterium* strain KD7 *in vitro* showed antibacterial activities against *E. amylovora,* indicating that the bioactive compounds are more likely extracellular secondary metabolites and that these metabolites are hydrophobic, which is the characteristics of *Bacillus* lipopeptides (Romero et al., 2007). Further TLC assay confirmed that the methanolic extracts contained amino acids. Our findings are in accordance with previous studies (Mohammad et al., 2018; Medhioub et al., 2022). The major spot was observed with Rf value of 0.71, suggesting the presence of lipopeptides in the fraction. Similar Rf value (Rf = 0.7) was reported for surfactin lipopeptide by Romero et al. (2007) and Jakinala et al. (2019). Next, two lipopeptides

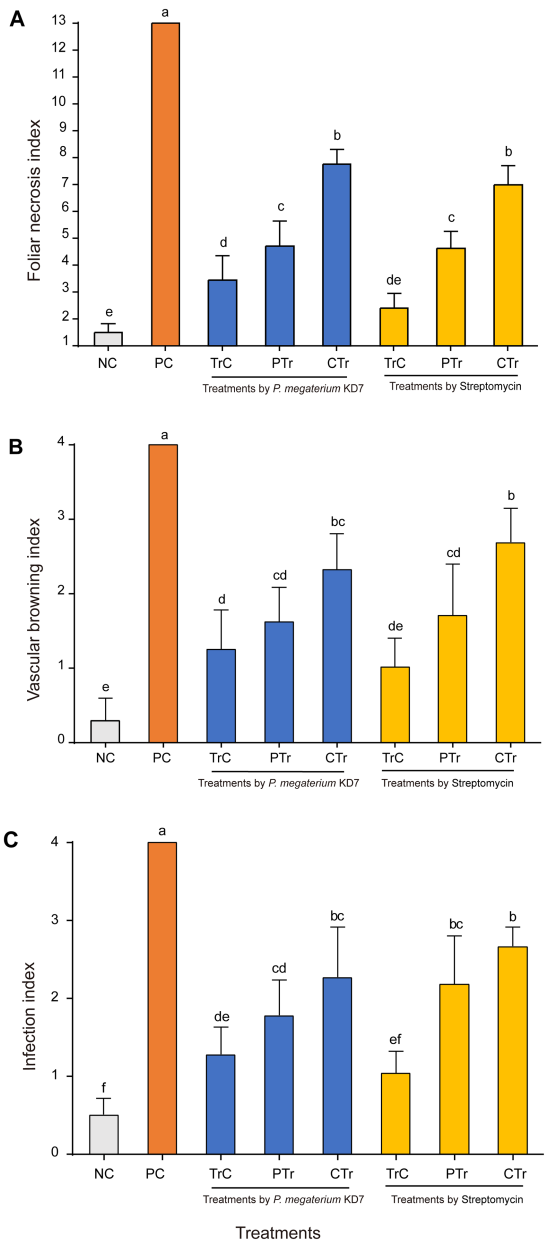

FIGURE 6
The disease index of fire blight in detached pear tissues treated with *P. megaterium* strain KD7 after 15days post-inoculation (dpi): **(A)** leaves, **(B)** twigs and **(C)** fruits. NC, negative control; PC, positive control; TrC, treatment control; PTr, protective treatment; CTr, curative treatment. Means with the same letter are not significantly different at $p \leq 0.05$.

families were identified with HRMS(ESI) analysis, including surfactin and iturin A, indicating that strain KD7 secreted these lipopeptides responsible for antibacterial activities against *E. amylovora*.

Many bacteria have acquired resistance to some common antibiotics and give them the ability to survive antibiotic treatment. So, antibiotics sensitivity evaluation is important in determining if bacteria are resistant to certain antibiotics. Streptomycin is the most effective and widely used chemical control in many countries for fire blight. Previous study (Patel et al., 2017) demonstrated that combining biocontrol agents and antibiotics are non-compatible in biocontrol strategies. In this study, strain KD7 had high levels of multi-resistance to antibiotics, but sensitive

to streptomycin, indicated that strain KD7 and streptomycin were non-compatible and should be applied separately in fire blight control.

In recent years, several authors reported the effect of bacterial antagonists against *E. amylovora* on detached plant tissues. *Lactobacillus plantarum* strains have been demonstrated to be effective in controlling fire blight on pear leaves, flowers and fruits, as well as in whole plants (Roselló et al., 2013). *Pantoea agglomerans* P10c and *B. subtilis* QST713 significantly reduced the incidence of fire blight on detached pear blossoms, and reduced blossom infection under field conditions (Bahadou et al., 2017). The bacterial antagonists (*Pseudomonas vancouverensis* L16, *P. congelans* 35 M, and *Enterobacter ludwigii* 43 M) had high protective ability on apple branches, blossoms and shoots, and even more effective than the copper product (Mikiciński et al., 2019). In this study, *P. megaterium* strain KD7 exhibited significant inhibitory activity against *E. amylovora* on detached pear leaves, twigs and fruits. However, further studies are needed to investigate the efficacy of strain KD7 against fire blight disease under natural environmental conditions.

Conclusion

From the soil environment of pear orchard in China 16 microbial isolates were obtained. By plate confrontation method, some *Priestia*, *Bacillus* and *Serratia* species revealed inhibitory effect against *E. amylovora*. The strain 8 (*P. megaterium* strain KD7) was evaluated *in vitro* and *in vivo* study on detached pear tissues, which is a potential source of a new biocontrol agent to control fire blight.

Data availability statement

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

Author contributions

ZC, LS, and LH designed the work. ZC and LH performed the experiments. WM, LZ, and DG analyzed the data. LS and ZC wrote and revised 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1099664/full#supplementary-material

SUPPLEMENTARY FIGURE S1
HRMS (ESI) analysis of the crude methanolic extract from strain KD7. **(A)** surfactin and **(B)** iturin A.

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AMF species do matter: *Rhizophagus irregularis* and *Funneliformis mosseae* affect healthy and PVY-infected *Solanum tuberosum* L. in a different way

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Arbuscular mycorrhizal fungi (AMF) were documented to positively influence plant growth and yield, which is extremely important for the production of many crops including potato. However, the nature of the interaction between arbuscular mycorrhiza and plant virus that share the same host is not well characterized. In this study, we examined the effect of different AMF, *Rhizophagus irregularis* and *Funneliformis mosseae*, on healthy and potato virus Y (PVY)-infected *Solanum tuberosum* L. The analyses conducted included the measurement of potato growth parameters, oxidative stress indicators, and photosynthetic capacity. Additionally, we evaluated both the development of AMF in plant roots and the virus level in mycorrhizal plants. We found that two AMF species colonized plant roots to varying degrees (ca. 38% for *R. irregularis* vs. 20% for *F. mosseae*). *Rhizophagus irregularis* had a more positive effect on potato growth parameters, causing a significant increase in the total fresh and dry weight of tubers, along with virus-challenged plants. Furthermore, this species lowered hydrogen peroxide levels in PVY-infected leaves and positively modulated the levels of nonenzymatic antioxidants, i.e., ascorbate and glutathione in leaves and roots. Finally, both fungal species contributed to reduced lipid peroxidation and alleviation of virus-induced oxidative damage in plant organs. We also confirmed an indirect interaction between AMF and PVY inhabiting the same host. The two AMF species seemed to have different abilities to colonize the roots of virus-infected hosts, as *R. irregularis* showed a stronger drop in mycorrhizal development in the presence of PVY. At the same time, arbuscular mycorrhiza exerted an effect on virus multiplication, causing increased PVY accumulation in plant leaves and a decreased concentration of virus in roots. In conclusion, the effect of AMF-plant interactions may differ depending on the genotypes of both symbiotic partners. Additionally, indirect AMF-PVY interactions occur in host plants, diminishing the establishment of arbuscular mycorrhiza while changing the distribution of viral particles in plants.

KEYWORDS

***Solanum tuberosum* L., potato virus Y (PVY), *Rhizophagus irregularis*, *Funneliformis mosseae*, mycorrhiza level, oxidative stress, photosynthesis**

Introduction

Under specific environmental conditions, plant growth and physiological traits strongly depend on their interactions with symbiotic and/or pathogenic microorganisms and biotic factors such as viruses. Endophytes inhabiting plants can provide their host with some advantages to survive many environmental stresses (e.g., drought and salinity) and resist phytopathogens. Arbuscular mycorrhizal fungi (AMF), commonly found in soil, create symbiotic associations called arbuscular mycorrhiza (AM, which is a type of endomycorrhiza) with the roots of ~80% of land plants (Brundrett and Tedersoo, 2018). AMF, as biotrophs, depend on carbohydrates and lipids provided by plants for the completion of their life cycle. Host plants benefit from AMF mainly under low nutrient supply in soil (i.e., insufficient P and N availability), as AMF hyphae transfer minerals from the soil to plant roots (Bowles et al., 2018; Averill et al., 2019; Chowdhury et al., 2022). Furthermore, mycorrhizal networks extending outside the rhizosphere provide many more advantages to plants, including more efficient water acquisition, translocation of signaling compounds, and enhancement of plant defense mechanisms (Bitterlich and Franken, 2016). Hence, AMF are considered to function as plant bioprotectors and are increasingly used for plant pathogen control (Dey and Ghosh, 2022; Weng et al., 2022).

Endomycorrhizal fungi are essential for supporting soil ecosystem services. AMF used as biofertilizers were documented to positively influence plant growth and yield, which is extremely important for the production of many crops (Tang et al., 2022; Wu et al., 2022), including wheat (García de León et al., 2020), soybean (Marro et al., 2020), pepper (Guzman et al., 2021), and potatoes (Hijri, 2016). Potatoes, one of the most important crop plants worldwide, were reported to establish AM with different AMF species that are an inherent part of the agricultural soil microbiome, i.e., *Rhizophagus irregularis* (basionym *Glomus irregulare*, syn. *R. irregulare*), *Rhizophagus intraradices* (basionym *Glomus intraradices*), and *Funneliformis mosseae* (basionym *Endogone mosseae*, syn. *Glomus mosseae*) (Douds et al., 2007; Lone et al., 2015; Chifetete and Dames, 2020; Deja-Sikora et al., 2020). The application of AMF-based inocula in pot, greenhouse, and field experiments confirmed that arbuscular mycorrhiza can increase potato tuber biomass (Davies et al., 2005; Hijri, 2016; Lombardo et al., 2021) and enhance potato resistance to many different phytopathogens, e.g., *Rhizoctonia solani* (Yao et al., 2002) and *Phytophthora infestans* (Gallou et al., 2011). However, the nature of the interaction between AM and potato viruses, the prominent pathogens of potatoes, is not well characterized (Deja-Sikora et al., 2019). Sipahioglu et al. (2009) suggested that inoculation with *R. intraradices* induced more severe symptoms of disease in plants infected with potato virus Y (PVY). In contrast, in studies by Deja-Sikora et al. (2020), it was found that *R. irregularis* caused no exacerbation of PVY infection but reduced oxidative stress in plants impacted by the virus. Similarly, the results of other studies using different combinations of solanaceous host plants, AMF species, and virus groups/genotypes were also inconclusive (Shaul et al., 1999; Miozzi et al., 2011). Therefore, the role of AMF in the course of plant viral disease development remains unclear. Based on data published to date, it seems that AM consistently contributed to increased photosynthetic activity in virus-challenged plants (Sipahioglu et al., 2009; Miozzi et al., 2011; Deja-Sikora et al., 2020). In some investigations, improved photosynthetic parameters corresponded to a more severe impact of the virus and to elevated accumulation of viral particles in mycorrhizal plants (Miozzi et al., 2011). In other studies, despite higher photosynthesis levels, viral infection remained asymptomatic in AMF-colonized plants (Deja-Sikora et al., 2020). It was proposed that there may exist a different level of functional compatibility between symbiotic partners (measured as an amount of P transport by fungal hyphae to plant), which vary with the AMF species and host genotype (Ravnskov and Jakobsen, 1995; Duffy and Cassells, 2000; Singh et al., 2012; Yang et al., 2012). This means that specific host varieties may interact with AMF species in a different way (Fiorilli et al., 2022). Furthermore, host–AMF compatibility may be modulated by soil properties, showing that multiple factors influence the final effect of plant–fungus interactions (Duffy and Cassells, 2000). As this interesting phenomenon is not fully clarified, we decided to check how the same host interacts with two different AMF species, especially when the host is additionally impacted by the phytovirus.

The aim of this investigation was to compare the effect of two AMF species, i.e., *R. irregularis* and *F. mosseae,* on growth parameters, oxidative stress level, and photosynthetic capacity in potato cv. Pirol infected with PVY. The fungal species used in our study belong to different taxonomic groups (i.e., genera). We expected that the outcome of plant–AMF interactions would change because of different levels of mycorrhiza. Based on the obtained results, we wanted to evaluate which of the two AMF species provides more benefits to the host impacted by viral pathogens. Additionally, we intended to examine the interaction between AMF and PVY inhabiting the same plant, as all biotic factors sharing the same host may influence not only the host but also each other. We hypothesized that PVY would contribute to worsening host quality. This was expected to disturb arbuscular mycorrhizal development in plant roots resulting in (i) decreased AMF colonization intensity and (ii) lowered influence of AMF on plant condition. At the same time, we assumed that arbuscular mycorrhiza would affect virus multiplication and distribution in plant organs.

Materials and methods

Experimental design

Potato plants used in the experiment originated from *in vitro* cultures. After acclimatization, each plant was transferred to a pot with sterile sand, and the experiment started. Pots were put together in pairs consisting of one PVY-free (PVY–) and one PVY-infected (PVY+) potato plant. Plants from one pair were placed into the same isolated tray compartment. The roots of both plants were separated from each other by a stainless steel grid (pore size 30 μm) replacing one of the pot walls and covering the pot bottom. Grid pores were sufficiently large to allow AMF hyphae to pass through (Supplementary Figure 1). Control plants were grown without fungi, while the other plants were inoculated with spores of AMF. Thus, we received six experimental variants, i.e., two controls marked as P^{PVY-} and P^{PVY+}, and four inoculated with two species

of AMF (*F. mosseae* and *R. irregularis*) marked as P^{PVY-}+ Fm, P^{PVY+}+ Fm, P^{PVY-}+ Ri, and P^{PVY+}+ Ri. Each pair of variants was analyzed in four biological replicates consisting of six technical replicates of plants.

Biological material

Potato virus Y-infected and virus-free plantlets of potatoes (*Solanum tuberosum* cv. Pirol) were grown *in vitro* on solid MS medium under the following conditions: light intensity of 200 μmol m^2 s^{-1}, long photoperiod of 16 L/8 D, and temperature of 17.5–18°C. Two species of AMF, i.e., *R. irregularis* and *F. mosseae*, were purchased from INOQ GmbH (Schnega, Germany). AMF spores were prepared for inoculation according to the instructions provided by the manufacturer. The PVY strain used in the experiments belonged to the N:O/N-Wilga group and caused systemic infection in the host (Deja-Sikora et al., 2020).

Pot experiment

Two-week-old potato plantlets with developed roots (at least one, 1-cm long) were removed from the MS medium and rinsed with sterile water until the roots were free of agar. Plantlets were transferred to 0.5-liter pots filled with sand that were sterilized two times in an autoclave (121°C, 45 min). The experiment consisted of six variants, as shown in Figure 1. The inoculation of plants with AMF was performed by applying 50 mg of powder inoculum (number of propagules–250 million/kg) directly onto the root during plantlet transfer from *in vitro* culture to the sand. Plantlets were watered with 50 ml of Hoagland medium with a 10-fold decreased concentration of phosphate (P 0.1 mM) and covered with a foil tent to enable acclimatization. The adaptation period lasted 3 weeks. The foil tent was perforated in the last week to provide air exchange. After acclimatization, plants were watered three times per week with Hoagland medium with increasing P concentrations from 0.15 mM in the fourth week to 0.5 mM in the ninth week. Then, the concentration of 0.5 mM P was maintained until the end of the experiment. Plants were grown for 12 weeks.

Plant growth parameters

Twelve-week-old potato plants were carefully removed from the substrate, and roots were rinsed with tap water. The growth parameters measured during sampling included the fresh weight of shoots, roots, and tubers, shoot length, and the number of nodes and tubers. After 48 h of incubation at 75°C, the dry weights of shoots, roots, and tubers were determined.

Photosynthetic gas exchange

The rate of photosynthetic gas exchange in leaves was examined in the last week of the experiment with a Ciras-3 Portable Photosynthesis System (PP Systems, Amesbury, MA, USA). The measurements were conducted using a PLC3 universal leaf cuvette (leaf area 4.5 cm^2) and a PLC3 LED light unit. During measurements, the following parameters were applied: ambient temperature (~20°C), sunlight-simulating RGBW settings (red: 38%, green: 37%, blue: 25%, and white: 0%), and CO_2 reference–390 μmol mol^{-1}; H_2O reference–70%; PAR internal—the series of three consecutive values of 1,500, 1,800, and 2,200 μmol m^2 s^{-1}. For each plant, the photosynthetic activity of the fourth leaf was analyzed for 10–15 min. When the stabilization of the CO_2 assimilation rate (A—μmol CO_2 m^2 s^{-1}) was obtained at every next PAR value, 5–10 measurements were recorded.

For the A parameter, two variables, reflecting the change in the amount of assimilated CO_2, were calculated according to the formulas: $dA1 = A_{1,800}\text{-}A_{1,500}$ and $dA2 = A_{2,200}\text{-}A_{1,500}$ to compare plant reactions to light intensity changes and reduce the number of pairs during statistical analysis.

Microscopic analysis of mycorrhiza

The intensity of mycorrhizal colonization in potato roots was determined by microscopic analysis using the aniline blue staining/vinegar method for the visualization of fungal structures. The arbuscular mycorrhiza rate was quantified according to the Trouvelot et al. (1986) method. For each plant, 30 images were taken at 100× magnification using a Leica LMD6 microscope (Leica Microsystems, Wetzlar, Germany). Parameters, i.e., the intensity of mycorrhiza (%M) and arbuscule abundance (%A) in the root system, were calculated with Mycocalc software (https://www2.dijon.inrae.fr/mychintec/Mycocalc-prg/download.html).

Oxidative stress indicators: H_2O_2, ascorbate (ASC), total glutathione (GSH) and lipid peroxidation

H_2O_2 levels in plant roots and leaves were examined with the colorimetric method described by Junglee et al. (2014) without freezing immediately after tissue acquisition. Briefly, 150 mg of tissue was homogenized in 1 ml of 0.1% TCA for 5 min. Homogenate (0.25 ml) was added to the reaction mixture containing 0.5 ml of 1 M KI and 0.25 ml of 10 mM potassium phosphate buffer (pH 7.0). For every sample, a background color control was prepared in such a way that water (0.5 ml) was used instead of KI. All samples were protected from light. After centrifugation, clear homogenate (0.2 ml) was transferred to a clean tube and incubated in darkness for 20 min at 20°C. Each series of reactions consisting of three replicates was blanked with its background color controls. The absorbance was measured at 350 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The total pool of ascorbate (tot ASC) and reduced ascorbate (red ASC) were examined according to Gillespie and Ainsworth (2007). A total of 100 mg of plant leaf was homogenized in ice-cold 1 ml of 6% TCA and centrifuged for 5 min at 13,000 *g* and at 4°C. The clear homogenate used for the total ASC assay (200 μl) was mixed with 100 μl of 75 mM potassium phosphate buffer (pH 7.0) and 100 μl of 10 mM DTT (Sigma-Aldrich, Saint Louis, MO,

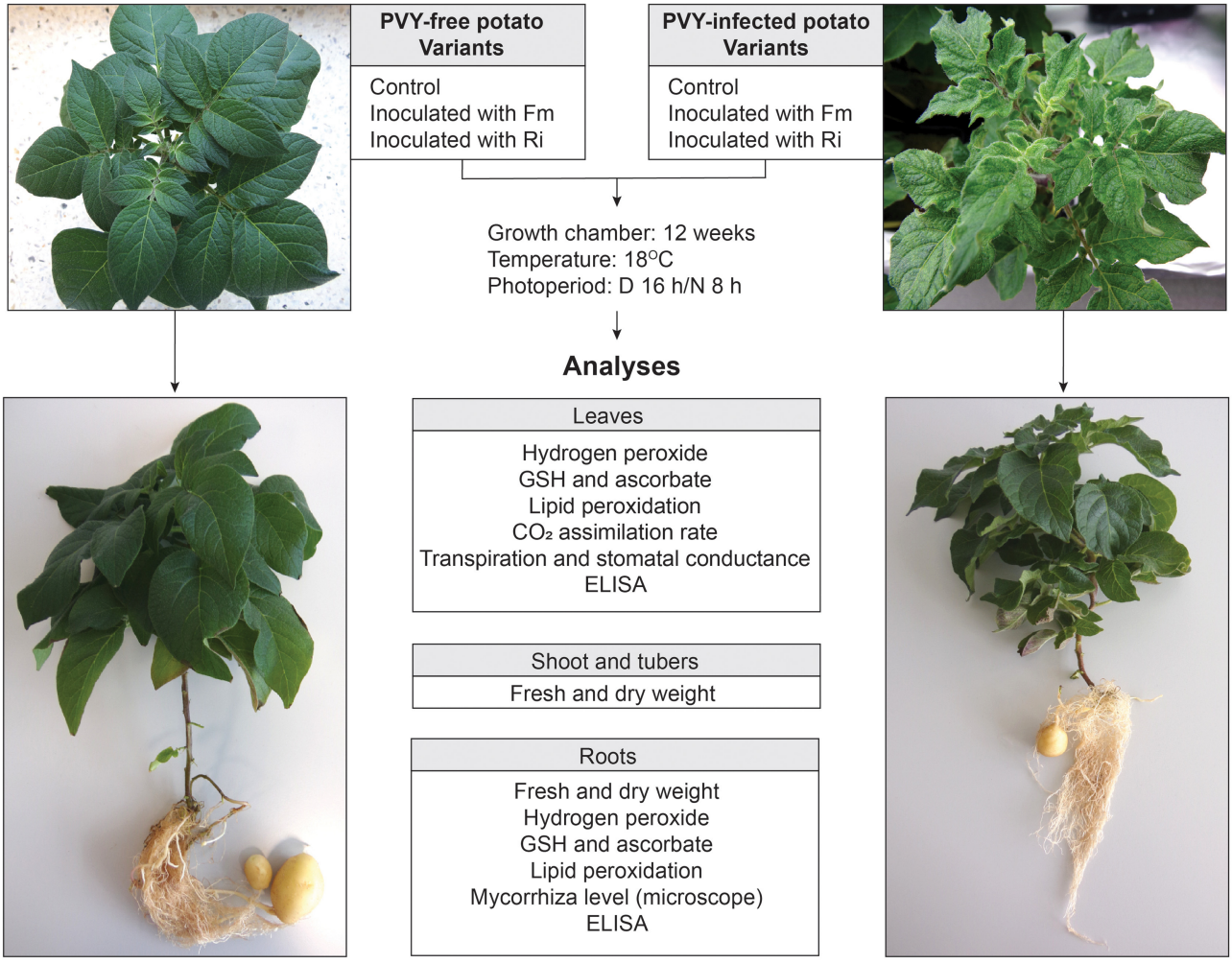

FIGURE 1
Experimental design. The model consisted of healthy and PVY-infected potato cv. Pirol inoculated with two AMF species—*Rhizophagus irregularis* and *Funneliformis mosseae*. Control plants were grown without mycorrhizal fungi. After 12 weeks of incubation plants were uprooted and analyzed according to the included scheme.

USA). After a 10-min incubation at RT, 100 µl of 0.5% NEM was added and further incubated for 1 min. The homogenate used for the reduced ASC assay was mixed with 100 µl of 75 mM potassium phosphate buffer (pH 7.0) and 200 µl of water. Then, both samples were processed in the same way. The following compounds were added to each assay tube: 500 µl of 10% TCA, 400 µl of 43% H_3PO_4, 400 µl of 4% α-α′-bipyridyl, and 200 µl of 3% $FeCl_3$. After incubation for 1 h at 37°C, the absorbance was measured at 525 nm using a Hitachi U-2900 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Oxidized ascorbate (DHA, dehydroascorbate) was calculated from the difference between total and reduced ASC.

The total glutathione was examined with a kinetic method using a Glutathione Assay Kit (Sigma-Aldrich, Saint Louis, MO, USA). The reaction product (TNB) was measured colorimetrically at 412 nm using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA).

Lipid hydroperoxides were checked colorimetrically with a PeroxiDetect Kit (Sigma-Aldrich, Saint Louis, MO, USA). The absorbance was measured at 560 nm using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA).

TAS-ELISA

Potato virus Y levels in the leaves and roots of virus-inoculated potato plantlets were examined using a commercial kit, i.e., ELISA Reagent Set for potato virus Y (Agdia, Inc., Elkhart, IN, United States). The analysis included a commercial positive control for PVY provided with the reagent set. Positive control was used as a standard to prepare a standard curve by two-fold serial dilutions. The range of absorbance, in which the reaction was linear, was used to quantify the virus concentration in plant tissues. The obtained values were relative and calculated based on the standard curve equation but without absolute assessment of viral particles. Values allowed us to determine the degree of change in the PVY level. The ELISA protocol was as follows. A total of 50 mg of leaves from

the fifth node and the same amount of roots were homogenized in the general extract buffer (GEB) at a 1:10 ratio. Then, the leaf homogenate was diluted 10 times, and the root homogenate was diluted two times to fall into the linear reaction range. The assay was performed according to the manufacturer's recommendations. For each series of analyses, one of the standards and a negative control were processed along with the samples.

Statistical analyses

Raw datasets were checked with the chi-square test for observations lying beyond the 75th percentile (Outliers package in R). These values were detected as outliers and removed from further analysis. The data were checked to follow a normal distribution with the Shapiro-Wilk W-test. The homogeneity of group variances was checked with the Levene's test. Two-way ANOVA was applied to check the effect of AMF inoculation on growth and stress parameters in healthy and virus-infected plants. Two-way ANOVA (type II) was calculated in R using the "car" package. A *post hoc* Tukey test was applied to check significant differences between variant pairs. Plots were prepared with R using the "ggplot2" package. In box plots, the following elements are shown: the medium line inside the box indicates the mean value and the whiskers present the standard deviation. Letters assigned to each box mark ANOVA groups. Groups not sharing letters are significantly different ($p \leq 0.05$). A *t*-test was applied to check differences within pairs of observations for photosynthetic parameters. Calculations were performed using R (R Core Team, 2022).

Results

Symptoms of PVY infection in potato

$PVY^{N:O}$-T1 strain infecting potato plantlets (cv. Pirol) under *in vitro* conditions was asymptomatic, as virus-positive plants were indistinguishable from healthy plants. During the pot stage of the experiment, the symptoms of PVY infection appeared on leaves and included a mosaic pattern, leaf crinkling, and stunting (Figure 1). The virus caused neither veinal necrosis nor tuber disease. Arbuscular mycorrhiza had no visible effect on the severity of expressed symptoms.

Mycorrhiza level in potato roots

The presence of AMF spores and hyphae on plant roots was checked at the end of the 12-week incubation. Before the microscopic evaluation of the arbuscular mycorrhiza level, we noticed many spores covering the root surface, which indicated the development of a symbiotic association between the host and fungi (Supplementary Figures 2, 3). Microscopic analysis revealed that the colonization level depended on the fungus used and the presence of PVY infection. After 3 months of plants' incubation, the average mycorrhiza level in PVY-negative plant roots was estimated to be slightly above 38% for *R. irregularis* and nearly 20% for *F. mosseae* (Figure 2A). The arbuscule amount for *R. irregularis* reached 11.8% vs. 1.2% for the other fungal species (Figure 2B). The observed differences were statistically significant, indicating that the two tested fungi colonized potato cv. Pirol roots to varying degrees. In PVY-positive plants, the root colonization level decreased by nearly 10% for each AMF species (for *R. irregularis* from 38.3 to 28.2% and for *F. mosseae* from 19.9 to 10%). At the same time, the arbuscule amount was four- to six-fold reduced, and the negative effect of the virus on plant-*R. irregularis* symbiotic association was stronger (11.8 vs. 1.9%).

The effect of PVY and AMF on potato growth parameters

Potato virus Y itself had no influence on the shoot and root fresh and dry weight (Figures 3A–D). A significant effect of the virus was noticed for the fresh and dry weights of tubers, both of which were lowered by over 60% (Figures 3E, F). In the presence of PVY, tuber yield was dramatically decreased. Similarly, the virus significantly reduced the total dry weight of infected plants by 22.3% compared to healthy plants (Figure 4A). Interestingly, the presence of PVY in plants was associated with a higher total FW/DW ratio (Figure 4B), which showed that PVY-positive plants had a higher water content in tissues.

Surprisingly, AMF contributed to decreased shoot FW (*F. mosseae* by 41% and *R. irregularis* by 30.8%) and shoot DW (*F. mosseae* by 32% and *R. irregularis* by 21.4%) only in virus-free plants. When PVY infection and arbuscular mycorrhiza interacted with the host at the same time (variants PVY+/Fm and PVY+/Ri), plant shoot FW and DW did not significantly differ from those of the healthy control variant (PVY–; Figures 3A, B). The parameters of potato roots colonized with AMF depended on fungal species, as *F. mosseae* and *R. irregularis* acted differently. The first species caused a drop in root FW by ∼23% for both variants PVY– and PVY+; however, the result was statistically insignificant. When noninoculated variants PVY– and PVY+ were combined and analyzed with a t-test against joined variants PVY–/Fm and PVY+/Fm, the drop in root FW was significant ($p = 0.00053$). At the same time, *F. mosseae* elevated root DW by 19% (for PVY–) to 56% (for PVY+). Analysis of combined noninoculated and inoculated variants also revealed significance ($p = 0.0008$). *R. irregularis* caused a significant decrease in root FW (by 31%−38%), irrespective of virus presence, and a drop in root DW (by 23%), but only in healthy plants.

The positive interaction between plants and arbuscular mycorrhiza was strongly reflected by the FW and DW of tubers (Figures 3E, F). Inoculation with AMF improved both parameters, alleviating the negative impact of the virus on tuber development (2.8-fold yield loss in PVY+ compared to PVY– plants). The effect of *R. irregularis* was more pronounced and significant each time, causing up to a three-fold increase in tuber FW and up to a four-fold increase in tuber DW, especially for PVY-infected plants.

Irrespective of AMF species colonizing potato roots, arbuscular mycorrhiza significantly improved the total DW of plants by

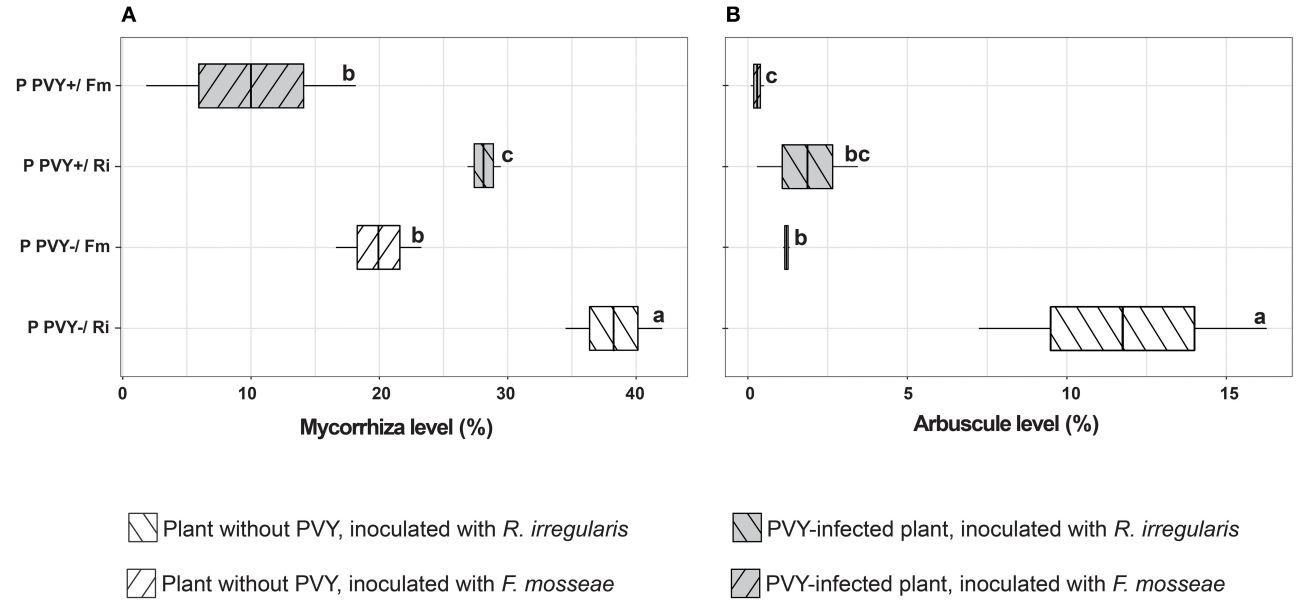

FIGURE 2
Level of mycorrhiza **(A)** and arbuscules **(B)** in healthy and PVY-infected potatoes after 12 weeks of incubation. The statistically significant differences are marked with letters. The same letters indicate no significant differences ($p > 0.05$).

19%−34.5% (Figure 4A), which resulted in a decreased total FW/DW ratio (Figure 4B).

The effect of PVY and AMF on oxidative stress in potatoes

In the absence of arbuscular mycorrhiza, PVY seemed to exert a minimal effect on potato roots. The virus did not influence the H_2O_2 concentration (Figure 5A), total GSH content, or lipid peroxidation level (Figures 6A, C) in this plant organ, yet caused a dramatic decrease in the total ascorbate level (by 40.5%). However, it did not affect the reduced ascorbate pool, which was maintained at the same level as in healthy plant roots (Figures 5C, E). PVY induced different reactions in plant leaves. Virus presence caused significant elevations in the following parameters: leaf H_2O_2 concentration (by 69.4%), total leaf GSH content (by 12.6%), and leaf lipid peroxidation (by 55.4%) (Figures 5B, 6B, D). At the same time, PVY evoked a decrease in leaf total and reduced ascorbate levels (by 26.4 and 40%) (Figures 5D, F).

Potato colonization with *F. mosseae* did not affect the total concentrations of H_2O_2 and GSH, both in roots and leaves, irrespective of the absence or presence of viral infection. Some tendencies were observed; however, the final result was not significant. A minor positive effect of this AMF species was observed for the ascorbate level in PVY-positive plants. Arbuscular mycorrhiza increased the total ascorbate concentration in roots (by 19.2%) and leaves (by 39%) and at the same time elevated the pool of reduced ascorbate in leaves (by 65.5%). Importantly, *F. mosseae* significantly lowered lipid peroxidation levels in the roots (by nearly 39%) and leaves (by 56%) of virus-infected plants.

Rhizophagus irregularis seemed to exert stronger and more diverse effects on oxidative stress parameters in both healthy and PVY-infected potato cv. Pirol. In healthy plants, this species was shown to significantly decrease H_2O_2 (by up to 55%) and total ascorbate content (by up to 40%), for both analyzed organs. Additionally, inoculation with *R. irregularis* lowered pools of reduced ascorbate and total GSH in leaves only. This symbiont exerted no significant effect on the lipid peroxidation level, which was comparable to that of healthy control plants; however, a slight decrease in the value of this parameter could be detected (Figures 6C, D). In virus-positive plants, almost all observed effects of arbuscular mycorrhiza were significant (excluding total root ASC, which was maintained at the same level as in the control). After inoculation, lower levels of H_2O_2 and lipid peroxidation were noticed in the roots and leaves of PVY-bearing plants. The fold change in H_2O_2 was similar to that in virus-negative plants, while lipid peroxidation was reduced by 28%−42.7%. Similarly, the total GSH content in leaves decreased by nearly 20%. Contrary to these observations, other parameters, including the total ascorbate pool in leaves and total GSH content in roots, were increased by 39 and 36%, respectively.

PVY and AMF effects on the photosynthetic capacity of potatoes

Potato virus Y impacted plant parameters related to photosynthesis. A negative effect of the virus was observed for the CO_2 assimilation rate, transpiration rate, and stomatal conductance at high light intensities exceeding 2,000 μmol m^2 s^{-1} (Figures 7A–C). PVY-infected plants showed a very weak reaction

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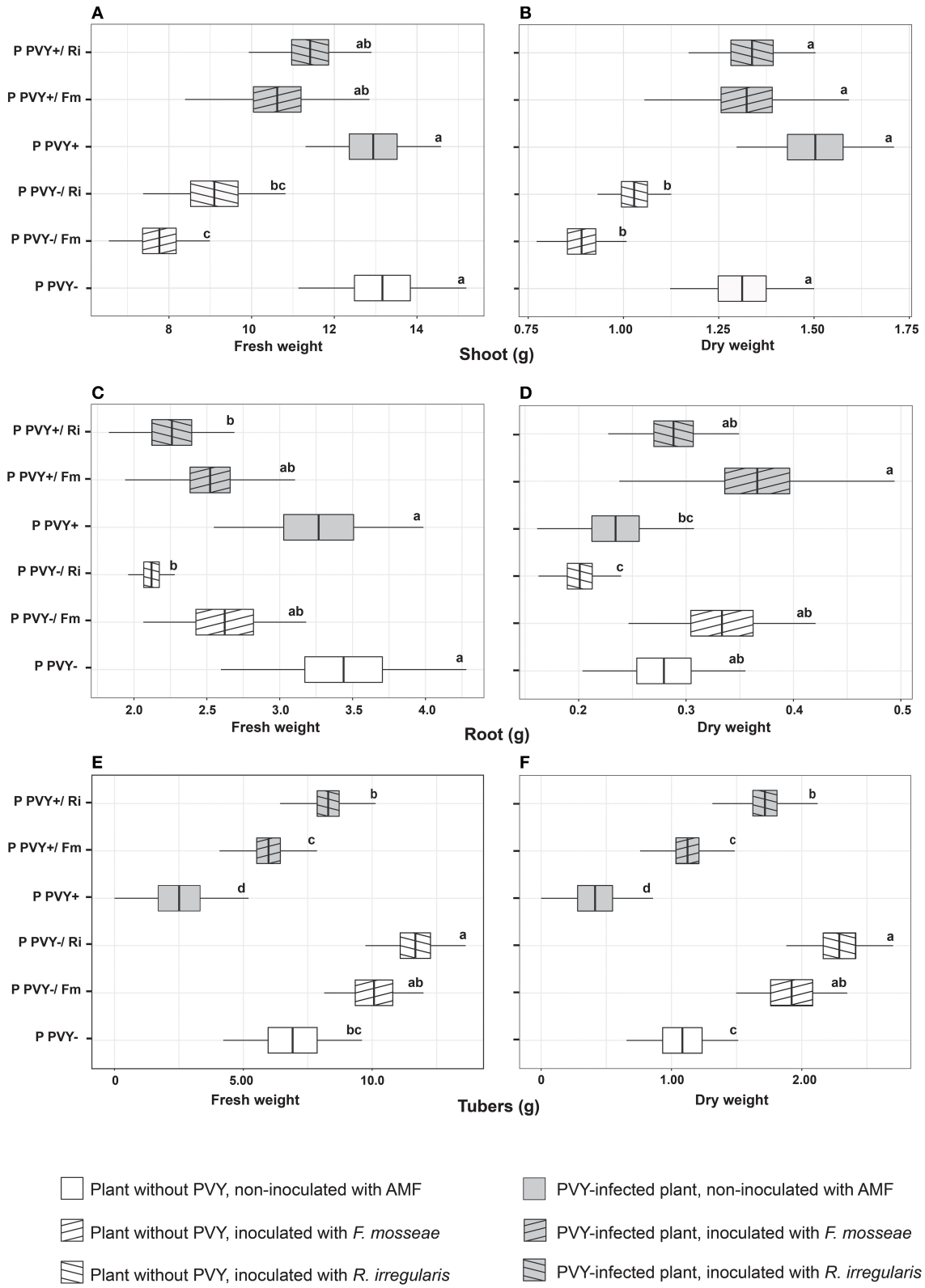

FIGURE 3
The combined effect of AMF inoculation and PVY infection on growth parameters of potato shoots **(A, B)**, roots **(C, D)**, and tubers **(E, F)**. The statistically significant differences are marked with letters. The same letters indicate no significant differences ($p > 0.05$).

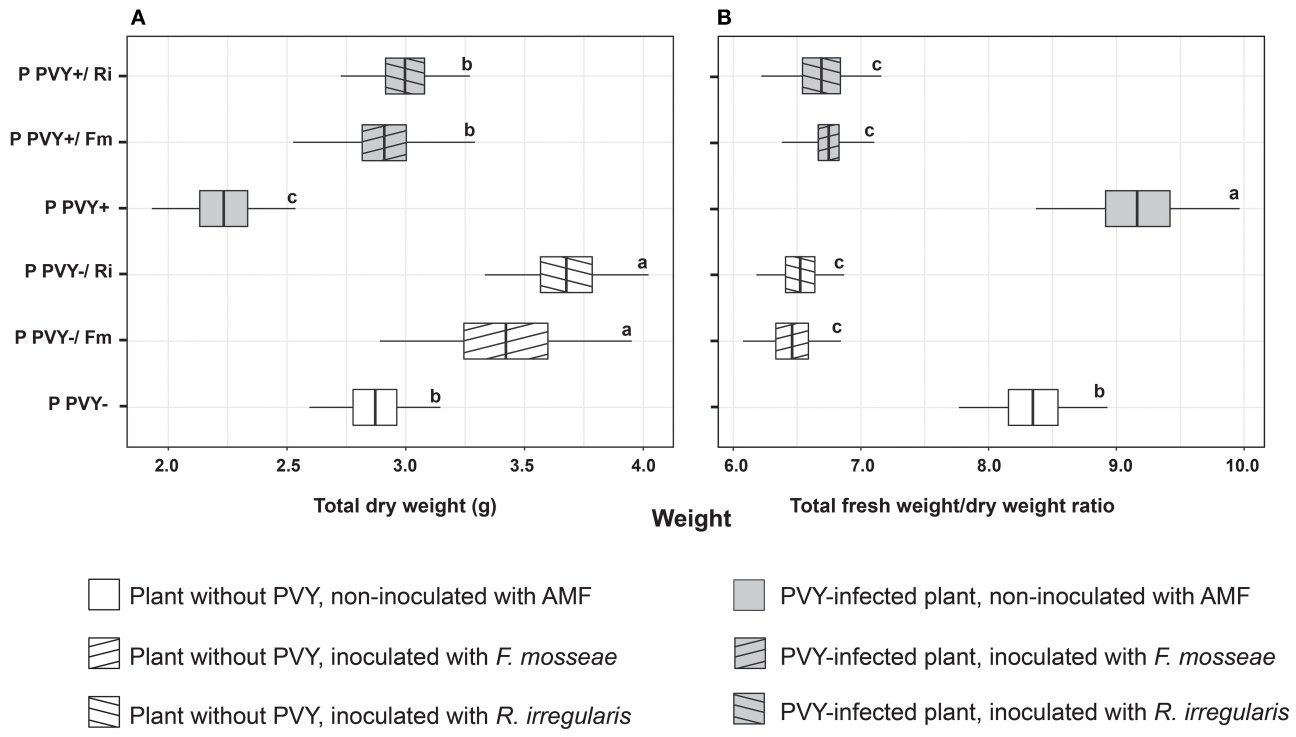

FIGURE 4
The combined effect of AMF inoculation and PVY infection on potato total dry weight **(A)** and ratio of total fresh to dry weight **(B)**. The statistically significant differences are marked with letters. The same letters indicate no significant differences ($p > 0.05$).

(value dA2) to light intensity change (only a 1.5-fold increase) compared to healthy plants (a 4.5-fold increase).

Inoculation of roots with AMF, irrespective of fungal species, significantly improved the photosynthetic capacity of both healthy and virus-bearing plants. Positive effects were noticeable for both species and all photosynthetic parameters. *Funneliformis mosseae* seemed to exert a slightly stronger influence on plant photosynthetic capacity than *R. irregularis*; however, most of the differences were statistically insignificant and could be treated at best as a trend. Interestingly, all plant variants mycorrhized with *F. mosseae* showed the most pronounced increase in the dA2 parameter, reaching the same level of photosynthesis regardless of virus presence (a 4.3-fold increase for healthy plants and a 29-fold increase for infected plants). PVY+/Fm plants regained their photosynthetic capacity as they reacted comparably to PVY–/Fm. Similarly, the transpiration rate in virus-positive individuals was restored to the level measured in healthy individuals upon interaction with *F. mosseae* (reaching a value of nearly 2 mmol H_2O m^{-2} s^{-1} at a light intensity of 2,200 μmol m^2 s^{-1}). At the same time, stomatal conductance followed the same trend; however, the effect of *F. mosseae* was the strongest in virus-free plants.

The effect of AMF on PVY level in potatoes

The PVY content in noninoculated plants (variant PVY+) differed depending on the plant organ and was 13 times higher in leaves than in roots (2.36 vs. 0.166, respectively), which was expected due to virus characteristics. In general, both AMF species influenced the level of viral particles in the host in a similar way (Figures 8A, B), causing an increase in the PVY content in leaves and a simultaneous decrease in the PVY content in roots. Nevertheless, the significance of the observed differences depended on the fungus interacting with the plant. For *R. irregularis*, we noticed only a tendency with no statistical significance, while *F. mosseae* exerted a significant effect on PVY content. This species caused a more than 1.5-fold increase in virus levels in plant leaves (from 2.36 to 3.86) and a 1.32-fold decrease in roots (from 0.166 to 0.126) compared to nonmycorrhizal plants (Figure 8C).

Discussion

Under environmental conditions, plants can host many different types of microorganisms and biotic factors, including viruses. Thus, plant shape and environmental fitness are modulated by multiple interactions between endophytes and phytopathogens inhabiting specific organs of the host. In this study, we demonstrated that AMF and PVY had an impact on both their host and each other. This tripartite interaction resulted in changed potato growth parameters, reduced mycorrhiza levels in roots, and altered accumulation of viral particles in roots and leaves.

Interestingly, we found that PVY infection can negatively impact potato-*R. irregularis* association, causing a dramatic drop in the number of developed arbuscules. At the same time, a similar but less pronounced effect was found for potato-*F. mosseae* symbiosis.

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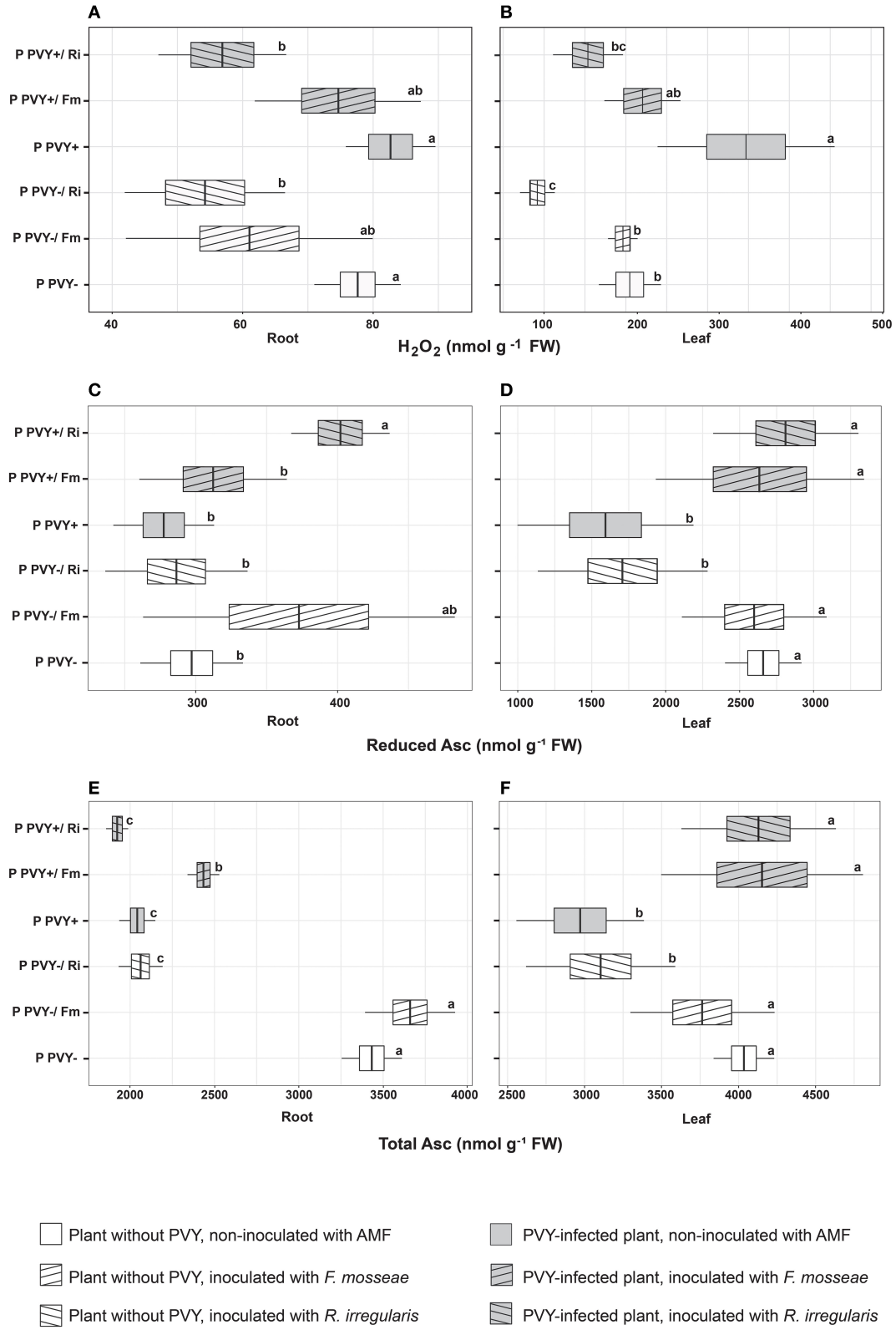

FIGURE 5
The combined effect of AMF inoculation and PVY infection on oxidative stress indicators—hydrogen peroxide **(A, B)**, reduced ascorbate **(C, D)** and total ascorbate level **(E, F)** in roots and leaves of in healthy and virus-infected potatoes. The statistically significant differences are marked with letters. The same letters indicate no significant differences ($p > 0.05$).

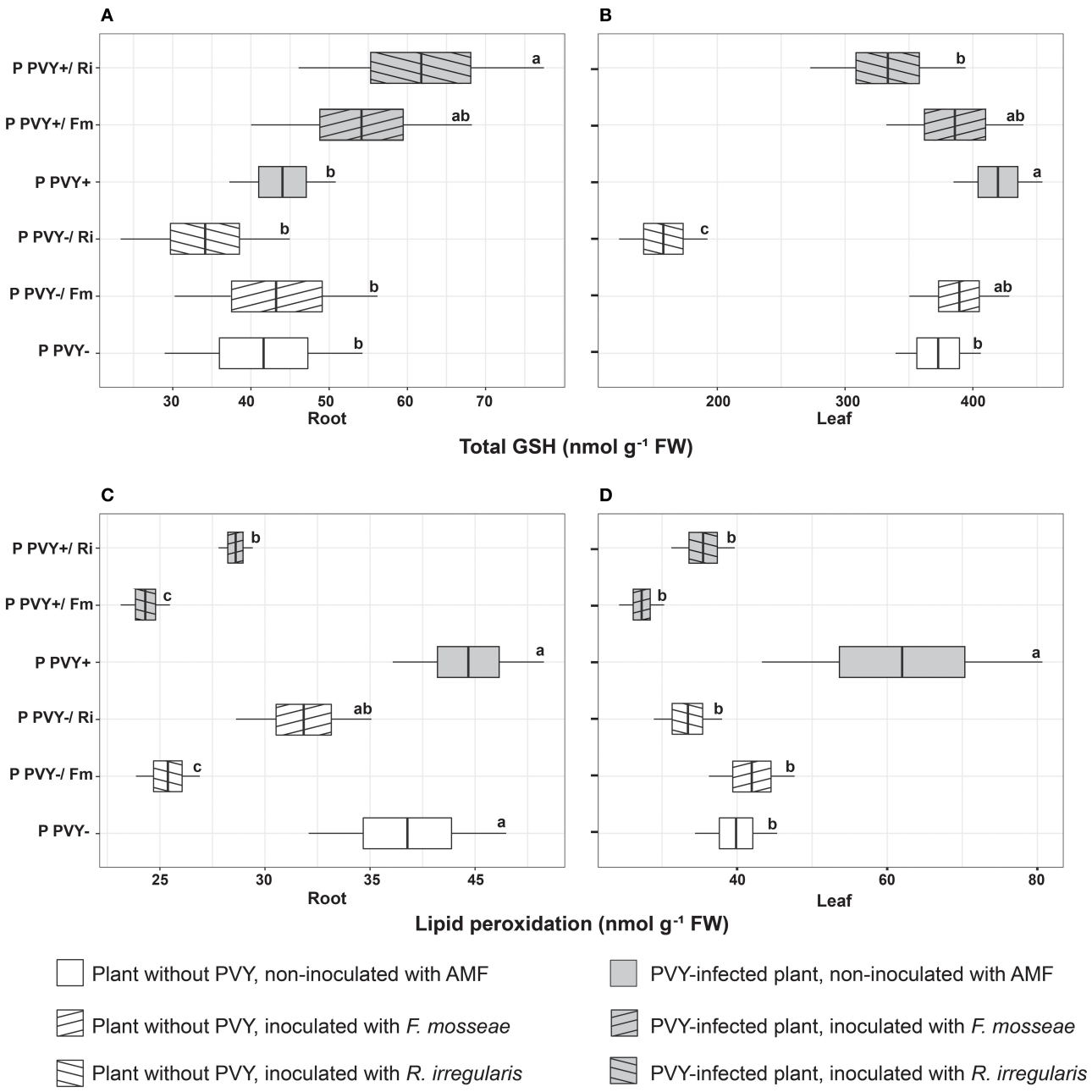

FIGURE 6
The combined effect of AMF inoculation and PVY infection on oxidative stress indicators in healthy and virus-infected potatoes. Measured parameters included total root and leaf glutathione **(A, B)** and lipid peroxidation level in root and leaf **(C, D)**. The statistically significant differences are marked with letters. The same letters indicate no significant differences ($p > 0.05$).

This observation suggests that the two strains of AMF could have different levels of susceptibility to PVY. It is also possible that PVY induced some changes in host plant roots that greatly disturbed potato-*R. irregularis* compatibility. Moreover, the tested AMF species had different capabilities to induce virus translocation from roots to leaves. These phenomena may be associated with the activation of specific plant defense mechanisms, which in turn affect virus concentrations in host organs. The conclusion is based on the altered accumulation of the virus, depending on AMF species colonizing host roots. We found that *F. mosseae* induced greater changes, i.e., a stronger decrease in PVY content in roots and simultaneously higher accumulation of pathogen in leaves. We compared our observations with the outcomes of other studies. The number of articles on plant virus–AMF interactions is limited. Furthermore, these studies involved different plant-virus-AMF models. Some previous reports indicated that AMF lowered virus concentrations in plant organs (Maffei et al., 2014). In other reports, AM fungi were associated with elevated virus levels (Daft and Okusanya, 1973; Miozzi et al., 2011). Our finding is consistent with the results described by Sipahioglu et al. (2009),

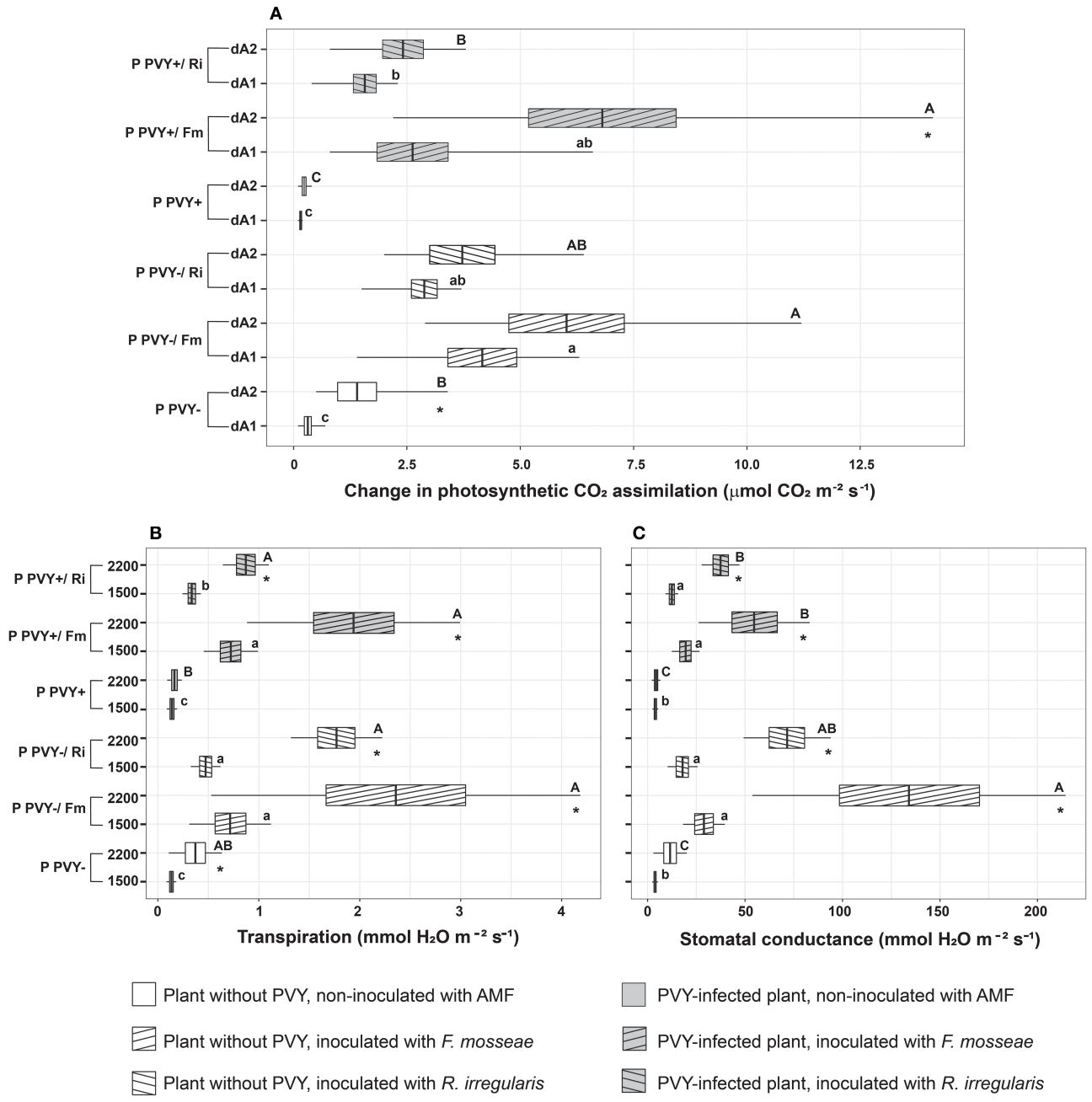

FIGURE 7
The combined effect of AMF inoculation and PVY infection on photosynthetic capacity in healthy and virus-infected potatoes. Measured parameters included CO_2 assimilation rate **(A)**, transpiration **(B)**, and stomatal conductance **(C)**. Values dA1 (A_{800}-A_{500}) and dA2 ($A_{1,200}$-A_{500}) show changes in the assimilation rate between two different light intensities. The statistically significant differences are marked with letters. The same letters indicate no significant differences ($p > 0.05$). The asterisk means significant difference between each two parameters measured for the experimental variant (for **A**: dA1 and dA2; for **B, C**: 1500 and 2200).

where *R. irregularis* stimulated an increase in the PVY reproduction and accumulation rate in leaves of potato cv. Marfona. Moreover, *F. mosseae* also induced long-term accumulation of phytovirus (tomatoes spotted wilt virus) in leaves of other solanaceous plants, i.e., tomatoes (Miozzi et al., 2011). However, none of these studies evaluated the effect of AMF on virus content in plant roots, which are the target organ for arbuscular mycorrhiza. We demonstrated that AM-induced changes in phytopathogen levels occurred in both leaves and roots. On the other hand, the lower the concentration of the virus in roots, the lower the impact of this pest on the symbiotic plant-fungus association. Interestingly, several mechanisms were proposed to link virus accumulation with arbuscular mycorrhiza (Hao et al., 2019). One of them involves viral multiplication due to increased P supply in plants expressing mycorrhiza-specific phosphate transporters PT4 (Daft and Okusanya, 1973; Lacroix et al., 2017). Better nutritional status

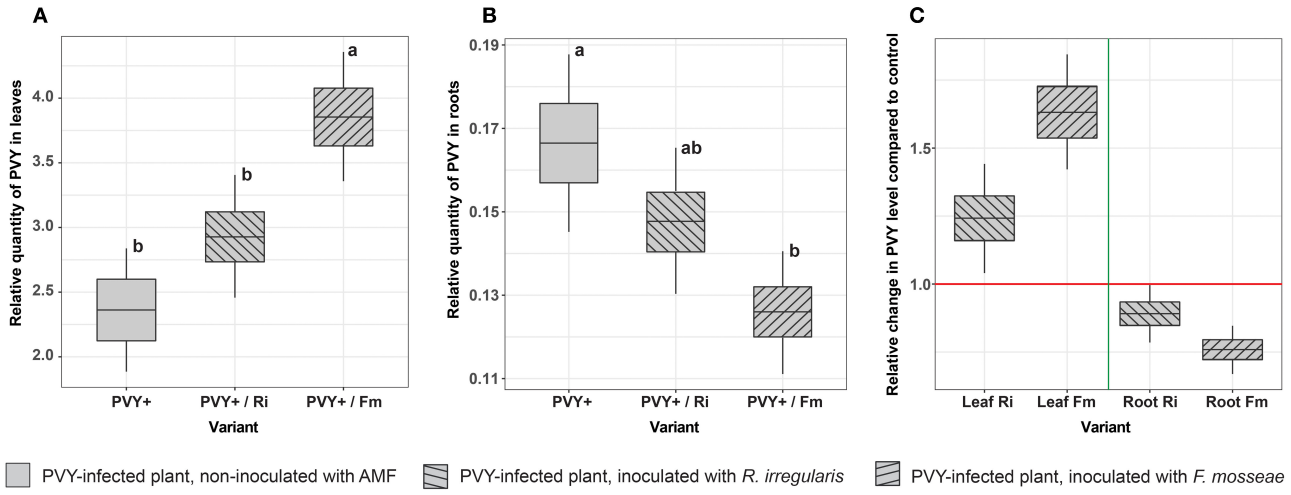

FIGURE 8
Effect of AMF on the relative quantity of PVY in leaves **(A)** and roots **(B)** in virus-infected potatoes. Each value is calculated as a ratio of the PVY level in mycorrhizal plants to that in nonmycorrhizal (control) plants. **(C)** Shows the fold change in the PVY level between mycorrhizal and nonmycorrhizal plant roots. The statistically significant differences are marked with letters. The same letters indicate no significant differences ($p > 0.05$).

of the mycorrhizal plant can enhance plant tolerance to virus pressure by compensating for the harmful effects of the pathogen (Hao et al., 2019). The other mechanism may be related to the downregulation of specific defense factors, e.g., pathogenesis-related (PR) proteins, heat-shock (HS) proteins, and glutathione S-transferase (GST) in virus-infected mycorrhizal tomatoes as was reported by Miozzi et al. (2011). Authors noticed that reduced activity of defense proteins was linked to increased infectivity of the virus in tomatoes and corresponded to virus accumulation in plant leaves. Finally, enhanced multiplication of the virus in mycorrhizal plants may be related to AMF-dependent improvement in their photosynthetic capacity. It was shown that infection sites can function as photosynthetic carbon sinks (Herbers et al., 2000; Zanini et al., 2021) to provide resources for virus replication. Thus, more efficient assimilation of CO_2 due to mycorrhiza could result in more photosynthates, which in turn may positively affect virus development in potatoes.

Contrary to the results of the *in vitro* experiment (Deja-Sikora et al., 2020), where the colonization of potato cv. Pirol with *R. irregularis* did not influence shoot and root fresh weights, the effect obtained in the pot experiment was different. We noticed no positive effect of the two tested AMF species on either shoot or root growth (i.e., FW and DW). Interestingly, *R. irregularis* seemed to induce a stronger decrease in the values of the mentioned parameters than *F. mosseae*. On the other hand, healthy and PVY-infected plants inoculated with AMF produced significantly higher tuber yields, reflected by increased tuber FW and DW, and the effect was greater for *R. irregularis*. Furthermore, the total DW of PVY-free and PVY-positive mycorrhizal plants was significantly enhanced compared to that of the control and did not differ between the tested AMF species. The obtained results showed that AM improved the growth capacity of potatoes, independent of virus presence, but the effect could be seen mostly for belowground parts of plants, especially tubers. Our finding is in agreement with other communications, indicating that AMF improved potato yield quality and quantity (Duffy and Cassells, 2000; Douds et al., 2007; Hijri, 2016). Hijri (2016), who performed 3-year field trials, reported that *R. irregularis*-treated potato seeds gave nearly 10% higher yields than noninoculated controls. Similarly, Douds et al. (2007) revealed a 10 to 20% higher potato yield after the application of *R. irregularis* inoculum. Finally, Duffy and Cassells (2000) concluded that the final effect of plant–AMF interactions depends on the species used and can result in either a yield increase or decrease, as host–fungus functional compatibility is an important factor modulating an outcome (Ravnskov and Jakobsen, 1995; Lone et al., 2020; Santander et al., 2021; Fritz et al., 2022). We expect that the gain in tuber biomass observed in our experiment resulted from the better nutritional status of plants associated with AMF, as indicated by Liu et al. (2018) and Yang et al. (2020). The authors showed that arbuscular mycorrhiza was involved in enhanced phosphorus, nitrogen, and potassium acquisition by potatoes. P and N are key for plant biomass development, while K is important for plant osmoregulation (Clark and Zeto, 2000).

Surprisingly, the positive effect of arbuscular mycorrhiza on potato yield can be dramatically changed by phytoviruses. Sipahioglu et al. (2009) observed an 85% drop in tuber weight only when PVY and *R. irregularis* were present at the same time. Potato cv. Marfona almost completely inhibited tuber development as a result of virus–fungus interactions. In our study, the PVY-infected potato cv. Pirol reacted positively to both AMF species. As expected, the virus itself disturbed tuber production causing more than two-fold yield loss. The application of *F. mosseae* elevated tuber biomass to the level reached by healthy plants, while inoculation with *R. irregularis* showed an even stronger effect. In addition, we noticed no symptoms of viral disease exacerbation. We conclude that the positive effect of AM on PVY-infected potatoes is directly related to lowered levels of oxidative stress and improved photosynthetic capacity in mycorrhizal plants.

We measured different indicators of oxidative stress to assess the physiological condition of plants colonized with PVY and AMF. In our experiments, *F. mosseae* seemed to have a minimal

effect on the oxidation status in healthy plants, as only a slightly lowered concentration of root H_2O_2 was noticed. Nevertheless, this change corresponded to a decreased lipid peroxidation in root cells. Again, *R. irregularis* regulated H_2O_2 levels and elements of the host antioxidative system much more strongly. In the absence of PVY, *R. irregularis* significantly reduced root and leaf levels of H_2O_2. This entailed further changes comprising decreases in total GSH (leaf), total ascorbate content (leaf and root), and lipid peroxidation (root). The measured parameters suggest that AMF, especially *R. irregularis,* alleviated oxidative stress in potatoes. Interestingly, the two AMF species interacted with the same host with different intensities. The degree of induced changes varied, depending on the species used, which was also noticed in other plant-AMF models (Cao et al., 2020; Malicka et al., 2021; Guo et al., 2022).

In our experiment, host plants responded to PVY with enhanced accumulation of ROS in leaves. This phenomenon was previously reported by many authors (Otulak and Garbaczewska, 2010; Deja-Sikora et al., 2020; Lukan et al., 2020). Additionally, the virus negatively impacted the total ascorbate pools in leaves and roots and simultaneously increased the GSH content, but the difference was significant only in leaves. All changes in the antioxidative system induced by PVY seemed to disrupt the oxidative balance in host plants as strongly increased levels of lipid peroxidation in leaves were apparent. Similarly, antioxidative imbalance and a higher lipid peroxidation rate, which are typical for susceptible host–virus interaction, were described by García-Marcos et al. (2009) in *Nicotiana benthamiana* infected with PVY and PVX. Several studies have reported that the accumulation of ROS upon systemic viral infection can contribute to disease symptom development including mosaic spots and plant deformations resulting in abnormal growth (Riedle-Bauer, 2000; Díaz-Vivancos et al., 2008; García-Marcos et al., 2009). Increased generation of ROS in leaves may be specifically linked to disturbed chloroplast metabolism, i.e., elevated electron leakage from photosynthetic electron transport chains, resulting from the inhibition of PSI and PSII (García-Marcos et al., 2009). This suggestion is supported by our observation of the adverse effect that PVY exerted on photosynthetic efficiency in potato cv. Pirol. Furthermore, Kogovšek et al. (2010) announced that PVY could upregulate the expression of antioxidant system enzymes, i.e., ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione S-transferase (GST), and the effect depended on virus aggressiveness and host genotype. In this study, we noticed significantly increased leaf GSH content, which suggests a rather mild virus–host interaction.

In contrast to previously described results, both AMF species showed more diverse and pronounced effects on PVY-infected potatoes. As leaves are a target for PVY and roots are a place where mycorrhiza is developed, plant antioxidative responses were noted in both organs. In general, potatoes reacted to the virus and AMF with decreased levels of leaf and root H_2O_2. Arbuscular mycorrhiza additionally contributed to the elevation of the total ascorbate pool in leaves, while *R. irregularis* also lowered the GSH content in these organs. In roots, AM seemed to specifically increase GSH levels and either total (*F. mosseae*) or reduced (*R. irregularis*) ascorbate content. Changes in the oxidation status of PVY-positive plants associated with arbuscular mycorrhiza resulted in greatly diminished lipid peroxidation in root and leaf cells, which indicates AMF-induced alleviation of virus-caused stress. Nonenzymatic antioxidants, i.e., ascorbate and GSH, are linked to each other *via* the ascorbate-glutathione pathway used by plants for H_2O_2 reduction in water (Noctor et al., 2012). These results may suggest elevated activity of APX and GR in mycorrhizal plants infected with PVY; however, further confirmation is needed.

Finally, we found that PVY negatively impacted photosynthetic parameters including CO_2 assimilation rate, transpiration, and stomatal conductance during increasing light intensity. The weaker photosynthetic activity of infected plants is possibly related to the photosystem inhibition mentioned earlier. Nevertheless, mycorrhiza improved the photosynthetic capacity of potatoes to such a level that PVY-infected plants reached similar values of the abovementioned parameters as healthy plants. This finding is, in general, in agreement with numerous studies where AMF positively influenced photosynthesis and protected the photosynthetic apparatus, especially under different types of stresses (Chandrasekaran et al., 2019; Gavito et al., 2019; Mathur et al., 2019; Popescu and Popescu, 2022). However, direct improvement of CO_2 uptake efficiency, transpiration, and stomatal conductance in mycorrhizal PVY-infected plants was not demonstrated. Some indirect evidence, based on chlorophyll content analysis, was previously described (Sipahioglu et al., 2009; Deja-Sikora et al., 2020). It was only shown that AMF-colonized healthy potato cv. Pirol had a higher chlorophyll content than the control, but no such result was found in the presence of the virus (Deja-Sikora et al., 2020). Sipahioglu et al. (2009) reported no significant difference in this parameter for mycorrhizal PVY-infected potato cv. Marfona. This means that chlorophyll content may not adequately reflect the photosynthetic capacity of mycorrhizal plants. More reliable results are obtained by recording the CO_2 assimilation rate, which reveals that AMF significantly contributes to the improvement of the photosynthesis process. It is not clear what exact mechanism underlies this finding; however, disinhibition in PSI and PSII (in spite of virus presence) is concluded.

Conclusion

This study indicated that multipartite interactions can take place in plant hosts inhabited by phytopathogens and endophytes. We demonstrated that plant viruses exerted a negative impact on arbuscular mycorrhizal development in host roots. At the same time, AM was associated with an increased rate of virus multiplication in host leaves, possibly due to improved plant nutritional status, lowered activity of defense proteins, or increased plant photosynthetic capacity. Arbuscular mycorrhiza positively affected tuber yield in potatoes, alleviating the negative effect of PVY; thus, the application of AMF inoculum can reduce economic losses caused by the virus. Finally, AMF contributed to a decrease in virus-induced oxidative stress and protected cell lipids from peroxidation. Interestingly, the two tested AMF species interacted with the host variety at different intensities, suggesting that plant–fungus compatibility may be critical for obtaining benefits.

Data availability statement

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

Author contributions

ED-S was responsible for the original manuscript preparation, text review and editing, the design and maintenance of the pot experiment, analyses of plant growth parameters, analyses of oxidative stress indicators, photosynthetic activity measurements, ELISA, and statistical analyses. KW assisted with the *in vitro* cultures of plants and AMF and participated in analyses of the plant growth parameters. KH conceptualized the study, supervised, reviewed the manuscript, and was responsible for the funding acquisition. All authors read and approved the final 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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Supplementary material

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Interaction between the flagellum of *Candidatus* Liberibacter asiaticus and the vitellogenin-like protein of *Diaphorina citri* significantly influences *C*Las titer

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Huanglongbing (HLB) is a global devastating citrus disease that is mainly caused by "*Candidatus* Liberibacter asiaticus" (*C*Las). It is mostly transmitted by the insect Asian citrus psyllid (ACP, *Diaphorina citri*) in a persistent and proliferative manner. *C*Las traverses multiple barriers to complete an infection cycle and is likely involved in multiple interactions with *D. citri*. However, the protein–protein interactions between *C*Las and *D. citri* are largely unknown. Here, we report on a vitellogenin-like protein (Vg_VWD) in *D. citri* that interacts with a *C*Las flagellum (flaA) protein. We found that *Vg_VWD* was upregulated in *C*Las-infected *D. citri*. Silencing of *Vg_VWD* in *D. citri via* RNAi silencing significantly increased the *C*Las titer, suggesting that *Vg_VWD* plays an important role in the *C*Las–*D. citri* interaction. *Agrobacterium*-mediated transient expression assays indicated that Vg_VWD inhibits BAX- and INF1-triggered necrosis and suppresses the callose deposition induced by flaA in *Nicotiana benthamiana*. These findings provide new insights into the molecular interaction between *C*Las and *D. citri*.

KEYWORDS

CLas, Huanglongbing, *Diaphorina citri*, flagella, vitellogenin

1. Introduction

Citrus Huanglongbing is the most devastating citrus disease worldwide and is associated with the phloem-colonizing pathogenic α-proteobacterium "*Candidatus* Liberibacter spp." Among the three known species "*Ca.* L. asiaticus" (*C*Las), "*Ca.* L. americanus" (*C*Lam), and "*Ca.* L. africanus" (*C*Laf) (Bové, 2006; Andrade et al., 2020), *C*Las is the most aggressive and is transmitted by the Asian citrus psyllid (ACP, *Diaphorina citri*) in a persistent-propagative manner (Andrade et al., 2020; Zhou, 2020). After being ingested by *D. citri* from the phloem sap of diseased citrus, *C*Las initially infects the intestinal epithelium and then crosses the basal lamina into the midgut visceral muscles, from where it spreads into the hemolymph, followed by the salivary glands, and then back into citrus with the *D. citri* feeding process (Wei and Li, 2016; Chen et al., 2022). The protein–protein interactions between the host and pathogen are

extremely important for pathogen colonization, movement, and transmission. Studying the interaction mechanisms between *C*Las and *D. citri* is important for finding solutions for this disease. However, little has been reported on the abovementioned issue.

The bacterial flagellum is a complex organelle embedded within the cell envelope that has long extracellular helical filaments (flagellar filaments). The flagellum is critical for bacterial motility, niche colonization, and pathogenesis (Subramanian and Kearns, 2019). As a transboundary pathogen, *C*Las retains a complete family of 30 flagella-encoding genes on its significantly reduced genome, among which the flagellin (flaA, CLIBASIA_02090) gene encodes monomers of the filaments (Duan et al., 2009). *C*Las *flaA* encodes 452 amino acids and contains a conserved 22-amino acid domain (flg_{22}) at positions 29 to 50 of the N terminus. It has pathogen-associated molecular pattern (PAMP) activity and induces plant innate immunity, including plant cell death and callose deposition (Zou et al., 2012). The expression pattern of *C*Las flagellar region genes varies in different hosts, with high expression in *C*Las-infected *D. citri* and low or no expression in susceptible *Citrus* plants (Yan et al., 2013; Andrade et al., 2020). No flagellar morphology was observed in *C*Las from citrus samples, and flagella-like structures in *C*Las were observed from the midgut of the *C*Las-infected *D. citri* (Andrade et al., 2020). *C*Las traverses multiple barriers in *D. citri* to complete the infection cycle, and the flagella enhance its motility to a favorable location for better colonization. Genomic and transcriptomic studies indicate that *D. citri* does not have an intact immune system like model insects such as *Drosophila* (Wulff et al., 2014; Vyas et al., 2015; Wang et al., 2017). Due to the lack of adaptive immunity and immunity pathways against Gram-negative bacteria, it is susceptible to infection by *C*Las, which can easily replicate and spread *in vivo* (Vyas et al., 2015; Arp et al., 2017).

Vitellogenin (Vg) belongs to the large lipid transfer protein (LLTP) superfamily (Sarkar and Ghanim, 2022). As a yolk precursor protein, Vg is present in almost all oviparous organisms, including insects, crustaceans, fishes, birds, amphibians, and reptiles (Zhang et al., 2011; Kruse et al., 2018). Vg was originally thought to be a female-specific protein. However, research has shown that Vg is not only involved in yolk protein formation but also plays a sex-independent role associated with immune function in non-mammalian vertebrates and invertebrates. In insects, Vg is generally synthesized in the fat body and secreted into the hemolymph and other tissues to perform functions. Vg usually serves as a pattern recognition molecule to recognize pathogens (Liu et al., 2009; Arrese and Soulages, 2010). Recent reports have shown that Vg can interact with PAMPs such as bacterial outer membrane proteins, flagella, and pili and acts as one of the pattern recognition receptors (PRRs) inducing host immunity (Li et al., 2008; Zhang et al., 2011; Li et al., 2017; Sarkar and Ghanim, 2022). Hemocyte-produced Vg interacts with rice stripe virus (RSV) and is positively correlated with RSV survival in *Laodelphax striatellus* (Huo et al., 2018), acts as an antioxidant for immunity in bees and *Caenorhabditis elegans* (Nakamura et al., 1999; Seehuus et al., 2006), and inhibits *Staphylococcus aureus* by binding to the lipoteichoic acids of the bacterial surface in *Homarus* (Hanada et al., 2011). Moreover, Vg also acts as a salivary protein involved in the insect–plant host interactions (Jaiswal et al., 2021). Vg generally contains three conserved domains: the lipoprotein amino-terminal region located at the N terminus, the DUF of unknown function, and the VWD domain at the C terminus. LPD_N is a very conserved and characteristic domain, DUF is a domain whose function is still unknown, and the VWD domain is rich in cysteines, which are associated with the formation of disulfide bonds (Qiao et al., 2021). The VWD domain has been found in the Vg of several vertebrates, crustaceans, and insects, and the domain induces the binding of oocyte membrane receptors to Vg (Opresko and Wiley, 1987; Wu et al., 2018; Faiz et al., 2019). In addition, VWDs are also present in several other proteins such as mucins and banded adhesion proteins, where the sphericity of this domain enhances the function of mucins and underlies the adhesion function in other proteins such as integrins and zonadhesins (Toribara et al., 1997; Qiao et al., 2021).

In this study, we screened the *D. citri* membrane protein library using *C*Las flagellin (flaA) as bait and found that flaA interacted with a vitellogenin-like protein (Vg_VWD Gene ID: LOC103523873), which was further confirmed by glutathione S-transferase (GST) pull-down and co-immunoprecipitation (Co-IP) assays. The transcription level of *Vg_VWD* was upregulated in *C*Las-infected *D. citri* and was highly expressed in the fat body and salivary glands. Silencing the expression of *Vg_VWD* significantly increased the *C*Las titer at different time points. Vg_VWD could suppress BAX- and INF1-triggered hypersensitive cell death and inhibit flaA-induced callose deposition in *Nicotiana benthamiana*.

2. Materials and methods

Overall, all the gene constructs were confirmed by Sanger sequencing. The primers used in this study are listed in Supplementary Table 1. The strains and plasmids used in this study are listed in Supplementary Table 2.

2.1. Insect rearing and plant growth

Uninfected *D. citri* used were reared in a greenhouse at the National Navel Orange Engineering Research Center, Gannan Normal University, Ganzhou, China. *C*Las-infected *D. citri* were collected near Tandong orchard (latitude 25°47′5″ north and longitude 114°52′4″ east). Infected *D. citri* and uninfected *D. citri* were reared separately in cages (60 cm × 60 cm × 90 cm). For this experiment, one to two pots of healthy citrus or *Murraya exotica* were maintained in the rearing cages under the conditions of 27 ± 1°C and RH of 70 ± 5%, with a 14-h light/10-h dark cycle. Wild-type (WT) *N. benthamiana* plants were grown in a growth chamber maintained at 25 ± 2°C and an RH of 70 ± 5%, with a 16-h light/8-h dark cycle.

2.2. Gene cloning and vector construction

The full length of *Vg_VWD* (Gene ID: LOC103523873, XP_008487105.1, 557 aa) was obtained by quantitative real-time (qRT)-PCR using total RNA isolated from *D. citri*, cloned into the pCE2 TA/Blunt-Zero vectors (Vazyme, China), and then sequenced. The ClonExpress II One Step Cloning Kit (Vazyme, China) was used to insert *Vg_VWD* into the prokaryotic expression vector (pGEX-4T-1, GST-tagged protein), yeast two-hybrid (Y2H) vector PPR3-N (prey), vector pgR107 of potato virus X (PVX), and pull-down vector

PMAL-C2X (PBM-tagged protein). The sequence of the *C*Las flagellin (*flaA*) was derived from the whole-genome sequence of the strain Psy62 (taxid: 537021, GenBank accession no. CP001677) (Duan et al., 2009). It was cloned into the Y2H vectors pBT3-N, pBT3-STE, pDHB1 (bait plasmid), pull-down vector (pGEX-4T-1, GST-tagged protein), and PVX vector pgR107.

2.3. Yeast two-hybrid (Y2H) assay

The full *flaA* gene (Gene ID: CLIBASIA_02090, 1359 dp) was cloned in-frame into the vector (pBT3-N, pBT3-STE, and pDHB1) as the bait. The self-activation and functional validation of the bait plasmids referred to the methods of Than et al. (2016) and Liang et al. (2022). In brief, the NMY51 yeast cells containing the plasmid were transferred to DDO (SD/-His/−Leu with agar), TDO (SD/-His/−Leu/−Trp with agar), and QDO (SD/-His/−Leu/−Trp/−Ade with agar) for observation and recording of the growth. The certified bait plasmids were transformed into NMY51 yeast cells according to the manufacturer's instructions to produce the yeast receptor cells containing the decoy plasmids. The *D. citri* membrane library plasmids were transferred into receptor cells, coated on QDO-medium, and incubated at 28°C for 3–5 days, following which the plasmids were extracted and sequenced from the grown single colonies. The obtained candidate proteins were verified by Y2H protein–protein, and the interaction intensity was verified by the β-galactosidase colorimetric reaction.

2.4. Protein expression and GST pull-down analysis

The full *flaA* (CLIBASIA_02090, 1,359 dp) was cloned into pGEX-4T-1 for fusion with the GST tag, and *Vg_VWD* was cloned into PMAL-C2X for fusion with the MBP tag. The recombinants were transformed into competent *Escherichia coli* Rosetta (DE3) cells for expression and purified with the GST Tag Protein Purification Kit (Beyotime Biotechnology, China) after IPTG (1 mM, 20°C for 8 h). For the experimental and control groups, 500 μg of GST and GST-flaA were added to fully bind the solutions with 50% glutathione-agarose resin at 4°C. The supernatant was removed by centrifugation, and 1 mL of PBST was added to wash off the unbound protein in the resin, following which 500 μg of the MBP/His-Vg_VWD protein was added and incubated overnight at 4°C. The products were rinsed three times with 1 mL of pre-cooled PBST, and RIPA-buffered cell lysate and loading buffer were added and mixed well, boiled for 5–10 min, and then the supernatant was collected by centrifugation. The supernatant was separated by SDS-PAGE and subjected to immunoblotting analysis.

2.5. *In vivo* Co-IP assay

The Co-IP assays were performed as previously described with a slight modification (Wang et al., 2016). In brief, His and Flag were tagged to the N terminus and C terminus of *Vg_VWD*, respectively, and HA was tagged to the N terminus of flaA. The construct fusion plasmids pFastBac1-Vg_VWD and pFastBac1-flaA were transfected into Sf9 cells to detect protein expression. The fusion plasmids were then co-transfected into Sf9 cells, and the expression of the co-transfected proteins was detected. The co-transfected cell lysate samples were collected for subsequent experiments. First, the co-transfected cell lysate was pretreated, the lysate was incubated with the protein A magnetic column to prevent non-specific binding between the lysate and the magnetic column, and the incubated flow-through was taken for Co-IP experiments. Second, the experimental and control groups were set up: the flag antibody or IgG antibody was hung onto the protein A magnetic column, then the supernatant was lysed after incubation with mixed magnetic beads (4°C, 3 h), followed by washing thoroughly with 1 mL of ice-cold PBS buffer for three times to elute the protein, which was collected for Western blot detection.

2.6. Collection of *Diaphorina citri* tissues

According to the morphological differentiation method for *D. citri* nymphs, 3–5 instar nymphs were prepared, with three biological replicates for each instar and 15 nymphs per replicate. For the collection of different gender adults, the 5th instar nymphs were selected and transferred to a single *M. exotica* seedling for feeding. Then, the adults were collected on days 3, 5, and 10 after emergence, respectively. Adults of different genders were distinguished under a microscope and were separately collected. There were three biological repeats and five male or female adults of *D. citri* per replicate.

The *C*Las-infected adults of *D. citri* were collected from orchards, where *C*Las-positive rates ranged between 80% and 95% (Supplementary Figure 2A). Six organs including the midgut, bacteriomes, testis, ovary, fat body, and hemolymph were dissected under the insect-dissecting microscope, and the hemolymph was collected according to Kruse et al.'s method (2018). Tissues collected from 30 *D. citri* adults (male:female = 1:1) were taken as one biological replicate, and three biological replicates were used in experiments. In addition, the salivary glands and Malpighian tubes were collected with three biological replicates each from 100 adults *D. citri* (male:female = 1:1). Dissected tissues were transferred to 1.5-mL centrifuge tubes (Trizol, 500 μL) using forceps. Subsequently, the total nucleic acids were extracted.

2.7. *C*Las titer quantification within *Diaphorina citri*

The DNA extraction of a single *D. citri via* method A was used to detect the *C*Las-infection rate of *D. citri* in the field and method B was used to detect the *C*Las titer in infected *D. citri*. Method A is performed according to the instructions for the Animal Tissue Direct PCR Kit as follows: (1) the *D. citri* were kept on ice for 3–5 min; (2) each *D. citri* was transferred *via* forceps to PCR tubes with premixed 12.5 μL buffer AL and 0.5 μL Foregene protease; (3) mashed with a small sterilized grinding rod homogenate, and the homogenate was treated under the conditions of 65°C for 20 min and 95°C for 5 min; and (4) centrifuged at 12,000 rpm for 5 min. The PCR detection system has been carried out according to Shen et al. (2022). Two μL of the supernatant obtained from method A was aspirated as the PCR template with primers OI1 and OI2 (Supplementary Table 1). Method B extracted DNA from *D. citri*

according to the CTAB method, and the specific steps were performed according to Quintana et al. (2022). A two-step assessment of *C*Las titer in a single *D. citri* was addressed: (1) the presence of *C*Las was detected by ordinary PCR; and (2) the *C*Las titer in infected *D. citri* was quantified by qPCR.

To perform qPCR, the total DNA of a single *C*Las-infected *D. citri* was diluted to 100 ng/μL, and the copy number of *C*Las per 100 ng of total DNA was detected by absolute quantification. Probe qPCR experiments were performed with Premix (TakaRa, Dalian, China) using probe primers HLBr and HLB4G, and sequence information is provided in Supplementary Table 1. A mixture (20 μL) of Premix (10 μL), probe (0.3 μL), each primer (0.4 μL), and *D. citri* template DNA [1 μL (100 ng)] was reacted on a Light Cycler 96 SYBR Green I Master (Roche). The qPCR conditions were as follows: 3 min at 95°C; 40 thermal cycles (10 s at 95°C; 30 s at 60°C); and 30 s at 37°C. At least three technical replicates were performed for each sample. The equation for absolute quantification of *C*Las was $y = -4.11x + 55.508$ ($R^2 = 0.9964$), where y is the Ct value, x is the copy number, and the *C*Las titer is 10x per 100 ng of the *D. citri* DNA.

2.8. RNA extraction and RT-qPCR analysis

The *D. citri* total RNA was extracted using Trizol regent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer protocol. The synthesis of cDNA was performed by using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) with 1 μg of total RNA reverse transcribed for each age of *D. citri* and 0.5 μg of total RNA reverse transcribed for each tissue. The cDNA was diluted 3–5 times and then subjected to RT-qPCR, and a reaction system (20 μL) was applied, including 10 μL of 2 × TB Green Premix (TaKaRa, Dalian, China), 7 μL of DNase/RNase-free water, 0.8 μL of each primer (10 μM), 0.4 μL of ROX reference, and 1 μL of diluted cDNA template. The PCR cycling consisted of an initial activation step at 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s, and a single collection at 72°C for 30 s. The *DcGAPDH* gene was used as an internal control. The relative expression value was calculated with three biological and technical replicates, and the calculation was accordant with the $2^{-\Delta\Delta CT}$ quantification method (Livak and Schmittgen, 2001). The primers are listed in Supplementary Table 1.

2.9. DsRNA synthesis and RNAi silencing

The primers specific (Supplementary Table 1) for *Vg_VWD* and *GFP* were designed to synthesize dsRNA by using the T7 High Yield Transcription Kit (Thermo Scientific, Wilmington, DE, United States) according to the manufacturer's instructions. The *D. citri* adults addressed for dsRNA injection were *ca.* 95% of *C*Las infection rates (Supplementary Figure 2B). The dsRNAs of *Vg_VWD* and *GFP* were injected as experimental and control groups, respectively, and the injected *D. citri* were transferred to *M. exotica* seedlings for rearing (Supplementary Figure 3). The total DNA of *D. citri* was extracted and quantified to 100 ng/μL at 6, 12, and 24 h after injection. The titer of *C*Las per 100 ng total DNA of treated *D. citri* was determined. The experiments for each group were designed with at least three biological replicates (each with five males or females) and three technical replicates.

2.10. Agro-infiltration assay in *Nicotiana benthamiana*

The full-length sequences of *flaA* (1,359 dp) and *Vg_VWD* (1,674 dp) were inserted into the binary vector potato virus X (PVX) digested by *Cla*I/*Sal*I (Liu et al., 2019). PVX-GFP was used as a negative control and PVX-BAX and PVX-NIF1 as positive controls. The plasmids PVX-GFP, PVX-BAX, PVX-NIF1, PVX-flaA, and PVX-Vg_VWD were transformed into *A. tumefaciens* GV3101 for culturing, which then were centrifuged and resuspended with the buffer [10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM $MgCl_2$, and 100 μM acetosyringone] to $OD_{600} = 0.6$. After maintaining the suspension at room temperature and in the dark for 2 h, it was infiltrated into four to six leaves of *N. benthamiana* using sterile syringes. The symptoms were observed and photographed later.

For the callose deposition assay, the sampled *N. benthamiana* leaves were incubated in a mixed solution of acetic acid:glycerol:ethanol (1v:1v:3v) until the green color faded away, and then rinsed with 150 mM K_2HPO_4 for 30 min. Finally, samples were stained with aniline blue solution (150 mM K_2HPO_4, 0.05% w/v aniline blue) for 2 h, and the callose deposition was observed *via* fluorescence microscope using a DAPI filter (excitation filter 390 nm; dichroic mirror 420 nm; emission filter 460 nm) (Schenk and Schikora, 2015).

2.11. Phylogenetic analysis

A phylogenetic tree of *D. citri* for Vg_VWD and 11 other insect Vg-VWDs was constructed using the neighbor-joining (NJ) method with 1,000 bootstrap replicates in MEGA 7. Sequence information is provided in Supplementary Table 3.

2.12. Statistical analysis

All statistical analyses were performed using SPSS 24.0 software. A one-way ANOVA was used, followed by least-significant difference (LSD) multiple comparisons tests. Pairwise comparisons were performed by an independent samples *t*-test. Graphs were illustrated using GraphPad Prism 8.2.1 software. Data were expressed as means ± standard deviation.

3. Results

3.1. FlaA interaction with Vg_VWD

To find *D. citri* proteins targeting *C*Las flagellum proteins, flaA was used as bait. The pDHB1-flaA plasmid passed the self-activation assay and functional validation (Supplementary Figure 1), and a Y2H screen was performed on the *D. citri* membrane library. As a result, 16 candidate proteins with potential interactions with flaA, of which 11

were annotated and five were unannotated (Supplementary Table 3). Following structural domain analysis and functional prediction, a vitellogenin-1-like protein XP_008487105.1 (named Vg_VWD thereafter) containing a conserved domain-VWD with 189 amino acids was selected for further analysis (Figure 1A). The phylogenetic analysis showed that the Vg-VWD sequences of *D. citri* and other representative Hemiptera insects clustered together. In addition, the Vg_VWD sequences of *D. citri* and the potato psyllid (*Bactericera cockerelli*) sequence were closest in the evolutionary relationship (Figure 1A).

The verification of the flaA and Vg_VWD cotransformants showed that the yeast grew well and formed a certain gradient on DDO and QDO (Figure 1B). The point-to-point Y2H method and β-galactosidase assays showed that flaA-Vg_VWD interacted in the yeast system (Figures 1C,D). Subsequent GST pull-down and Co-IP assays further confirmed the interaction between flaA and Vg_VWD (Figures 1E,F). Taken together, these results indicate that flaA interacts with Vg_VWD *in vivo* and *in vitro*.

3.2. CLas acquisition induces Vg_VWD upregulation in *Diaphorina citri*

Quantitative RT-PCR was used to measure the transcript levels of the *Vg_VWD* gene in *D. citri*. In uninfected *D. citri*, the expression level of *Vg_VWD* gradually decreased in 3–5 instar nymphs, and Vg-VWD expression levels increased in females and decreased in males at 3–7 days after emergence (Figure 2A). The transcript abundance of *Vg_VWD* in *C*Las-infected *D. citri* was much higher than that in uninfected *D. citri* ($p < 0.05$), especially in females. This induction was 74,227-fold (*C*Las-infected/uninfected) in females, but only 38.3-fold (*C*Las-infected/uninfected) in males. In addition, the transcript abundance of *Vg_VWD* in *C*Las-infected females was 36,289-fold higher than that in *C*Las-infected males. These results indicate that *Vg_VWD* is expressed at significantly higher levels in females than males after infection ($p < 0.05$) (Figures 2B,C).

The proteomics analysis showed that *Vg* was upregulated in the hemolymph of the *C*Las-infected *D. citri* (Kruse et al., 2018). Here, we further analyzed the expression patterns of the *16srRNA* and *Vg_VWD* genes of *D. citri* in the midgut, salivary glands, malpighian tubules, bacteriomes, testes, ovaries, fat body, and hemolymph of the *C*Las-infected *D. citri* using RT-qPCR. The gene expression levels of 16S rRNA in the midgut and salivary gland were significantly higher than those in the other tissues ($p < 0.05$), while the transcription of *Vg_VWD* was highest in the fat body, followed by the salivary glands and bacteriomes (Figure 2D).

As insect hemolymph and salivary glands play important roles in the transmission of pathogens (Jiang et al., 2019; Chen et al., 2021), we next compared the transcription of *Vg_VWD* in the salivary glands and hemolymph of infected and uninfected *D. citri*. The results showed that the expression levels of *Vg_VWD* in the salivary gland and hemolymph were 338.39-fold and 61.67-fold higher in infected *D. citri* than those in uninfected *D. citri*, respectively (Figure 2E). Collectively, these results demonstrate that *C*Las acquisition induces *Vg_VWD* transcription in *D. citri*, with significant differences between males and females ($p < 0.05$).

3.3. Reducing Vg_VWD increased the titer of CLas in *Diaphorina citri*

Next, according to the characteristics of *Vg_VWD* expression (Figure 2A), we constructed *Vg_VWD*-silenced *D. citri* using *dsVg_VWD*-mediated RNAi silencing to determine whether Vg_VWD is involved in *C*Las proliferation. After *dsVg_VWD* injection in *C*Las-infected *D. citri*, the expression of *Vg_VWD* was reduced by 70% compared with the same *dsGFP* injection dose for 24 h (Figure 3A). The *C*Las titer was detected in the *C*Las-infected male and female of *D. citri* at 6, 12, and 24 h after the injection. The results showed that the *C*Las titer of female *D. citri* was significantly different from the control group at 12 and 24 h (Figure 3B), with an increase of 1.76 and 1.58 times (*C*Las copies in 100 ng of female *D. citri* DNA), respectively. There was a significant difference in the *C*Las titer in male *D. citri* at 24 h compared with the control group, which was 1.98-fold higher (*C*Las copies in 100 ng of male *D. citri* DNA), whereas no significant differences were observed at 6 and 12 h (Figure 3C).

3.4. Vg_VWD suppresses BAX- and INF1-triggered hypersensitive cell death and inhibits flaA-induced callose deposition in *Nicotiana benthamiana*

Since *Vg_VWD* was highly expressed in the salivary glands of the *C*Las-infected *D. citri* and directly interacts with *C*Las flagellin, we speculated that Vg_VWD may attach to the *C*Las flagellin protein during psyllid feeding along with the secretion of psyllid saliva into the citrus phloem, which is likely to be a stress factor. The mouse BAX and *Phytophthora infestans* INF1 are well-known inducers of cell death and are widely used to identify the PCD inhibitors of pathogens (Kamoun et al., 1998; Lacomme and Cruz, 1999). Vg_VWD was transiently expressed in *N. benthamiana* to evaluate its inhibitory effect on PCD, and the results showed that it could inhibit the BAX- and INF1-induced hypersensitivity response (Figures 4B,C). Callose deposition is one important indicator of plant immunity reaction, and the *C*Las flagellin has PAMP-triggered immunity and causes *N. benthamiana* leaf necrosis and callose deposition (Zou et al., 2012). Our results showed that Vg_VWD attenuates flaA-induced callose deposition in *N. benthamiana* (Figure 4D). Therefore, these results suggest that Vg_VWD can suppress *N. benthamiana* immunity and inhibit the phenomenon of callose deposition induced by flaA.

4. Discussion

Flagella are bacterial motor organs associated with tropism. Increasing numbers of studies have now shown that flagella play a central role in many bacterial infection processes, such as surface adhesion, biofilm formation, and the induction of host immunization (Duan et al., 2013; Chaban et al., 2015). *C*Las flagella genes have different expression patterns in different hosts, such as the flagella-like structures present in *C*Las cells isolated from *D. citri*, but they are not found in cells isolated from susceptible citrus (Andrade et al., 2020). Here, we found that the *C*Las flagellum (flaA) protein interacted with Vg_VWD in *D. citri*. This is one of the few reports of the

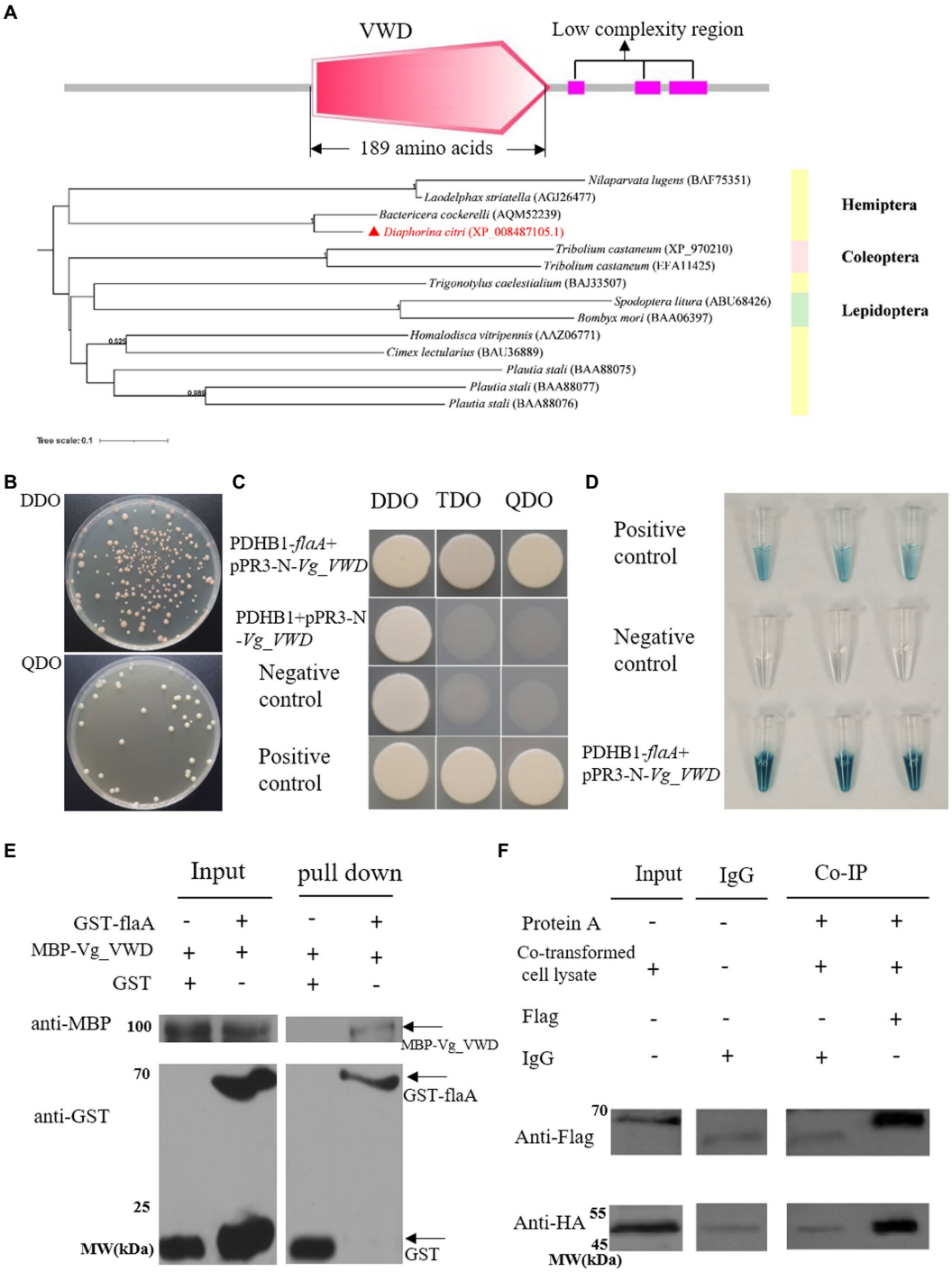

FIGURE 1
*C*Las flagellum (flaA) interacts with *D. citri* vitellogenin-like (Vg_VWD) and its molecular characterization. **(A)** The structural domain analysis of the Vg_VWD protein sequence and a phylogenetic tree containing Vg_VWD and other selected homologs from analysis by SMART software showed a VWD-conserved structural domain. **(B)** Yeast-two hybrid (Y2H) assay for the growth of yeast strain co-transformed with the bait and prey plasmids on DDO and QDO media. **(C)** The bait plasmid PDHB1-*flaA* and the prey-plasmid pPR3-N-*Vg_VWD* were co-transferred into NMY51 yeast, spotted on DDO, TDO, and QDO, and grow normally on selection medium TDO and QDO. PDHB1+pPR3-N-*Vg_VWD* was verified for self-activation of the prey-plasmid and failed to grow on selection medium TDO and QDO. **(D)** Yeast cultures were blue in the β-galactosidase assay. The well-grown test yeasts were selected separately and subjected to overnight incubation in a DDO liquid medium, after which crude protein was extracted and the concentrations tested. Approximately 75μL of crude protein was added to 5μL of x-gal and incubated at 37°C for 2h to observe the color change and photograph. **(E)** GST pull-down assay demonstrating the interaction of flaA with Vg_VWD. GST-flaA was the bait, and GST alone served as the control, with MBP-Vg_VWD serving as the prey. The bait protein or the GST control was incubated with MBP-Vg_VWD protein. Input and pull-down samples were probed with antibodies against GST or MBP for Western blot assays. **(F)** Co-IP detection of flaA interacting with Vg_VWD in Sf9 cells. Sf9 cells were transfected with the indicated plasmid combinations and the proteins were present in the lysate supernatant of Sf9 cells. The input group detected the target proteins in the lysate of co-transfected cells: Vg_VWD+Flag and HA+flaA with protein molecular weights of 65.1kDa and 51.2kDa, respectively. The IgG group excluded the heavy chain interference generated by protein A and IgG antibodies. The co-IP group was immunoprecipitated with Flag antibody (murine monoclonal antibody), IgG was used as control, and after WB analysis, the arrow marks the position of the target band.

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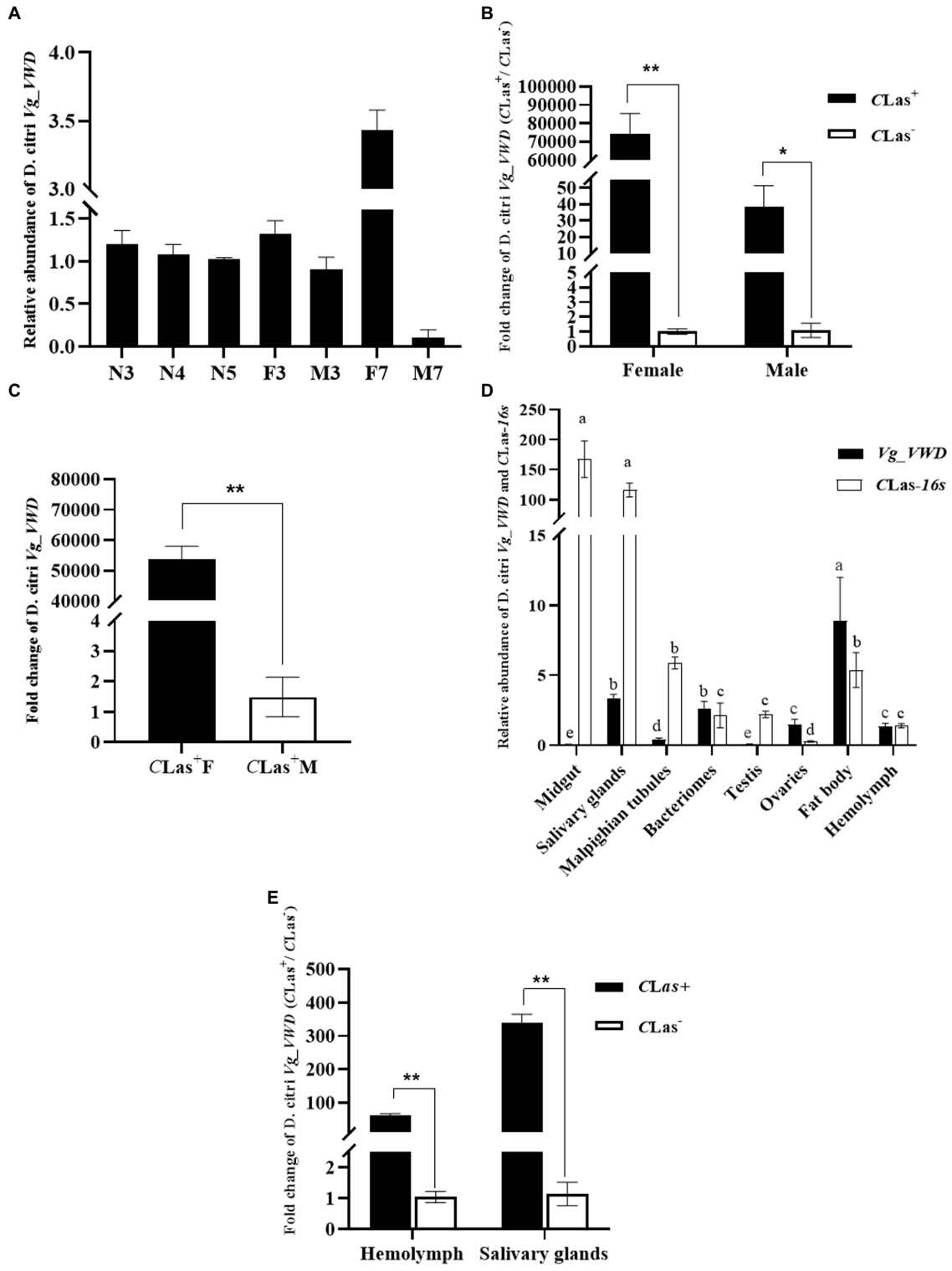

FIGURE 2
Expression profiles of *Vg_VWD* in different tissues of male and female individuals of *D. citri*, and sampled at different developmental stages. **(A)** Relative expression of *Vg_VWD* at different developmental stages (N3–N5, 3rd- to 5th instar nymphs; 3–7, 3, and 7days after emergence as adults; F; female, M: male). **(B)** Relative expression of *Vg_VWD* between male and female of *C*Las-infected and uninfected *D. citri*. **(C)** Relative expression of *Vg_VWD* expression between male and female of *C*Las-infected *D. citri*. **(D)** Relative expression of *C*Las-*16s* and *Vg_VWD* expression in different tissue of *C*Las-infected *D. citri* **(E)** Relative expression of *Vg_VWD* in the hemolymph and salivary glands of *C*Las-infected and uninfected *D. citri*. Data were analyzed using SPSS software, multiple comparisons using the one-way ANOVA followed by least significant difference (LSD) *post-hoc* tests at a *p*-value of 0.05. Pairwise comparisons were performed by independent samples *t*-test. The significant differences are indicated by * ($p<0.05$) or ** ($p<0.01$). Different letters in **(E)** indicate that the genes in the same middle are different in different types of samples ($p<0.05$), and the gene expression is adjusted by $\log_{10}$ and then subjected to the one-way ANOVA. GraphPad software was used for drawing.

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protein–protein interaction between *C*Las and *D. citri* to our knowledge. Protein–protein interactions are an important way to understand the interactions between *C*Las and *D. citri*, but studies on which are lacking. Using protein interaction reporter technology, Ramsey et al. (2017) found that *D. citri* hemocyanin protein physically interacted with the *C*Las coenzyme A (CoA) biosynthesis enzyme phosphopantothenoylcysteine synthetase/decarboxylase, and *D. citri* myosin protein with the *C*Las pantothenate kinase. These interaction proteins may be able to provide important clues about the interaction relationship between *C*Las and *D. citri*.

Over the past decade, insect Vg has been found to play important roles in reproductive development, immunity, and antioxidant activity (Park et al., 2018; Salmela and Sundström, 2018; Saleh et al., 2019). The Vg is usually synthesized and cleaved in the fat body of an insect (Dittmer et al., 2019; Wu et al., 2021). Vg, which also acts as a pattern recognition molecule to recognize pathogens, can interact with PAMPs such as bacterial outer membrane proteins, flagella, and pili, and acts as a PRR to induce host immunity (Liu et al., 2009; Arrese and Soulages, 2010). With the advancement of high-throughput sequencing and proteomic technologies, Vg has recently been discovered in insect salivary glands (Ji et al., 2013; Huang et al., 2018). Vg is also used as a saliva protein (Huang et al., 2021; Ji et al., 2021; Zeng et al., 2023). Transcriptome and hemolymph protein studies have revealed that Vg is upregulated in *C*Las-infected *D. citri* (Kruse et al., 2018; Jaiswal et al., 2021). This corroborates our detection of *Vg_VWD* expression in *C*Las-infected and uninfected *D. citri*. In addition, we found that *Vg_VWD* expression levels were significantly higher in females than in males, implying a sex bias. Reducing the expression of *Vg_VWD* in *D. citri* by RNAi interference led to a significant increase in *C*Las titers independent of insect sex, suggesting a general role in defense against *D. citri* infection. No significant difference was observed in the *C*Las infection rates between differentially *Vg_VWD*-expressed female and male *D. citri* collected in the field, which may indicate a neutral impact of *Vg_VWD* on the acquisition of *C*Las by *D. citri* (Figure 5A). Surprisingly, only a slightly higher titer of *C*Las in males than in females was detected here (Figure 5B) and in a previous study (Hosseinzadeh et al., 2019). *Vg_VWD* regulation-related immune pathways may play a balancing role between *C*Las and *D. citri* vector capacity, and therefore, we could also assume that the female is more susceptible to *C*Las than the male and thus maintains higher levels of Vg_VWD expression to counteract *C*Las. These viewpoints, however, require further validation.

Vg_VWD expression was confirmed by RT-qPCR to be highly abundant in the salivary glands and hemolymph of the *C*Las-infected *D. citri*, and the hemolymph detection result was consistent with that from a previous study (Kruse et al., 2018). We found that *Vg_VWD* was specifically highly expressed in the salivary glands of *C*Las-infection *D. citri*. In addition, it interacts with flaA, so we speculate that Vg_VWD may enter the plant host phloem by attaching to *C*Las in the absence of signal peptides and secretion. Therefore, we directly examined the influence of *Agrobacterium*-mediated transient expression of *Vg_VWD* in *N. benthamiana* as a substitute model plant for citrus. Transient expression showed that Vg_VWD inhibited the hypersensitivity reaction of *N. benthamiana* leaves caused by BAX and INF1, and also inhibited the phenomenon of leaf necrosis caused by BAX and INF1, confirming its function as a salivary protein. Recent studies

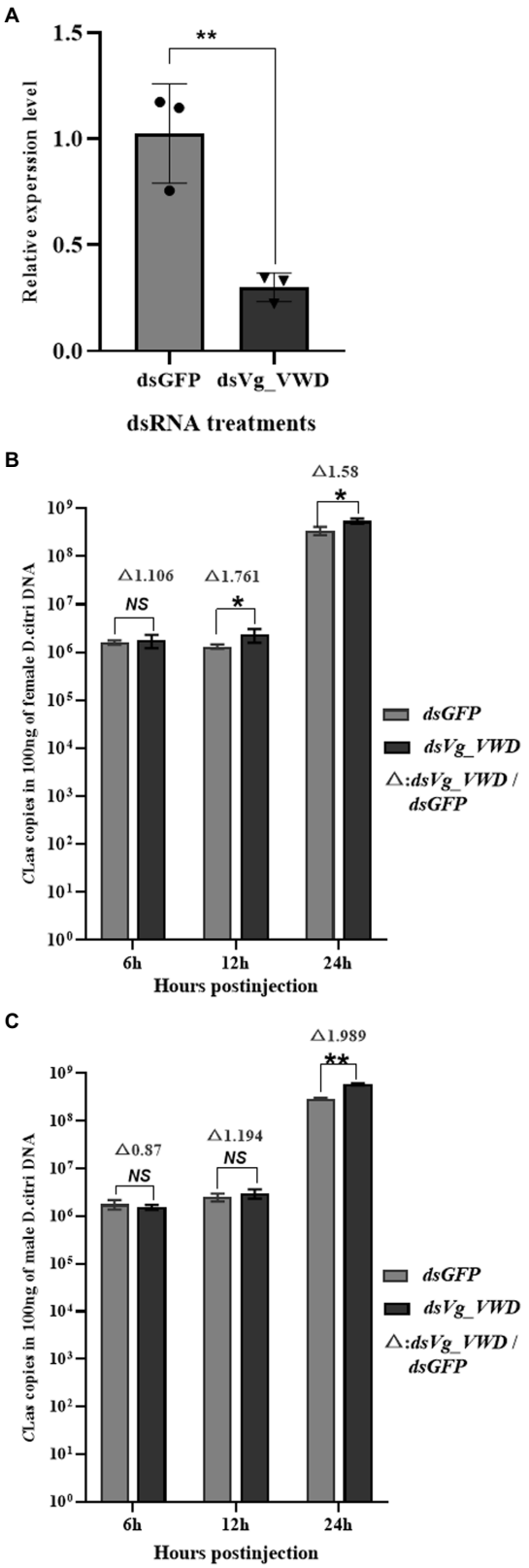

FIGURE 3
D. citri Vg_VWD silencing efficiency and detection of changes in *C*Las titer after silencing. **(A)** *Vg_VWD* silencing efficiency of female *D. citri*. **(B,C)** Are the detection of changes in *C*Las titer at 6, 12, and 24h after the silencing, respectively. Data analysis was performed using SPSS software for independent samples *t*-test, the significant differences are indicated by * ($p<0.05$) or ** ($p<0.01$).

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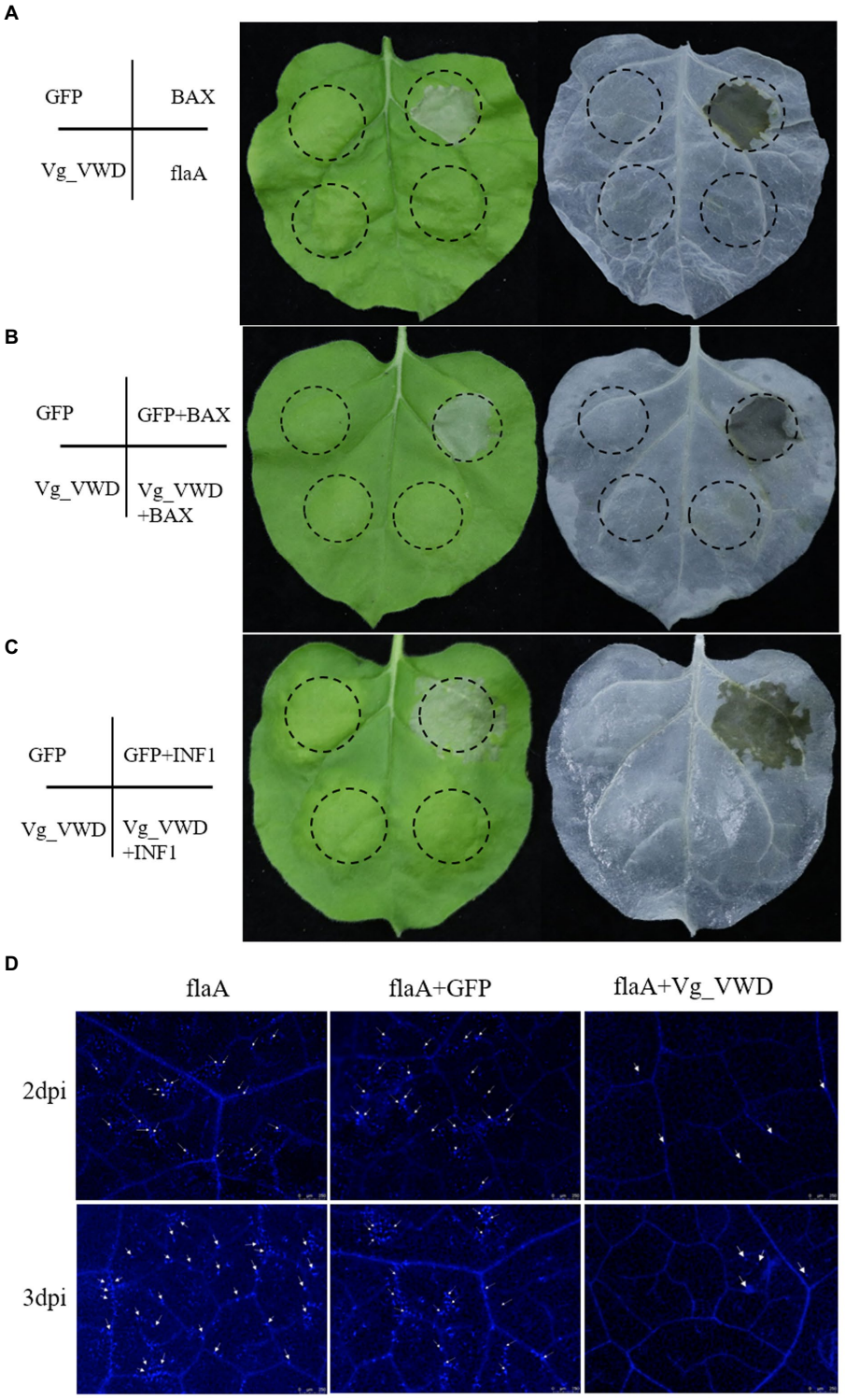

FIGURE 4
Transient expression of the Vg_VWD and flaA in *N. benthamiana*. **(A)** Symptoms on leaves with *GFP*, *BAX*, *Vg_VWD,* and *flaA* at 6days postinoculation (dpi), where Vg_VWD and flaA postinoculation leaves showed no obvious symptoms. The *Agrobacterium* GV3101 (pJIC SA_Rep) strain harboring *GFP*, *Vg_VWD*, *flaA,* and *BAX*, resuspension with buffer, were infiltrated into the leaves of *N. benthamiana*. **(B,C)** The *Vg_VWD* suppressed the hypersensitive cell death triggered by *BAX* and *INF1* in *N. benthamiana* infiltrated with GV3101 carrying *GFP* and *Vg_VWD*, and followed 24h later with *BAX* or *INF1* within the same regions (marked by the dashed circle). The leaves were harvested at 6days after postinoculation of *BAX* and *INF1*, followed by photography and decolorization with ethanol. **(D)** Vg_VWD suppressed accumulation of callose caused by flaA. Infiltrated leaves with flaA after 2 and 3days were sampled for Aniline-blue staining and photographed. Scale bars represent 250μM. The experiment was repeated three times with at least 6 *N. benthamiana* leaves each time.

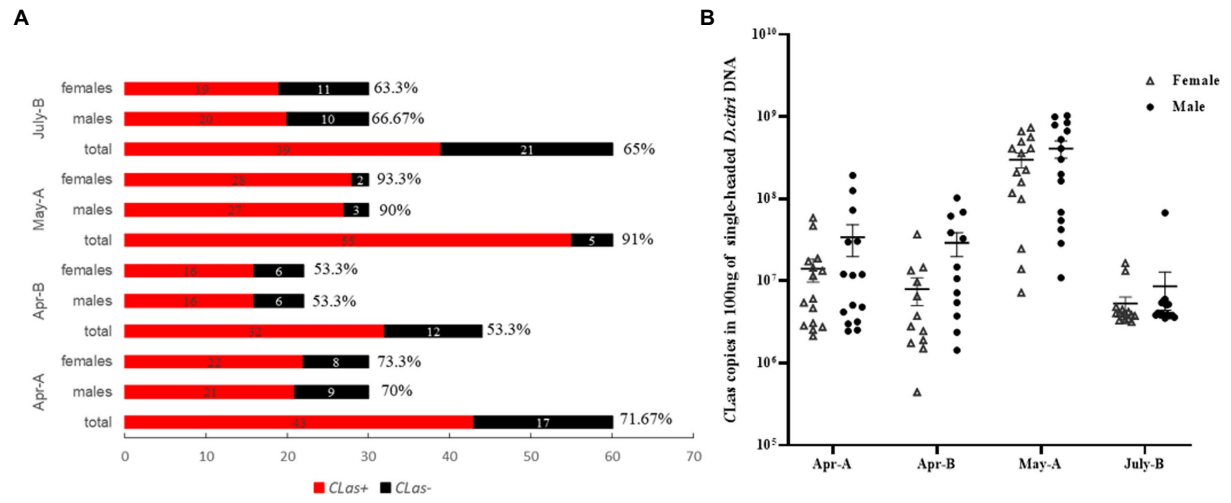

FIGURE 5
Detection of infection rate and titer of *C*Las in males and females of *D. citri* in the field. **(A)** Single male and single female were tested for *C*Las-infection rate in the field *D. citri*, and the percentages indicate the *C*Las-infection rates in each group. **(B)** *C*Las titers per 100ng of DNA in males and females of the *C*Las-infection *D. citri*.

have also reported that Vg in the salivary gland of the rice *planthopper* can inhibit plant immunity and the production of H_2O_2 during the feeding process as well as improve feeding fitness (Ji et al., 2021). FlaA has a conserved flg22 structural domain, which acts as a typical PAMP molecule and can induce plant immunity. In the present study, Vg_VWD was able to inhibit the plant immune response and suppress the phenomenon of callose deposition induced by flaA. From this perspective, Vg_VWD enters the plant by interacting with flaA, which may enhance the adaptation of *D. citri* feeding and create a favorable environment for *D. citri*. Conversely, both *C*Las and *C*Las flagellin can stimulate plant immunity (Zou et al., 2012; Ma et al., 2022). We speculate that the interaction of Vg_VWD with flaA may reduce the immune response induced by *C*Las invasion into plants, thus enhancing the early colonization of *C*Las in plants. The significance of this interaction is positive for both *D. citri* feeding and *C*Las early colonization, but negative for the plant. Meanwhile, the introduction of the *Vg_VWD* gene into a citrus genetic system could be used to study the relationship among *D. citri* feeding, *C*Las early colonization, and immune resistance of citrus in future, thereby providing new ideas for the prevention and control of Huanglongbing.

In summary, we present evidence that *C*Las–flaA interacts with *D. citri* Vg_VWD. As a regulatory factor, *Vg_VWD* was upregulated in *C*Las-infected *D. citri* compared with uninfected ones, and the *C*Las titer increased significantly after *Vg_VWD* was silenced. Furthermore, we also found that *Vg_VWD* was highly expressed in the salivary glands of the *C*Las-infected *D. citri*. In *N. benthamiana* plants, Vg_VWD inhibits plant immunity and plays a function similar to salivary proteins. This study not only deepens our understanding of the molecular interaction between *C*Las and *D. citri* but also provides a foundation for studying the roles that flaA and Vg_VWD may play together or separately in insect and plant hosts.

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

TP, CZ, XW, and HY designed the experiments. TP and YY performed the experiments. CY participated in the rearing and collection of test insects. TP analyzed the data and wrote the manuscript. JH, XW, and CZ revised and embellished the manuscript. AH, SD, and LY provided the appropriate experimental apparatus and assistance. 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/fmicb.2023.1119619/full#supplementary-material

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A biotroph sets the stage for a necrotroph to play: '*Candidatus* Phytoplasma solani' infection of sugar beet facilitated *Macrophomina phaseolina* root rot

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'*Candidatus* Phytoplasma solani' (stolbur phytoplasma) is associated with rubbery taproot disease (RTD) of sugar beet (*Beta vulgaris* L.), while *Macrophomina phaseolina* is considered the most important root rot pathogen of this plant in Serbia. The high prevalence of *M. phaseolina* root rot reported on sugar beet in Serbia, unmatched elsewhere in the world, coupled with the notorious tendency of RTD-affected sugar beet to rot, has prompted research into the relationship between the two diseases. This study investigates the correlation between the occurrence of sugar beet RTD and the presence of root rot fungal pathogens in a semi-field '*Ca*. P. solani' transmission experiment with the cixiid vector *Reptalus quinquecostatus* (Dufour), in addition to naturally infected sugar beet in the open field. Our results showed that: (i) *Reptalus quinquecostatus* transmitted '*Ca*. P. solani' to sugar beet which induced typical RTD root symptoms; (ii) *Macrophomina phaseolina* root rot was exclusively present in '*Ca*. P. solani'-infected sugar beet in both the semi-field experiment and naturally infected sugar beet; and that (iii) even under environmental conditions favorable to the pathogen, *M. phaseolina* did not infect sugar beet, unless the plants had been previously infected with phytoplasma.

KEYWORDS

phytoplasma fungus complex, stolbur phytoplasma, RTD, rubbery taproot disease, *Reptalus quinquecostatus*, *Beta vulgaris* (sugar beet), charcoal rot

Introduction

Rubbery taproot disease (RTD) of sugar beet in Serbia and the Pannonian Plain has been associated with the plant pathogenic microorganism '*Candidatus* Phytoplasma solani' (Mollicutes, Acholeplasmataceae) (Quaglino et al., 2013; Ćurčić et al., 2021a,b). '*Candidatus* P. solani,' known by its trivial name "stolbur phytoplasma," is a fastidious, phloem-limited bacterium that infects a variety of cultivated plants across Europe, occasionally causing serious economic losses (Mitrović et al., 2013; EPPO, 2023). Several insect species of the family Cixiidae

(Hemiptera, Auchenorrhyncha) have been identified as vectors of '*Ca*. P. solani' (Jović et al., 2019; Kosovac et al., 2023). A particular cixiid planthopper, *Reptalus quinquecostatus* (Dufour) *sensu* Holzinger et al. (2003), has recently been revealed as a '*Ca*. P. solani' vector to sugar beet in Serbia and proposed culpable for the 2020 epidemic RTD outbreak recorded in Rimski Šančevi (Novi Sad, northern Serbia) (Kosovac et al., 2023). Symptoms of sugar beet RTD first appear in the second half of July, approximately a month after '*Ca*. P. solani' has been transmitted by vector(s) in the field. The symptoms begin with a loss of turgor in leaves during the hottest part of the day, followed by yellowing and necrosis of the oldest leaves. Eventually, all leaves become necrotic, which leads to the complete decline of the plant. At the same time, taproots of diseased plants wilt and become rubbery. Although initially without any rot symptoms, taproots begin to rot after aboveground parts of the plant have declined. As a consequence, some of the taproots completely rot before harvest (Ćurčić et al., 2021b; Kosovac et al., 2023).

Among other reported pathogens (*Fusarium* spp., *Rhizoctonia solani*), *Macrophomina phaseolina* (Tassi) Goid (Botryosphaeriaceae) is currently considered the most important root rot fungal pathogen of sugar beet in Serbia. In extreme environmental conditions (i.e., warm summers and severe droughts), it may cause losses of up to 100% (Jasnić et al., 2005; Stojšin et al., 2012; Budakov et al., 2015). *Macrophomina phaseolina* is a soil-borne, necrotrophic pathogen present all across the world, affecting more than 500 plant species (100 families) (Babu et al., 2007; Abass et al., 2021; Marquez et al., 2021). It is the causal agent of stem and root rot, seedling blight and charcoal rot. *Macrophomina phaseolina* survives for at least 2 years as sclerotia, formed in host plants, soil or leftover host tissue (Collins, 1988; Su et al., 2001). The fungus prefers temperatures in the range of 30–35°C, though some isolates have the greatest growth rate at 40°C (Manici et al., 1995). Under the conditions of high temperatures (30–35°C) and low soil moisture (below 60%), *M. phaseolina* may cause significant yield losses in soybean and sorghum. In extreme cases, 100% yield losses have been recorded in groundnut cultivars when the disease appeared at the pre-emergence stage (Kaur et al., 2012; Marquez et al., 2021). Taxonomically, *M. phaseolina* had been the only species in the genus *Macrophomina*, until recently when multilocus phylogenetic analysis allowed the description and distinction of four cryptic *Macrophomina* species—*M. pseudophaseolina*, *M. euphorbiicola*, *M. vaccinii*, and *M. tecta* (Sarr et al., 2014; Machado et al., 2019; Zhao et al., 2019; Poudel et al., 2021).

In addition to Serbia, *M. phaseolina* in sugar beet has been reported in the hot inland valleys of California (United States), India, Iran, Egypt, Russia, and some other countries of the former USSR, Greece, and Hungary. In these countries, it is generally considered a minor root rot pathogen of weakened, injured or stressed plants (Cooke and Scott, 1993; Karadimos et al., 2002; Jacobsen, 2006). Recent studies of microbial communities in both healthy and root rot-affected sugar beet in Austria and Germany, using conventional (isolation) and molecular techniques (including high-throughput sequencing), found *M. phaseolina* neither in healthy nor root rot-affected sugar beet, unlike other pathogenic or nonpathogenic fungi (Liebe et al., 2016; Liebe and Varrelmann, 2016; Kusstatscher et al., 2019).

The observed tendency of '*Ca*. P. solani'-infected sugar beet to rot, as well as the high prevalence of *M. phaseolina* root rot reported in sugar beet in Serbia (compared to its negligible impact in other regions across the world) prompted investigation into the relationship between the presence of '*Ca*. P. solani' and root rot fungi in sugar beet in Serbia. Therefore, the aim of this interdisciplinary study was: (i) to study the correlation between the occurrence of RTD of sugar beet and the presence of root rot fungal pathogens in a semi-field '*Ca*. P. solani' transmission experiment with vector *R. quinquecostatus sensu* Holzinger et al. (2003); (ii) to further assess and confirm the dominance of *M. phaseolina* root rot in '*Ca*. P. solani'-infected sugar beet in open-field conditions; and (iii) to characterize selected isolates of '*Ca*. P. solani' on the epidemiologically informative *tuf* and *stamp* genes, and to morphologically and molecularly characterize *M. phaseolina*.

Materials and methods

Semi-field '*Candidatus* Phytoplasma solani' transmission experiment

Our study of the relationship between '*Ca*. P. solani' infection of sugar beet and fungal root rot was conducted from May to November 2022, at a long-term experimental field in Rimski Šančevi (N 45°19′57″; E 19°49′58″) at the Institute of Field and Vegetable Crops, Novi Sad. The long-term experimental field was set up in 1965 as a four-field crop rotation scheme for sugar beet, corn, sunflower, and wheat, 2 ha each. For the semi-field experiment, two net cages (2 m × 2 m × 2.5 m) were installed in the sugar beet plot on May 15, covering 40 plants each, and subjected to the same agrotechnical protocol as the rest of the field. The aim of the semi-field experiment was to ensure a pool of RTD-affected sugar beet using a naturally infected population of a certain cixiid vector present *in situ*. An abundant population of *Reptalus* sp. aggregated in Rimski Šančevi on a parsnip field bordering the experimental sugar beet plot. When the first adults appeared at the beginning of June 2022, a total of 30 insects were caught. Species identity of collected males was determined by a specific morphological difference in the anal tube—a distinct process with a left orientation in *R. quinquecostatus*, but absent in its congeneric species *R. panzeri* (Holzinger et al., 2003). Genomic DNA was isolated from individual insects using a modified CTAB method (Gatineau et al., 2001), primarily to molecularly determine the identity of sampled females based on the internal transcribed spacer 2 (ITS2) (Bertin et al., 2010; Kosovac et al., 2023). After all 30 representative individuals were identified as *R. quinquecostatus sensu* Holzinger et al. (2003), insects were subjected to '*Ca*. P. solani' detection to confirm the infection status of the targeted population. Detection was performed by amplifying the '*Ca*. P. solani'—specific *stamp* gene in nested PCR assays, using Stamp-F/R0 and Stamp-F1/R1 primer pairs and following previously described conditions (Fabre et al., 2011). Each 25 µL PCR mix contained 20 ng of template DNA, 1× PCR Master Mix (Thermo Scientific, Vilnius, Lithuania) and 0.4 µM of each primer. Samples lacking template DNA were employed as negative controls. In total, 1 µL of direct PCR amplicon diluted 30× in sterile water was used as a template for nested PCR. Six microlitres of nested PCR products were then separated in a 1% agarose gel, stained by ethidium bromide, and visualized with a UV transilluminator. Amplification of the fragment of expected size, ~470 bp, was considered a positive reaction. A total of 250 *R. quinquecostatus* individuals, collected shortly afterward from the assessed population,

were released on June 9, 2022, into one of the two net cages described above, whereas the other cage without insects was used as a negative control.

Sugar beets in the semi-field experiment were visually evaluated for the development of RTD leaf symptoms once a week or more frequently. Sampling of the sugar beet root tissue was done depending on RTD and rot symptom severity and plant decline. The final sampling was done in the beginning of October 2022. All 80 experimental sugar beet from both cages, RTD and root rot-symptomatic, as well as the asymptomatic plants, were further subjected to phytoplasma and fungi assessment.

Open-field sugar beet assessment

Sampling of open-field sugar beet was conducted during November 2022 at three locations: Rimski Šančevi, where the semi-field experiment was performed, Banatsko Veliko Selo (N 45°47′56″; E 20°34′43″; ~80 km north-east of the experimental field) and Sremska Mitrovica (N 44°57′20″; E 19°40′24″; ~45 km south-west). A total of 180 sugar beet samples (60 per each field) were collected: (1) 20 with prominent RTD symptom rubbery taproot, but without rot; (2) 20 with charcoal root rot; and (3) 20 asymptomatic (without RTD and root rot). All field-collected samples were further subjected to phytoplasma and fungi assessment as described onward.

Phytoplasma assessment

Nucleic acid extraction from all sugar beet samples (semi-field and open-field) was performed from 0.5 g of taproot tissue, following the CTAB protocol (Doyle and Doyle, 1990). Total nucleic acids were precipitated with isopropanol, re-suspended in TE buffer (10 mM Tris pH 8 and 1 mM EDTA) and stored at −20°C.

For phytoplasma assessment in collected samples, amplification of the '*Ca*. P. solani'—specific *stamp* gene was performed in nested PCR assays as described above. To examine the presence of phytoplasmas other than '*Ca*. P. solani,' samples evaluated as negative in *stamp* PCR, were further subjected to a universal phytoplasma assay using the TaqMan real-time PCR protocol (qPCR), which targets the 16S rRNA gene of phytoplasmas and the 18S rRNA gene of plants (to confirm the presence of the DNA template and evaluate its quality) as described by Christensen et al. (2004, 2013) with a few modifications. Briefly, the final reaction volumes of 15 µL contained 1x TaqMan qPCR master mix (Nippon genetics Europe), 1 µL template DNA, 0.15 µL Uracil-N-Glycosylase (UNG), and 0.4 µM of each primer and probe. The qPCR was performed in a Magnetic Induction Cycler, Mic (Bio Molecular Systems, Upper Coomera, Australia). Each assay included a DNA-free blank reaction, a negative control corresponding to an RTD asymptomatic sugar beet, and a positive control of '*Ca*. P. solani,' strain 284/09 (Mitrović et al., 2014). Data evaluation was performed using micPCR© software Version 2.6.4 (Bio Molecular Systems, Upper Coomera, Australia).

All '*Ca*. P. solani'-positive samples were further subjected to characterization of the epidemiologically decisive *tuf* gene that indicates strains affiliation to a specific epidemiological cycle (Langer and Maixner, 2004; Aryan et al., 2014; Ćurčić et al., 2021b). To amplify the *tuf* gene, the Tuf1-f1/Tuf1-r1 (CACGTTGATCACGGCAAAAC/CCACCTTCACGGATAGAAAAC) and fTufAy/rTufAy primer pairs were used in nested PCR assays (Schneider and Gibb, 1997; Langer and Maixner, 2004; Kosovac, 2018). For differentiation of the *tuf* types (tuf-a, b, and d), the obtained *tuf* amplicons (fTufAy/rTufAy) were subjected to RFLP analyses with *Hpa*II and *Tai*I restriction enzymes (Thermo Scientific) in separate reactions, according to manufacturer's instructions (Langer and Maixner, 2004; Ćurčić et al., 2021b). Restriction products were separated in an 8% polyacrylamide gel, stained and visualized as described above. To check for the presence of additional variability in the *tuf* gene, six randomly selected sugar beets from the semi-field transmission experiment and from each of the assessed open fields (24 in total) were subjected to *tuf* gene sequence analyses. The fTufAy/rTufAy nested PCR products were sequenced in both directions with the primers applied for amplification, to yield a 2X consensus amplicon sequence, using a commercial service (Macrogen Inc., Seoul, Korea). The *tuf* sequences were then assembled using Pregap4 from the Staden program package (Staden et al., 2000) and subjected to multiple sequence alignment using ClustalX in MEGA X (Thompson et al., 1997; Kumar et al., 2018). Strains CrHo13_1183, CrHo12_601, CrHo12_650, and 429/19 corresponding to the previously described '*Ca*. P. solani' *tuf* genotypes tuf a, tuf b1, tuf b2, and tuf d, respectively (Aryan et al., 2014; Ćurčić et al., 2021b), were used for the comparison.

In all 24 '*Ca*. P. solani' strains selected for *tuf* gene sequence analyses, *stamp* gene was also sequenced in both directions as described above since its diversity follows up epidemiological divergence that *tuf* gene basically reveals (Fabre et al., 2011). The obtained sequences were assembled using Pregap4 from the Staden program package (Staden et al., 2000), manually inspected and compared with those of the publicly available strains representing previously described *stamp* genotypes (Pierro et al., 2018) using BLAST in the GenBank.

Fungal assessment

Sugar beet roots with two types of symptoms: root rot and rubbery taproots without rot, as well as asymptomatic roots, were assessed for the presence of fungi. Isolation was done from the margin of healthy and rotted tissue of roots with rot symptoms and from the internal portion of the roots without rot (rubbery taproot and asymptomatic). Root fragments were washed, disinfected in 70% ethanol and placed on potato dextrose agar (PDA, EMD, Darmstadt, Germany, pH 5.6 ± 0.2) in Petri dishes (90 mm). After 3–5 days of incubation at 24 ± 2°C in 12/12 h light/dark regime, developing fungal colonies were transferred to a pure culture and their morphology was assessed. Isolates with colony features typical for *Macrophomina* sp. (initially whitish colonies that become dark grey with age and develop numerous black sclerotia) (Sarr et al., 2014) were further subjected to molecular analyses for fungal species confirmation, whereas other isolates were identified at genus level based on morphology.

DNA was extracted from 7-day-old cultures of obtained isolates, according to the previously described CTAB protocol (Day and Shattock, 1997). The isolates were tested using *M. phaseolina*—specific primers for translation elongation factor 1α (TEF1-α) MpTefF/MpTefR, following previously described conditions (Santos et al., 2020). Amplification of the fragment of expected size, ~220 bp, was considered a positive reaction. A total of seven *M. phaseolina* isolates,

two per open-field locality and one from the semi-field experiment, were randomly selected for further molecular and morphological characterization. Five loci selected for characterization—internal transcribed spacer regions 1 and 2, including the 5.8S rRNA gene (ITS), translation elongation factor 1-α (TEF1-α), actin (ACT), calmodulin (CAL), and β-tubulin (TUB) genes—were amplified using primer pairs ITS1/ITS4 (White et al., 1990), EF1-728F (Carbone and Kohn, 1999)/EF2R (Jacobs et al., 2004), ACT-512F/ACT-783R (Carbone and Kohn, 1999), CAL-228F/CAL-737R (Carbone and Kohn, 1999), and T1 (O'Donnell and Cigelnik, 1997)/Bt2b (Glass and Donaldson, 1995), respectively. The PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s (ITS), or 55°C for 50 s (CAL), or 55°C for 1 min (TEF1-α, ACT, and TUB), and elongation at 72°C for 1 min, and a final elongation at 72°C for 10 min. Each 25 μL PCR mix contained 20 ng of template DNA, 1× PCR Master Mix (Thermo Scientific, Vilnius, Lithuania), and 0.4 μM of each primer. Samples lacking template DNA were employed as negative controls. PCR products (5 μL) were separated in a 1.5% agarose gel, stained and visualized as described above. Amplified products were purified and sequenced in both directions as described above. Sequences were assembled and deposited in the NCBI GenBank. Evolutionary history was inferred based on combined analyses of the five loci (ITS, TEF-1α, ACT, CAL, and TUB) of seven isolates obtained in this study, reference isolates of *Macrophomina* spp. and *Botryosphaeria dothidea* CBS115476 as an outgroup (Supplementary Table S1), using the Maximum Likelihood (ML) and Maximum Parsimony (MP) methods (MEGA X). For ML, the best nucleotide substitution model was determined using the "find best model" option in MEGA X. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The MP trees were obtained using the Tree-Bisection-Reconnection (TBR) algorithm with search level 3, in which the initial trees were obtained by the random addition of sequences (10 replicates). To estimate the statistical significance of the inferred clades, 1,000 bootstraps were performed.

Morphological characterization of the seven selected isolates was performed on PDA at 24°C for 3 days in the dark for macromorphology and on pine needle agar (PNA) at 24°C under 12/12 h light/dark regime for 4–8 weeks for micromorphology (Crous et al., 2006; Sarr et al., 2014). Morphology of sclerotia, conidiomata, and conidia was evaluated using the compound microscope Zeiss Axio Lab, Jena, Germany. Photographs and measurements were obtained using the camera Axiocam ERc 5 s, Zeiss and software ZEN 2 (blue edition), Jena, Germany.

Results

Reptalus quinquecostatus transmits '*Candidatus* Phytoplasma solani' to sugar beet, RTD develops, and root rot follows

Combination of morphology and molecular tools applied in identification of the 30 *Reptalus* sp. individuals (19 males and 11 females), sampled prior to the set-up of the semi-field experiment, confirmed presence of only *R. quinquecostatus sensu* Holzinger et al. (2003) aggregating on the bordering parsnip. As the '*Ca.* P. solani' infection rate of the analyzed *R. quinquecostatus* population was 63% which indicated its high potential to experimentally induce RTD in sugar beet, this population was further used in the semi-field sugar beet experiment.

The first RTD symptomatic sugar beet in the cage with released *R. quinquecostatus* were observed in mid-July (45 DAI). The symptoms included loss of turgor in leaves during the hottest part of the day, followed by yellowing and, later, necrosis of the oldest leaves, progressing from their margins. Eventually, all leaves became necrotic, which led to the decline of the plants. Out of 40 sugar beet exposed to *R. quinquecostatus*, 32 declined plants were collected on August 10 (62 DAI). The remaining eight plants (of which one had declined, three presented RTD leaf symptoms and four were asymptomatic), were finally collected on September 8 (91 DAI). The declined sugar beets exhibited different stages of charcoal root rot with root tissue color varying from light yellow and brown to black on cross section, usually starting from the tail (Figure 1). Some of the declined sugar beets had advanced stage of root rot and hence it was challenging to evaluate rubberiness of their taproots, whereas some of the declined plants with rubbery taproots had early stage of root rot, clearly visible just after cutting the taproot (Figure 1). The three sugar beets with RTD leaf symptoms had rubbery taproots without root rot, which on cross section were visually indistinguishable from healthy taproots and lacked discoloration. The four sugar beets collected as asymptomatic had neither rubbery taproots nor root rot. In the control cage without insects, all 40 sugar beets remained RTD-asymptomatic on their leaves and were collected in the beginning of October as free of rubbery taproots and root rot. Molecular analysis of sugar beet samples from the cage with *R. quinquecostatus* revealed '*Ca.* P. solani' infection in 36 out of 40 sugar beets, including all 33 declined plants and three RTD symptomatic lacking root rot. The remaining four asymptomatic sugar beet, as well as all 40 asymptomatic sugar beets (no RTD or root rot) from the control cage resulted negative in both the '*Ca.* P. solani'—specific PCR and universal phytoplasma qPCR assays (Table 1).

Macrophomina phaseolina is present only in root rot of sugar beet with '*Candidatus* Phytoplasma solani'

Among sugar beet from the *R. quinquecostatus* transmission cage, fungal assessment revealed the presence of *M. phaseolina* in all 33 declined plants, which were also '*Ca.* P. solani'-infected, whereas no *M. phaseolina* presence was confirmed in the seven sugar beet without rot, regardless of phytoplasma presence. Neither was *M. phaseolina* presence confirmed in any of the 40 asymptomatic sugar beet from the negative control cage. In sugar beet without '*Ca.* P. solani' infection, fungi other than *M. phaseolina* were sporadically isolated (*Fusarium* sp., *Penicillium* sp. and *Rhizopus* sp.; Table 1).

Similar to the semi-field experiment, samples collected from the open fields with charcoal root rot expressed also rubberiness, although evaluating rubberiness of the taproots was challenging in the declined sugar beet with advanced stage of root rot. Presence of '*Ca.* P. solani' followed the same occurrence pattern in the open-field samples as in the semi-field transmission

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FIGURE 1
Cross section of sugar beet infected with '*Candidatus* Phytoplasma solani' and *Macrophomina phaseolina*. **(A)** Early stage of charcoal root rot beginning from root tail; **(B)** Different stages of charcoal root rot; and **(C)** Advanced stage of charcoal root rot.

TABLE 1 Sugar beet root symptoms and presence of '*Ca*. P. solani' and *M. phaseolina* in the semi-field experiment with *R. quinquecostatus*.

Semi-field trial	*R. quinquecostatus* test cage			Negative control cage
Symptoms	RTD+root rot 33/40*	RTD 3/40	Asymp 4/40	Asymp 40/40
'*Ca*. P. solani'	33/33	3/3	0/4	0/40
M. phaseolina	33/33	0/3	0/4	0/40
Other fungi**	0/33	0/3	1/4 Fus	8/40 Fus 6/40 Rhi 4/40 Pen

*Number of samples in which the symptom or pathogen is present/total number of assessed.
**Fus, *Fusarium* sp.; Pen, *Penicillium* sp.; Rhi: *Rhizopus* sp.

experiment at each of the three assessed localities: 60 declined sugar beet with charcoal root rot and 60 RTD symptomatic ones (with rubbery, but not rotted taproots) were positive for '*Ca*. P. solani,' whereas all 60 asymptomatic sugar beets were negative for '*Ca*. P. solani' and universal phytoplasma (Table 2). Similarly, results of fungal assessment of open-field samples were comparable with results obtained in the semi-field experiment. *Macrophomina phaseolina* was detected in all 60 declined sugar beets with charcoal root rot, but not in any of the 60 rubbery taproot sugar beets without root rot or in any of the 60 asymptomatic plants regardless of phytoplasma presence. Moreover, as in the semi-field transmission experiment, in rubbery taproot sugar beet without rot and asymptomatic sugar beet, fungi other than *M. phaseolina* from the same genera (*Fusarium* sp., *Penicillium* sp. and *Rhizopus* sp.) were sporadically isolated regardless of phytoplasma presence (Table 2).

Molecular characterization of '*Candidatus* Phytoplasma solani'

The expected *tuf* gene amplicons were obtained for 148 out of 156 '*Ca*. P. solani' infected sugar beets (36 RTD symptomatic plants from the semi-field experiment and 120 plants from the open-field assessment). *Tuf* gene RFLP analyses revealed the presence of the tuf-d type in 33 out of 36 RTD symptomatic and '*Ca*. P. solani' positive sugar beets from the *R. quinquecostatus* transmission experiment that were amplified on the *tuf* gene, while in samples collected in field, the tuf-b type was also recorded. In Rimski Šančevi, 31 out of 38 sugar beet samples assessed for the *tuf* gene had the tuf-d type, six had the tuf-b type, while one sample showed mixed infection with the two tuf types. In Banatsko Veliko Selo the tuf-d type also dominated in analyzed samples and was found in 36 out of 38 sugar beet samples, with the tuf-b type present in only two sugar beets. In Sremska Mitrovica, the

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TABLE 2 Sugar beet root symptoms and presence of '*Ca*. P. solani' and *M. phaseolina* in the open field.

Locality	Rimski Šančevi			Banatsko Veliko Selo			Sremska Mitrovica		
Symptoms	RTD+root rot	RTD	Asymp	RTD+root rot	RTD	Asymp	RTD+root rot	RTD	Asymp
'*Ca*. P. solani'	20/20*	20/20	0/20	20/20	20/20	0/20	20/20	20/20	0/20
M. phaseolina	20/20	0/20	0/20	20/20	0/20	0/20	20/20	0/20	0/20
Other fungi**	0/20	9/20 Fus 1/20 Rhi 1/20 Pen	6/20 Fus 2/20 Rhi	0/20	5/20 Fus 2/20 Rhi	8/20 Fus	0/20	5/20 Fus 4/20 Rhi 7/20 Pen	3/20 Fus 3/20 Rhi 4/20 Pen

*Number of samples in which the pathogen is present/total number of assessed.
**Fus: *Fusarium* sp.; Pen: *Penicillium* sp.; Rhi: *Rhizopus* sp.

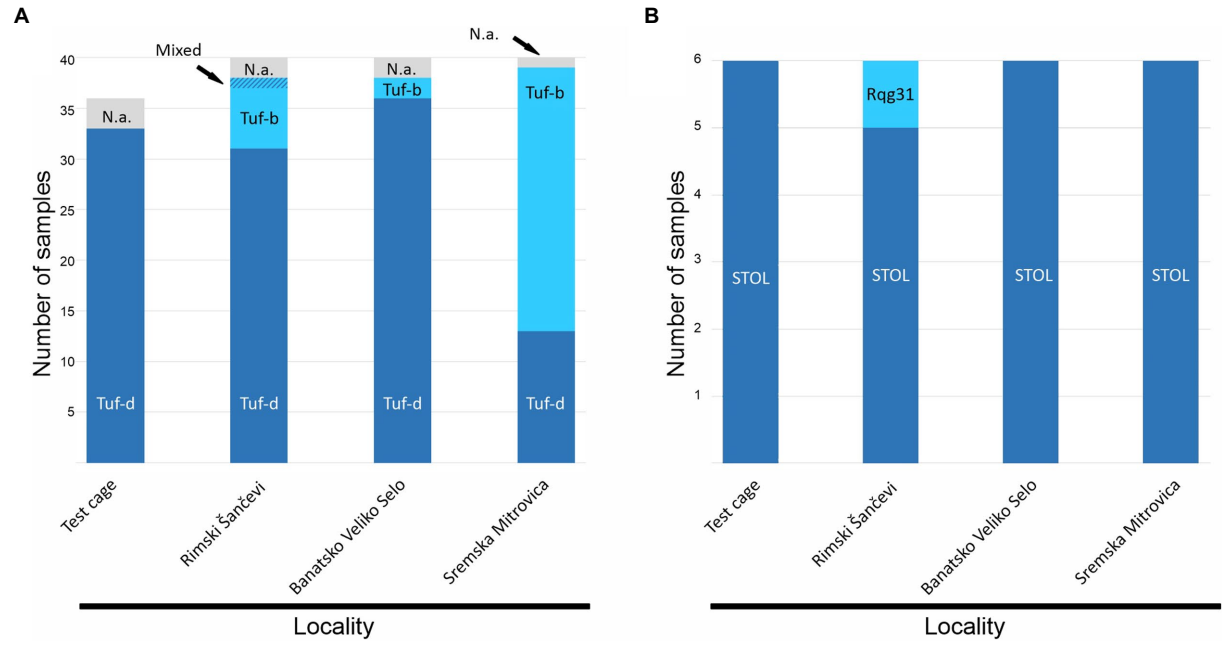

FIGURE 2
Molecular characterization of '*Candidatus* Phytoplasma solani' based on **(A)** *tuf* gene **(B)** *stamp* gene. N.a. not amplified.

majority of the analyzed plants, 26 out of 39, had tuf-b, whereas the tuf-d type was present in 13 sugar beet. Sequencing of the 24 randomly selected strains confirmed the presence of the tuf-d genotype in 23 out of 24 analyzed samples, whereas the tuf-b1 genotype was found in one sample from the field in Rimski Šančevi (Figure 2A).

The partial *stamp* gene sequences obtained from the same set of samples showed prevalence of the STOL (St4) *stamp* genotype in 23 out of 24 samples, whereas in Rimski Šančevi, only one sugar beet, with the tuf-b type, had the Rqg31 (St2) genotype (Figure 2B).

Molecular and morphological characterization of *Macrophomina phaseolina*

In all fungal isolates forming dark grey colonies with numerous black sclerotia on PDA, *M. phaseolina* was confirmed with *M. phaseolina*-specific primers (MpTefF/MpTefR) that generated amplicons of ~220 bp in PCR, whereas no amplification was observed in the negative controls. ITS, TEF1-α, ACT, CAL, and TUB amplicons of expected size (~600, 300, 300, 580, and 700 bp, respectively) were obtained for the seven selected isolates. Sequencing of the obtained amplicons yielded nucleotide sequences of 544–545 nt for ITS, 259–260 nt for TEF1-α, 260 nt for ACT, 544 nt for CAL, and 650 nt for TUB, which were deposited in the NCBI GenBank[1] under accession numbers provided in Supplementary Table S1. Six out of seven analyzed isolates were identical in all five assessed loci, while one (SR231) differed from the other six in all loci (2 nt in ITS and TEF1-α, 1 nt in ACT, 3 nt in CAL, and 4 nt in TUB). The combined dataset of the concatenated five locus alignments contained 2,064 characters, of which 79 were parsimony informative. MP analysis resulted in

1 www.ncbi.nlm.nih.gov/nucleotide

eight equally most parsimonious trees. The phylogenetic tree constructed by the ML method, using the Hasegawa-Kishino-Yano model, had the same topology as the MP tree. A representative phylogenetic tree is presented in Figure 3. Multilocus phylogeny confirmed the identity of the obtained isolates as *M. phaseolina* (Figure 3). Six isolates from sugar beet formed a subclade within *M. phaseolina*, while one isolate (SR231) clustered separately with the *M. phaseolina* isolate from *Helianthus annuus* from Australia (BRIP70730), from which it differed in 3 nt in CAL.

Macrophomina phaseolina colonies had even margins, were initially white with an abundant fluffy or flat aerial mycelium, and turned dark grey with age, developing dense, black sclerotial masses on PDA (Figure 4A). After 3 days on PDA, the average colony diameter was 68.11 ± 1.84 mm. Sclerotia were black, smooth, and hard (mean diam. ± SE of 169 sclerotia 108.5 ± 2.1 µm; Figure 4B). Conidiomata were dark brown to black, solitary or gregarious (Figures 4B,C). Conidiogenous cells were hyaline, short, obpyriform to subcylindrical (Figure 4D). Conidia (Figure 4E) were ellipsoid to obovoid, hyaline and with apical mucoid appendages, (20.82–) 23.78–26.48 (−30.19) µm long and (8.8–) 9.95–10.88 (−11.72) µm wide (mean ± SE of 100 conidia = 25.15 ± 0.2 × 10.4 ± 0.06 µm). Microconidia were aseptate, hyaline and smooth (mean ± SE of 30 microconidia = 5.8 ± 0.13 × 3.5 ± 0.12 µm; Figure 4F).

Discussion

This study investigates the relationship between the presence of fungal root rot pathogens and occurrence of '*Ca*. P. solani'-associated RTD of sugar beet in Serbia. In both the semi-field experiment and open-field assessment, *M. phaseolina* was found only in '*Ca*. P. solani'-infected sugar beet. Apart from *M. phaseolina*, which was predominant on RTD-affected sugar beet with root rot, few other fungi were found in sugar beet without root rot regardless of phytoplasma presence or RTD symptoms. *Macrophomina phaseolina* has been reported as the most significant root rot pathogen of sugar beet in Serbia, causing economic damage that exceeds the impact of other fungal pathogens (Budakov et al., 2015). On the other hand, '*Ca*. P. solani' causes the typical RTD symptom, rubbery taproot, which facilitates rotting of sugar beet, as reported in current and previous studies (Ćurčić et al., 2021a,b). Accordingly, a common trait of both sugar beet pathogens—to escalate during warm droughty summers (Marić, 1974; Budakov et al., 2015; Ćurčić et al., 2021b)—suggests a plausible correlation that has not been investigated to date.

Results obtained in the semi-field transmission experiment in Rimski Šančevi, involving *R. quinquecostatus sensu* Holzinger et al. (2003), corroborated the '*Ca*. P. solani' vectoring role of this cixiid planthopper in the sugar beet RTD context (Kosovac et al., 2023). The transmission experiment with *R. quinquecostatus* resulted in 90%

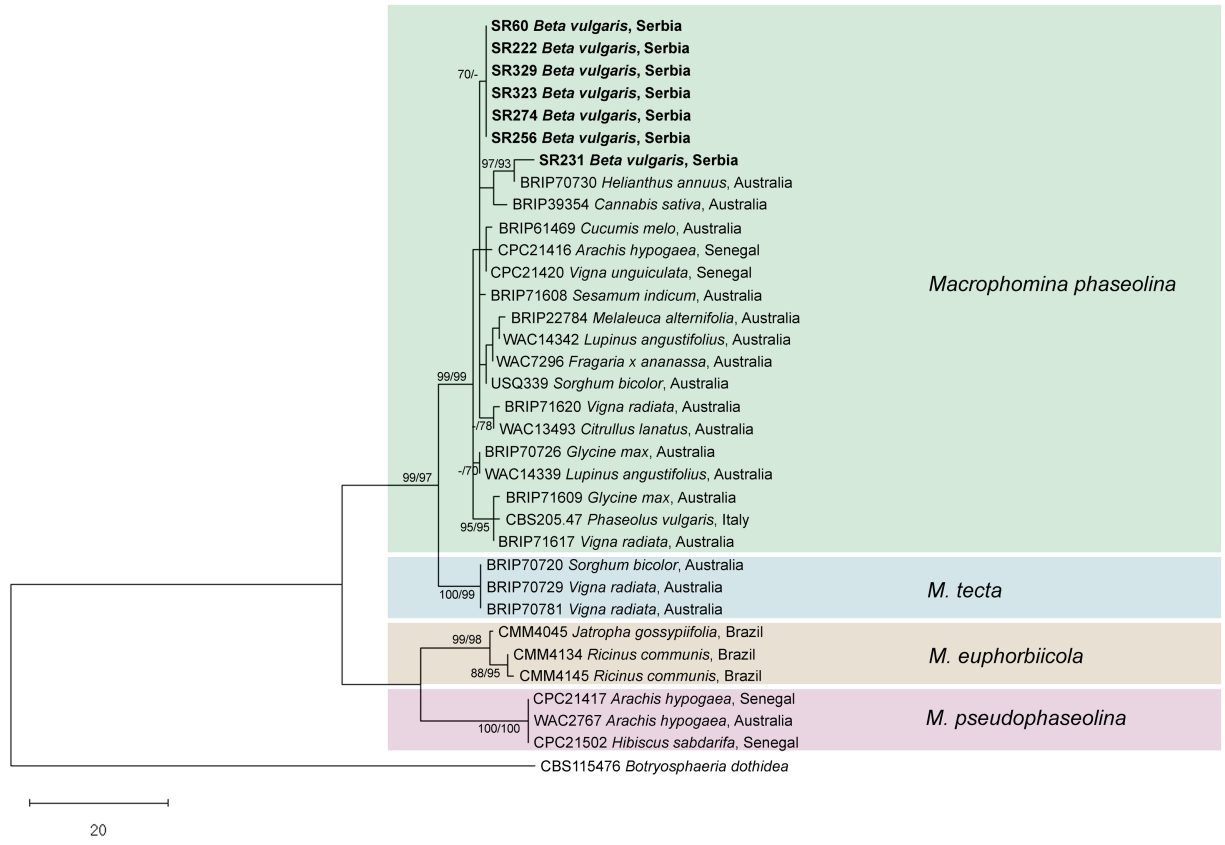

FIGURE 3
Phylogenetic tree resulting from the analysis of concatenated ITS, TEF1-α, ACT, CAL, and TUB sequences of *Macrophomina* spp. Numbers on the branches represent maximum parsimony and maximum likelihood bootstrap values (MP/ML) from 1,000 replicates. Values less than 70% are marked with "-." The tree was rooted to *Botryosphaeria dothidea*. The scale bar represents 20 nucleotide substitutions. Isolates obtained in this work are shown in bold.

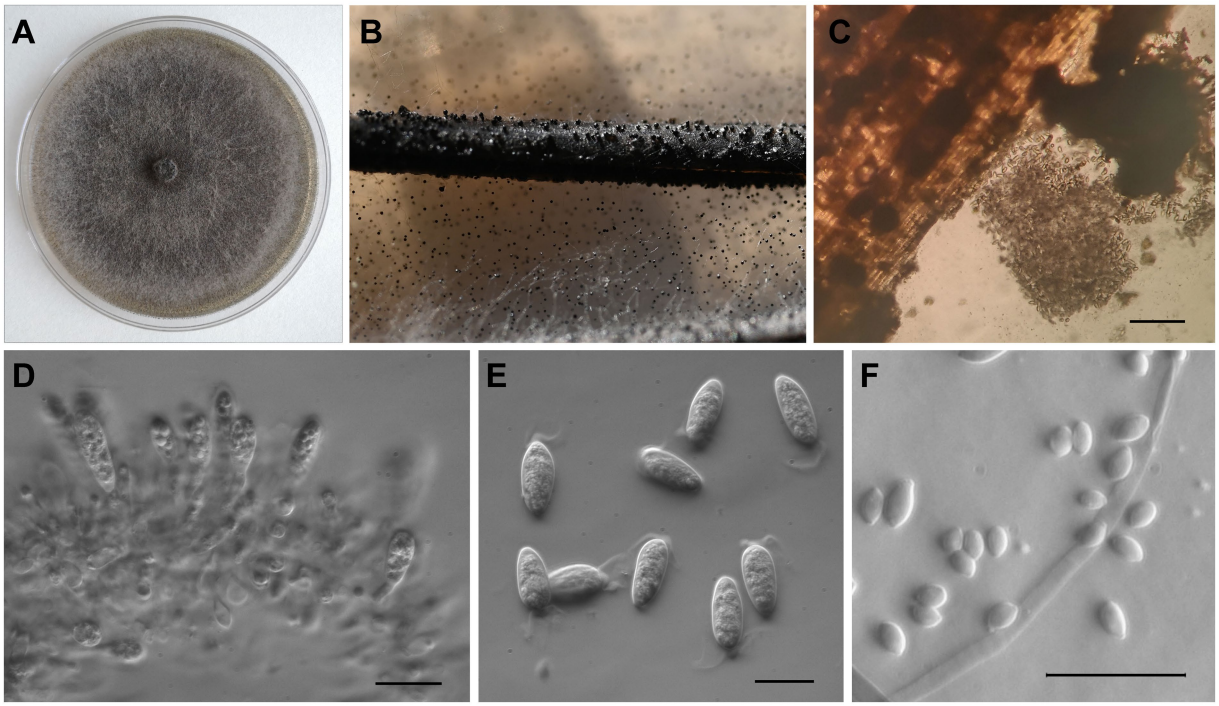

FIGURE 4
Morphological characteristics of *Macrophomina phaseolina* isolated from sugar beet in Serbia. **(A)** Colony on PDA; **(B)** Sclerotia and conidiomata on PNA; **(C)** Conidiomata and conidia; **(D)** Conidiogenous cells; **(E)** Conidia with apical appendages; and **(F)** Microconidia. In **(C)** scale bar=200μm, while in **(D–F)** scale bar=20μm.

'*Ca*. P. solani' infection rate of sugar beet in the experimental cage. Typical leaf RTD symptoms such as loss of turgor, wilting, yellowing, and necrosis, were previously reproduced in laboratory-controlled single-plant experiments using this insect vector, but ruberiness of the taproot had not developed in the test plants, likely because of an optimal watering regime (Kosovac et al., 2023). However, in the semi-field experiment, 36 out of 40 sugar beet expressed prominent rubbery taproot with or without root rot. Characterization of '*Ca*. P. solani' strains transmitted by *R. quinquecostatus* revealed the presence of only the tuf-d type in infected sugar beet, aligning with experimental results from the 2020 epidemic RTD occurrence on the same locality. Furthermore, all six selected strains characterized on the *stamp* gene belonged to the STOL (St4) genotype, previously reported as the only genotype associated with tuf-d (Ćurčić et al., 2021a,b; Kosovac et al., 2023). However, the presence of two tuf-types in the open–field assessment suggests involvement of vector(s) other than *R. quinquecostatus*.

Symptoms observed in the '*Ca*. P. solani' transmission cage—development of rubbery taproots, which are initially without rot, but eventually decline and rot—resemble those in the open fields. Root rot of '*Ca*. P. solani' infected sugar beet in the semi-field experiment was solely due to *M. phaseolina*. The strict correlation of *M. phaseolina* presence with '*Ca*. P. solani' infection, found on three localities in the open-field assessment, shows that *M. phaseolina* did not infect phytoplasma-free sugar beet, even under favorable environmental conditions. Our results suggest that *M. phaseolina* amplifies sugar beet yield losses initiated specifically by '*Ca*. P. solani,' which can be the reason for the discrepancy between reports of *M. phaseolina* as the most significant fungal root pathogen of sugar beet in Serbia and other reports, in which the fungus is described as a minor threat elsewhere (Cooke and Scott, 1993; Jacobsen, 2006; Budakov et al., 2015). RTD-affected sugar beet without root rot can still be used for processing in industry, providing the condition appears in no more than 2% of sugar beet, while root rot is tolerated in no more than 0.5% (National standard SRPS E.B1. 2002; Sugar beet-quality requirements and sampling).

Though our results suggest that '*Ca*. P. solani' infection renders sugar beet more susceptible to *M. phaseolina,* the mechanisms of interactions among the two plant pathogens (a biotroph and a necrotroph) and the plant host are currently unknown. However, it is clear that, because of synergistic interactions, the simple sum of single pathogen infections does not produce equally severe disease symptoms as does co-infection. A similar (bacterium-fungus) synergistic interaction, which leads to a disease complex, has been reported in sugar beet for *Leuconostoc* spp. and *R. solani* root rot (Strausbaugh, 2016). Moreover, such cases of complex diseases are not uncommon, as numerous disease complexes have been described in other hosts (reviewed in Agrios, 2005; Lamichhane and Venturi, 2015). Whereas RTD is associated exclusively with '*Ca*. P. solani,' charcoal root rot of sugar beet seems to be a complex disease that occurs as a consequence of RTD and is associated with two species belonging to separate phyla—'*Ca*. P. solani' and *M. phaseolina*. This is the first description of a phytoplasma-fungus disease complex that may have important implications in the development of an effective plant disease management strategy.

Fungi found in asymptomatic sugar beet were comparable to those isolated from sugar beet with RTD (rubbery taproots), but without root rot. This finding confirms the previously established

association of RTD solely with '*Ca*. P. solani,' without the involvement of fungi (Ćurčić et al., 2021a,b; Kosovac et al., 2023). Moreover, all of the fungi isolated from the healthy and rubbery sugar beet taproots without root rot in this study (i.e., *Fusarium* sp., *Penicillium* sp., and *Rhizopus* sp.) have already been reported as present in healthy sugar beet, and as postharvest pathogens (Liebe et al., 2016; Liebe and Varrelmann, 2016; Strausbaugh, 2018; Kusstatscher et al., 2019).

Multilocus phylogeny performed in this study resolved the previously described *Macrophomina* species and confirmed identification of sugar beet isolates from Serbia as *M. phaseolina*. Two haplotypes of *M. phaseolina* were detected in sugar beet from Serbia, which is in agreement with the previously described high level of intraspecific diversity within *M. phaseolina* (Poudel et al., 2021). Furthermore, to our knowledge, our research is the first to provide characterization of five loci (ITS, TEF1-α, ACT, CAL, and TUB) of European *M. phaseolina*, beside ex-type CBS 205.47 from Italy.

Considering the longevity of *M. phaseolina* sclerotia and an almost 60-year-long four-crop (sugar beet, sunflower, corn, and wheat) agricultural system in Rimski Šančevi, with all listed crops having been reported as hosts of this pathogen, it is likely that the experimental field is highly contaminated with the sclerotia of *M. phaseolina* (Jacobsen, 2006; Babu et al., 2007; Abass et al., 2021; Marquez et al., 2021). The crop rotation practice applied in the experimental field is similarly applied in the wider area of Serbia, producing an environment that contributes to the problem. The presence of '*Ca*. P. solani' (reservoir host plant(s) and efficient vector(s)), *M. phaseolina* contaminated soil and favorable weather conditions (temperature above 30°C and drought) represents a triangle that creates a "perfect storm" of critical factors causing high yield losses in Serbia. The lack of simultaneous impact of all these factors may explain why '*Ca*. P. solani' infection of sugar beet recorded in some other parts of Europe, such as France, Germany, and Austria (Sémétey et al., 2007; Ćurčić et al., 2021a), is not as devastating as in Serbia. However, the situation may differ in the future because of climate change or interference of other secondary pathogen(s).

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/genbank/, OQ420603, OQ420617, OQ421259, OQ420624, OQ420610, OQ420604, OQ420618, OQ421260, OQ420625, OQ420611, OQ420609, OQ420623, OQ421265, OQ420630, OQ420616, OQ420608, OQ420622, OQ421264, OQ420629, OQ420615, OQ420607, OQ420621, OQ421263, OQ420628, OQ420614, OQ420606, OQ420620, OQ421262, OQ420627, OQ420613, OQ420605, OQ420619, OQ421261, OQ420626, and OQ420612.

Author contributions

BD and ND managed the project and drafted the manuscript. ŽĆ and ER set up and maintained the experimental field. AK designed the transmission experiments and identified insects. AK, ŽĆ, ER, and BD conducted transmission experiments. ŽĆ, ER, BD, ND, and IV collected samples. JS, BD, and AK conducted the phytoplasma analyses. IV, NV, and ND conducted the fungi analyses. BD, JS, AK, ND, and IV contributed to the interpretation of the data. BD, ND, IV, and AK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1164035/full#supplementary-material

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Pseudomonas spp. can help plants face climate change

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Climate change is increasingly affecting agriculture through droughts, high salinity in soils, heatwaves, and floodings, which put intense pressure on crops. This results in yield losses, leading to food insecurity in the most affected regions. Multiple plant-beneficial bacteria belonging to the genus *Pseudomonas* have been shown to improve plant tolerance to these stresses. Various mechanisms are involved, including alteration of the plant ethylene levels, direct phytohormone production, emission of volatile organic compounds, reinforcement of the root apoplast barriers, and exopolysaccharide biosynthesis. In this review, we summarize the effects of climate change-induced stresses on plants and detail the mechanisms used by plant-beneficial *Pseudomonas* strains to alleviate them. Recommendations are made to promote targeted research on the stress-alleviating potential of these bacteria.

KEYWORDS

***Pseudomonas*, plants, climate change, abiotic stress, salt, drought, heat, flood**

1. Introduction

The surface temperature has increased by 1.1°C globally and by 1.6°C on land since the pre-industrial era (IPCC, 2021). This rise in temperature is highly likely to continue, and even accelerate in the coming decades, leading to more intense and frequent droughts, extremes of heat, and other major weather events (IPCC, 2021, 2022; Lesk et al., 2022). While mitigation of climate change has been deemed critical to hamper this global threat, adaptation is also required to minimize the vulnerability of the affected agroecosystems and of the societies that rely on them (IPCC, 2022).

Agriculture is increasingly affected worldwide by this changing climate, notably through droughts, heatwaves, increased soil salinity, and floods (Tomaz et al., 2020; Bezner Kerr et al., 2022). These extreme events generate stress in plants, i.e., changes in growth conditions altering or even disrupting the plant homeostasis (Shulaev et al., 2008). Agricultural yields are already affected, with impacts ranging from slowing to halting yield growth in regions such as Australia and Southern Europe. A 10–20% decrease in yields has even been reported for some crops in Western Africa (Bezner Kerr et al., 2022).

Plants may be able to acclimate and adapt to some extent to climate change with the help of their microbiome (Trivedi et al., 2022). Indeed, plants are closely associated with a myriad of microorganisms, including protists, fungi, and bacteria, which form its microbiome. This microbiome helps plants acquire nutrients, enhance growth-related physiological processes, promote defense against plant pathogens, and alleviate abiotic stresses (Rubin et al., 2017; Trivedi et al., 2020). These microbes mainly colonize two plant-dependent compartments: the phyllosphere and the rhizosphere, which refer to the external surface of leaves and to the volume of soil influenced by the roots, respectively (Zboralski et al., 2023).

Bacteria belonging to the genus *Pseudomonas* are often core members of the phyllosphere and rhizosphere microbiome, competitively colonizing these compartments and thriving in them (Trivedi et al., 2020; Zboralski and Filion, 2020). These bacteria are rod-shaped, Gram-negative, motile, non-sporulating, and mostly aerobic organisms (Palleroni, 2005). On average, the genomes of *Pseudomonas* type strains consist of 5.6 ± 1.0 Mb and contain 5,260 ± 928 genes (Hesse et al., 2018). These bacteria can also carry plasmids, even if they are not commonly encountered (Silby et al., 2011). More than 300 species of *Pseudomonas* have been described so far according to the List of Prokaryotic names with Standing in Nomenclature (Parte et al., 2020).

Numerous *Pseudomonas* strains have been studied over the last decades for their biocontrol and plant growth promotion abilities (Weller, 2007; Mercado-Blanco, 2015). These plant-beneficial strains produce a multitude of secondary metabolites, including cyclic lipopeptides, antibiotics, siderophores, effectors, and plant hormones (Gross and Loper, 2009; Ghequire and De Mot, 2014; Götze and Stallforth, 2020; Biessy and Filion, 2021). These compounds mediate several direct and indirect plant-beneficial effects of *Pseudomonas* spp., for instance through the modulation of plant hormone levels and improved nutrient availability in the soil, or through the inhibition of plant pathogens and improved plant resistance to infections.

Pseudomonas strains have received increasing attention for their potential to relieve plants from environmental stresses (Supplementary Tables S1, S2; Rajkumar et al., 2017). Whether they are used as single-strain inoculants or as members of microbial consortia, *Pseudomonas* spp. show promise in alleviating stresses in crop plants exacerbated by climate change. Therefore, and given their biocontrol capabilities, the use of *Pseudomonas* spp. as bioinoculants in agriculture could actively contribute to reducing input costs, chemical contamination, pesticide use and exposure, and improving resilience at multiple levels.

How do the climate-related stresses impact plants? What are the mechanisms involved in the plant stress-alleviating effects of *Pseudomonas* spp.? What are the pending issues that need to be addressed by researchers to better use *Pseudomonas* strains in efforts to adapt agriculture to climate change and extreme weather events? Through this review, we aim to answer these questions and contribute to supporting the work in progress on the development of bioinoculants to better adapt agriculture to climate change.

2. Abiotic stresses exacerbated by climate change affect plants in different ways

2.1. Drought and high salinity create osmotic stress

Stresses caused by drought and high salinity often originate from distinct causes but lead to similar impacts for the plant (Forni et al., 2017).

Drought is usually defined as a deficiency in rainfall resulting in water shortage (Wilhite and Glantz, 1985). In agriculture, drought can be more specifically defined as insufficient rainfall for a given period of time whereby crop water requirements can no longer be met by the available water supply (Kebede et al., 2019). Such a lack in water availability decreases the soil water potential, creating osmotic stress for the plant (Figure 1). It also leads to a higher soil hardness, which reduces soil penetrability for roots (Colebrook et al., 2014). Drought is considered the main cause of losses in agriculture (FAO, 2021b).

Salinity stress in plants is caused by an excess of soluble salts in the soil, especially sodium chloride (Munns and Tester, 2008). Such excess in soluble salts can originate from low drainage, saltwater intrusion in coastal regions due to the rising of sea levels, or irrigation and land clearing raising the water table (Munns and Tester, 2008). Worldwide, at least 4.4% of topsoil (0–30 cm) and more than 8.7% of subsoil (30–100 cm) land area is affected by salt (FAO, 2021a). This area is likely to expand and soil salinity to increase in several regions because of climate change (Hassani et al., 2021). High salt concentrations in the soil decrease the soil water potential, making it more difficult for the plant to take up water, leading to osmotic stress, similar to drought conditions (Figure 1). Additionally, the plant takes up an excess of salts, which accumulates in the leaves to toxic levels, leading to ionic stress and impairing photosynthetic capacity (Munns and Tester, 2008).

Leaf growth tends to be more affected by osmotic stress than root growth as the plant saves water by limiting evapotranspiration through stomatal closure and keeps exploring the soil through its roots in search of the valuable molecule (Forni et al., 2017). Drought can also accelerate the crop cycle by triggering the reproductive phase earlier, leading to lower yields (Desclaux and Roumet, 1996). If drought or high salt concentration in soils persist, cells may lose membrane integrity and water, impairing photosynthesis and generating reactive oxygen species (ROS), eventually leading to plant death (Forni et al., 2017; Shahid et al., 2020). Plants respond to drought and high salinity through the production of ROS-scavenging enzymes, osmolytes, and secondary compounds like anthocyanins and phenolics, which help them acclimate to the resulting stresses (Forni et al., 2017).

2.2. Heat stress produces a physiological shock

Heat stress can be defined as an increase in temperature above a given threshold over a period of time sufficient to induce irreversible damages to plants, reducing yields (Wahid et al., 2007). It is difficult to provide the reader with a specific threshold or even a range of temperatures above which heat stress occurs, because plant species and cultivars all have different optimal growth temperatures and sensitivity to temperature variation (Hatfield and Prueger, 2015). Also, the effects of heat stress on yields are not linear: each additional degree above the optimal growth temperature for a given crop results in a greater yield loss than the previous degree (Schlenker and Roberts, 2009). Although all the developmental stages of plants are vulnerable to heat stress, the extent of this vulnerability varies according to the stage (Jagadish et al., 2021). The flowering, gametogenesis, and pollination stages are especially sensitive to heat (Hatfield and Prueger, 2015; Jagadish et al., 2021). Heat affects membrane and cuticle integrity, and enzyme activity, leading to impaired photosynthesis as well as increased respiration and oxidative stress (Figure 1; López et al., 2022).

When water is available in the soil, plants can use it to cool down the leaves and limit the effects of heat (López et al., 2022). However, when drought and heat combine, which is expected to be increasingly

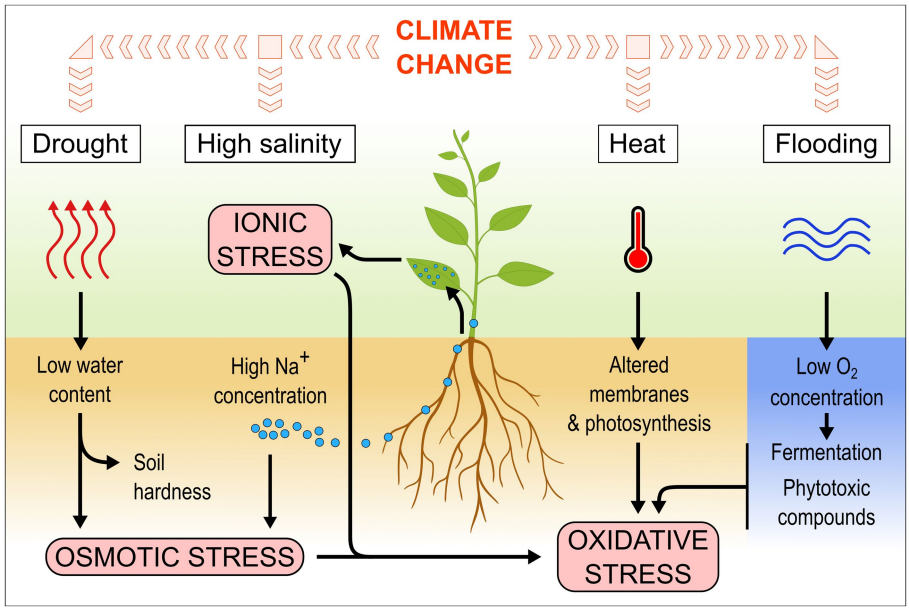

FIGURE 1
Climate-related stresses affect plants in different ways and lead to various cellular stresses.

common due to climate change (Cohen et al., 2021), leaf temperature can increase by 5°C to 10°C, causing severe damage to the whole plant and resulting in a sharp decline in crop yield (López et al., 2022). Plants can also acclimate to some extent to heat using mechanisms that resemble those used to cope with drought or high salinity conditions. They accumulate osmolytes such as polyol, proline, and ammonium compounds, produce secondary metabolites like isoprenoids or carotenoids, activate detoxification systems against ROS, and synthesize heat shock proteins to protect other proteins from denaturation (Wahid et al., 2007).

2.3. Flooding stress drastically reduces oxygen availability

Flooding can be broadly defined as excessively wet conditions in which water occupies volumes around roots and/or shoots that are normally filled with air (Sasidharan et al., 2017). It especially encompasses two phenomena: waterlogging and submergence. Waterlogging is a soil condition whereby excess water inhibits gas exchange with the atmosphere at the root level only (McFarlane et al., 1989; Sasidharan et al., 2017). In practice, waterlogging is happening when the water table is less than 30 cm below the soil surface, or when more than 90% of the soil pores are filled with water (Shaw and Meyer, 2015). Submergence occurs when free-standing water is above the soil surface, partially or completely submerging the plant (McFarlane et al., 1989; Sasidharan et al., 2017). Worldwide, flooding is considered the second most impactful disaster in agriculture, after drought (FAO, 2021b). Most crops are not adapted to grow in water-saturated soils, which can lead to severe yield losses depending on the crop, soil type, and the duration of the flood (Zhou, 2010). In coastal areas, flooding stress can combine with salt stress, exacerbating yield losses (Zhou, 2010).

Under water-saturated conditions, access to oxygen is limited because of its poor diffusion in water compared to air (Watanabe et al., 2013). The low amount of available oxygen is quickly consumed by the roots and microbes, leading to anoxic conditions and to the development of anaerobic microbes, which decrease the redox potential in the flooded soil (Figure 1; Laanbroek, 1990; Watanabe et al., 2013). Under prolonged flooding, phytotoxic reduced forms of inorganic compounds accumulate in the soil, such as sulfides (Singh et al., 2018). Without oxygen, plants are unable to perform respiration and use fermentation instead to generate usable forms of energy (Perata and Alpi, 1993). If the plants are entirely submerged, they receive less light, impacting photosynthesis and further reducing oxygen availability (Loreti et al., 2016). They actively degrade starch to maintain glycolysis and survive, but growth is generally strongly reduced, leading to yield losses (Loreti et al., 2016, 2018).

3. *Pseudomonas* spp. directly affect phytohormone levels to help plants cope with climate-related stresses

The mechanisms used by *Pseudomonas* spp. to help plants cope with abiotic stresses intensified by climate change are often not specific to a single stress and rather help the plant grow under various stressful conditions. They especially consist in producing compounds directly affecting the plant hormone levels, notably ethylene, auxin, gibberellins, and cytokinins.

3.1. Reduction of ethylene levels in plants

The reduction of plant ethylene levels is certainly one of the most studied bacteria-mediated plant stress relief mechanisms.

Ethylene is a chemically simple gaseous plant hormone that is particularly well known for its role in fruit ripening (Barry and Giovannoni, 2007). It also plays a central role in growth and response to biotic and abiotic stresses (Dubois et al., 2018). Ethylene concentration increases when plants face drought, heat, high salt, and flooding, resulting in reduced growth (Dubois et al., 2018; Pattyn et al., 2021).

Some bacteria, including multiple *Pseudomonas* strains belonging to diverse species, can lower the plant ethylene levels by producing the AcdS enzyme, a cytoplasmic deaminase that degrades the direct precursor of ethylene, 1-aminocyclopropane-1-carboxylate (ACC), into ammonium and α-ketobutyrate (Figure 2; Nascimento et al., 2014; Glick and Nascimento, 2021). ACC itself has recently been proposed as a signal molecule for plants, involved in various plant processes such as pathogen interactions and stress response (Polko and Kieber, 2019). Therefore, the activity of AcdS decreases the concentration of not only one, but two signaling molecules involved in plant stress responses. This leads to enhanced plant growth under stress conditions and generates usable sources of nitrogen and carbon for the bacteria (Glick and Nascimento, 2021).

The effect of the ACC deaminase produced by *Pseudomonas* strains on plants under stress conditions has been demonstrated in several plant species using reverse genetics approaches, especially in canola, cucumber, and tomato under salt stress, and in tomato under flooding stress (Glick and Nascimento, 2021). Some research teams also used a transgenic approach, transferring a *Pseudomonas acdS* gene directly into the genome of tomato and *Arabidopsis thaliana*, which then became more tolerant to a flooding period of 5–9 days (Grichko and Glick, 2001; Jung et al., 2018). These two approaches clearly demonstrated the active role played by ACC deaminase in plant stress alleviation.

3.2. Direct biosynthesis of phytohormones

Some *Pseudomonas* spp. have been shown to directly produce phytohormones, especially IAA, gibberellins, and cytokinins, while promoting plant growth under abiotic stress conditions at the same time (García de Salamone et al., 2001; Egamberdieva, 2009; Kang et al., 2014; Spaepen, 2015; Mekureyaw et al., 2022; Yasmin et al., 2022).

3.2.1. Auxin biosynthesis

Auxin—from the Greek word "auxein," "to grow"—usually refers to indole-3-acetic acid (IAA; Figure 3), the main plant auxinic compound (Enders and Strader, 2015). Its role in plants has historically been demonstrated in cell elongation and apical dominance, but this phytohormone is also involved in many other growth and development processes in plants, if not all (Zhao, 2010; Leftley et al., 2021). For the last decade, its role in the plant response to abiotic stresses has been further explored (Sharma et al., 2015). Auxin has notably been shown to contribute to the optimization of the root system architecture under environmental stress, especially osmotic stress (Leftley et al., 2021).

Many *Pseudomonas* spp. have been shown to produce IAA, and in some cases, to improve plant tolerance to climate-related stresses. For instance, several strains known to produce IAA can reduce the effect of high salinity on germination and seedling growth in wheat and cotton (Egamberdieva, 2009; Egamberdieva et al., 2015). Under drought conditions, other IAA-producing *Pseudomonas* strain were able to improve growth in different plant species, including wheat, jujube, and *A. thaliana* (Chandra et al., 2018; Raheem et al., 2018; Zhang et al., 2020; Yasmin et al., 2022). Heat stress could also be alleviated in wheat using an IAA-producing, thermotolerant strain (Ali Shaik et al., 2011). Using IAA-deficient mutants, some research

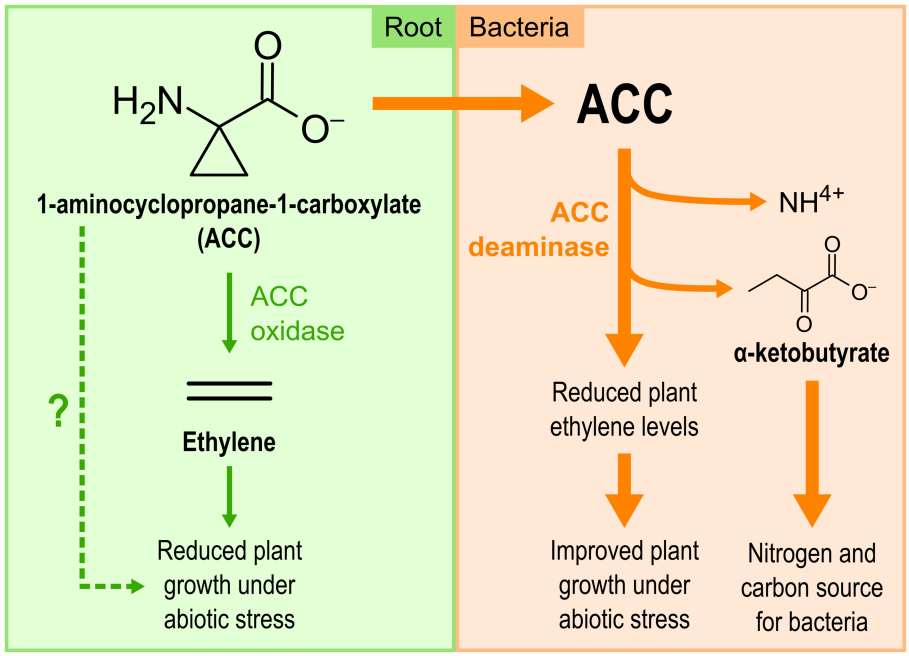

FIGURE 2
The 1-aminocyclopropane-1-carboxylate (ACC) deaminase produced by *Pseudomonas* spp. reduces ethylene levels in plants under climate-related abiotic stresses.

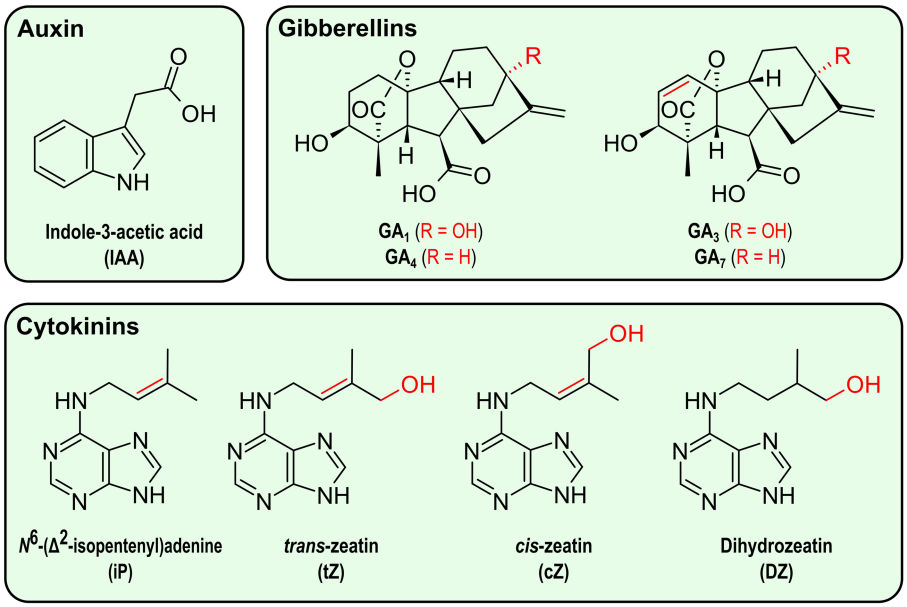

FIGURE 3
Structure of the main phytohormones produced by bacteria. Elements in red represent variations between compounds in the same category.

groups demonstrated that IAA produced by *Pseudomonas* strains played a substantial role in plant growth promotion under standard growth conditions (Patten and Glick, 2002; Ul Hassan and Bano, 2019). However, to our knowledge, such a clear causal link remains to be demonstrated under climate-related stress conditions. Interestingly, external IAA application on a strain belonging to another *Proteobacteria* genus, *Bradyrhizobium*, improves its tolerance to various abiotic stresses such as osmotic stress (Duca and Glick, 2020). IAA might also contribute to stress tolerance in *Pseudomonas* strains.

Three main IAA biosynthetic pathways have been uncovered in plant-beneficial *Pseudomonas* spp. and are all tryptophan-dependent (Duca et al., 2014, 2018). The indole-3-acetamide (IAM) pathway is mediated by enzymes encoded by the *iaa* gene cluster (Gross and Loper, 2009). Many *Pseudomonas* strains carry this cluster in their genome (Spaepen and Vanderleyden, 2011; Loper et al., 2012; Biessy et al., 2019). The indole-3-pyruvic acid (IPA) pathway, including the shortcut enabled by a tryptophan side-chain oxidase, has been found in *Pseudomonas* spp., but the genes involved have not yet been identified in strains belonging to this genus (Duca et al., 2014; Duca and Glick, 2020). The *ipdC* gene, encoding a key enzyme in the IPA pathway, has been detected in several bacterial genera (Duca et al., 2014). This gene had been identified in a single *Pseudomonas* strain, *P. putida* GR12-2 (Patten and Glick, 2002). However, the reported sequence turned out to originate from a strain belonging to another *Gammaproteobacteria* genus, *Enterobacter* (Gross and Loper, 2009). Moreover, BLASTn and BLASTp searches using *ipdC* sequences from various genera did not yield any significant results in the *Pseudomonas* genus, suggesting the implication of genes distinct from *ipdC* in this pathway. The third IAA pathway identified in *Pseudomonas* spp. is the indole-acetaldoxime/indole-3-acetonitrile (IAOx/IAN) pathway (Duca et al., 2014). The genes encoding the enzymes involved in this pathway have almost all been identified in *P. putida* UW4: *phe*, *nit*, *nthA*, *nthB*, and *ami* (Duca et al., 2018). Few other *Pseudomonas* strains have been shown to harbor homologous genes in their genome (Duca et al., 2014). Only the gene that encodes the enzyme mediating the first step of the pathway remains to be identified, along with the protein itself (Duca et al., 2018). All three pathways can be found in a single strain, for example in *P. putida* UW4, in which they are interrelated (Duca et al., 2018).

Some *Pseudomonas* strains harbor the *iac* gene cluster, responsible for IAA catabolism, sometimes harboring the IAA biosynthetic cluster as well (Loper et al., 2012; Biessy et al., 2019; Zboralski et al., 2022). This cluster allows its carrier strain to use IAA as the sole carbon and energy source (Leveau and Gerards, 2008). Since the optimal IAA concentration to favor plant growth is narrow (Persello-Cartieaux et al., 2003), plant-beneficial *Pseudomonas* must finely tune the external IAA concentration to benefit from plant growth. This catabolic pathway may contribute to such regulation. Under climate-related stresses, this balance between IAA biosynthesis and degradation in plant-beneficial *Pseudomonas* spp. and its role in stress-alleviation remains to be investigated.

3.2.2. Gibberellins biosynthesis

Gibberellins are tetracyclic diterpenoid carboxylic acids whose name originates from the fungus *Gibberella fujikuroi* (now *Fusarium fujikuroi*), a rice pathogen from which some gibberellins were first isolated for their role in shoot elongation (Hedden and Sponsel, 2015). These hormones control transitions between plant stages as well as cell division and elongation (Colebrook et al., 2014). The main bioactive gibberellins are GA_1, GA_3, GA_4, and GA_7 (Figure 3), with GA_1 and GA_4 being the most common ones in plants (Binenbaum et al., 2018). Under stress conditions such as flooding, drought and high salinity, the plant gibberellin signaling pathway is usually inhibited, allowing the plant to adapt its growth to the new conditions and prevent ROS accumulation (Achard et al., 2008; Colebrook et al., 2014).

Gibberellins biosynthesis has been reported in some plant-beneficial *Pseudomonas* strains (Kang et al., 2014, 2020; Pandya and Desai, 2014; Sharma et al., 2018; Yasmin et al., 2022). However, the methods used to detect and quantify gibberellins differ greatly, which can lead to unreliable claims (Bottini et al., 2004). These methods range from basic spectrophotometric approaches (Pandya and Desai, 2014; Sharma et al., 2018) to more robust methods involving analytical chemistry tools, including gas chromatography–mass spectrometry (Kang et al., 2014, 2019, 2020) and high-performance liquid chromatography (García de Salamone et al., 2001; Yasmin et al., 2022). Analytical approaches allowed to accurately detect different bioactive gibberellins in *Pseudomonas* strains that were able to promote the growth of soybean, *A. thaliana*, lettuce, and Chinese cabbage, in some cases under high salinity and drought conditions (Kang et al., 2014, 2019; Adhikari et al., 2020; Yasmin et al., 2022).

The gibberellin biosynthetic pathway has been elucidated recently in bacteria compared to its distinct counterparts in plants and fungi (Nett et al., 2017). The biosynthetic operon has been well described in rhizobia and notably contains three cytochrome P450 monooxygenases, which are each involved in multiple steps leading to the synthesis of GA_9 (Nett et al., 2017). The entire operon has been identified in two *Pseudomonas* strains only, *P. psychrotolerans* NS274 and *P. psychrotolerans* RSA46 (Nagel et al., 2018). Their operon is quite distant from other bacterial ones and include a fourth cytochrome P450 monooxygenase that is known to enable the conversion of GA_9 to the bioactive compound GA_4 (Nagel et al., 2018). These strains were both isolated from rice seeds and have not been assessed yet for potential plant-beneficial effects and gibberellin production (Midha et al., 2016). Interestingly, they seem to be the only known bacterial strains carrying this operon that are neither rhizobia nor known pathogens (Nagel et al., 2018). However, the nature of their potential relationship with plants remains to be assessed. Considering that hundreds of high-quality *Pseudomonas* genomes are now available and that several *Pseudomonas* strains are known to produce gibberellins, it is surprising that only two strains have been shown to display the biosynthetic operon in their genome. Sequencing the genomes of the strains that are already known to produce gibberellins would likely help identify the associated biosynthetic pathway. Also, this biosynthetic pathway may be different than the only one described to date in other bacteria (Nett et al., 2017). More research efforts are needed to elucidate this enigma and build gibberellin-defective mutants. These mutants will be needed to assess the exact role *Pseudomonas*-produced gibberellins may play in the alleviation of climate-related stresses in plants.

3.2.3. Cytokinins biosynthesis

Cytokinins are a family of adenine derivatives initially described for their roles in cell division, from which their name is derived, and growth (Skoog and Armstrong, 1970). They have later been characterized for their involvement in the plasticity of the root system architecture under drought conditions, in sodium exclusion under high salinity conditions, as well as in the plant response to heat stress (Cortleven et al., 2019; Li et al., 2022). Different cytokinins have been found in plants, but the main ones are isoprenoid cytokinins, including N^6-(Δ^2-isopentenyl)-adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DZ) (Figure 3; Sakakibara, 2006; Cortleven et al., 2019).

Cytokinin production has long been known to be associated with some plant-pathogenic *Pseudomonas* strains and has also been described in some plant-beneficial ones (García de Salamone et al., 2001; Pérez-Martínez et al., 2008; Großkinsky et al., 2016). The tZ-producing strain *P. putida* AKMP7 has been shown to alleviate heat stress in wheat (Ali Shaik et al., 2011; Raja Gopalan et al., 2022). Another cytokinin-producing *Pseudomonas* strain, *Pseudomonas* sp. G20-18, was shown to increase cytokinin concentrations in the rhizosphere of canola and to prime the tomato stress response to drought (Pallai et al., 2012; Mekureyaw et al., 2022). Interestingly, cytokinin biosynthesis was also demonstrated to induce plant defenses against *P. syringae* pv. *tomato* DC3000 when this pathogen and G20-18 were infiltrated in *A. thaliana* leaves (Großkinsky et al., 2016).

Two distinct cytokinin biosynthetic pathways have been described in bacteria: *de novo* biosynthesis from adenosine monophosphate initiated by an adenylate isopentenyl transferase (IPT), and synthesis from a modified adenosine in specific tRNA catalyzed by a tRNA-IPT (Großkinsky et al., 2016; Frébortová and Frébort, 2021). Wei et al. recently published a comparative analysis of the adenylate IPTs in plant-beneficial and plant-pathogenic bacteria (Wei et al., 2023). They showed that the genomes of plant-beneficial bacteria tended more often to harbor genes related to cytokinin degradation and metabolism in the vicinity of adenylate IPT than genomes of pathogens, which contained more adenylate IPT gene copies on average. This suggests that beneficial bacteria regulate cytokinin biosynthesis differently than pathogenic ones to optimize plant-beneficial effects (Wei et al., 2023). Homologs of the adenylate IPT gene were found in the genome of 90 bacteria, including only one *Pseudomonas* strain, *P. psychrotolerans* PRS08-11306 (Wei et al., 2023). This strain, which was isolated from rice seeds, was shown to improve rice growth but has not been tested yet for cytokinin production (Liu et al., 2017). The fact that only one *Pseudomonas* strain was shown to display an adenylate IPT gene in its genome suggests that the *de novo* cytokinin biosynthesis pathway may not be very common within this genus. The tRNA-IPT-encoding gene *miaA*, involved in the other cytokinin biosynthetic pathway, is found in almost all bacterial species, including *Pseudomonas* spp., probably because of its role in tRNA modification and in translation (Carpentier et al., 2020; Frébortová and Frébort, 2021; Nielsen et al., 2021; Wei et al., 2023). Also, mutations in *miaA* often result in pleiotropic effects (Gibb et al., 2020). This makes it difficult to study the effect of cytokinin-deficient mutants on plants, although a viable *miaA*-defective mutant was successfully engineered in *Pseudomonas* sp. G20-18 (Großkinsky et al., 2016). In the latter, the cytokinin production was not directly assessed, but its effect on cytokinin levels in *A. thaliana* was evaluated under standard conditions. It notably decreased the plant tZ and iP levels when compared to the wild type, suggesting a potentially lower cytokinin biosynthesis in this mutant (Großkinsky et al., 2016).

4. Volatile organic compounds mediate a remote stress-alleviating effect of *Pseudomonas* spp. in plants

Volatile organic compounds (VOCs) are low-molecular weight molecules displaying a high vapor pressure and a low boiling point, which allow them to diffuse easily in air and water (Netzker et al., 2020). Plant-beneficial *Pseudomonas* strains have been shown to

produce a multitude of them, including alkenes, amides, aromatic compounds, esters, ketones, and sulphur compounds, which are involved in antimicrobial activity, elicitation of the plant immune responses, and plant growth promotion (Garbeva and Weisskopf, 2020; Netzker et al., 2020). However, only few of these compounds have been demonstrated to influence plants at concentrations observed *in vivo*, especially under abiotic stresses.

Several *Pseudomonas* strains can produce VOCs that directly help plants cope with drought or high salinity. *P. chlororaphis* subsp. *aureofaciens* O6 produces 2R,3R-butanediol, which induces systemic tolerance to drought and high salinity in *A. thaliana* by triggering stomatal closure (Cho et al., 2008, 2012). Another *Pseudomonas* strains, *P. pseudoalcaligenes* SMR-16, can improve maize growth under drought using volatile compounds (Yasmin et al., 2021). The emitted compounds were characterized and included 2R,3R-butanediol along with several others, such as dimethyl disulfide and 2-pentylfuran. The potential role played by each one in growth promotion was unfortunately not assessed. Finally, a research group identified a strain named *P. simiae* AU that was able to improve soybean growth under salt stress through a mix of volatile compounds, which remain to be characterized (Vaishnav et al., 2015). The volatile compounds produced by this strain induced the accumulation of proline and a reduction in the sodium content of roots, helping the plants cope with osmotic and ionic stress, respectively.

Other volatile compounds produced by plant-beneficial *Pseudomonas* strains have been shown to have a direct effect on the growth of different plant species, for instance 2-butanone, N,N-dimethyl-formamide, formamide, 1-hexanol, indole, 2-methyl-n-1-tridecene, and 13-tetradecadien-1-ol (Blom et al., 2011; Park et al., 2015; Zhou et al., 2016). To our knowledge, the potential effects of such compounds on plants under abiotic stresses have however not yet been investigated.

Curiously, after decades of research on the effects of microbial VOCs on plants, the mechanism(s) mediating their perception by plants remains mostly unknown (Bailly, 2020). Even though these molecules have been shown to display a plethora of plant-beneficial effects, no specific receptor proteins actually binding these microbial compounds have been found, even for the long-known 2,3-butanediol (Bailly, 2020; Weisskopf et al., 2021). Such a knowledge gap represents a major barrier to a better use of VOCs in the development of effective microbial inoculants. Bailly (2020) suggested a practical approach to lift this barrier, which consists in the identification of reliable traits induced by these compounds, the construction of a classification of these compounds based on their bioactivity, and large-scale transcriptomic and proteomic approaches to uncover how plants react to these molecules.

5. A *Pseudomonas* strain strengthens the root apoplast barriers, countering the effects of high salinity in plants

In two recent studies, a research group discovered that a *Pseudomonas* strain known as *P. mandelii* IB-Ki14 was able to help wheat and pea better tolerate salt in the soil by increasing the deposition of suberin and lignin in xylem cell walls, suberin lamellae, and Casparian strips (Figure 4; Martynenko et al., 2022, 2023). These structures are important apoplastic barriers in the root endodermis controlling water and mineral influx into the root stele. The authors hypothesized that the strengthening of these barriers would decrease the hydraulic conductance from the roots to the leaves, but this was not the case. Instead, they showed that bacterial inoculation led to a dramatic increase in the amount of aquaporins in the roots, especially around the epidermis and endodermis, under normal growing conditions (Arkhipova et al., 2022). Under high salinity conditions, aquaporin activity may compensate the loss of apoplastic permeability by improving symplastic transport of water, enabling the plant to better control water and sodium influx.

Accumulation of abscisic acid in plants, a phytohormone involved in the plant stress response, usually leads to these effects on apoplastic barriers under salt stress conditions (Martynenko et al., 2023). However, the authors showed that there was no such accumulation in pea plants inoculated with *P. mandelii* IB-Ki14, which led them to suggest that these salt stress-alleviating effects may be ABA-independent (Martynenko et al., 2023). However, *P. mandelii* IB-Ki14 was able to improve IAA levels in roots, which may mediate the observed effects. Further investigation is needed to decipher the underlying mechanism.

Interestingly, accumulation of suberin in roots of wetland plant species has been suspected to improve their tolerance to waterlogging by reducing root oxygen loss (Watanabe et al., 2013). Such an accumulation occurs in the root outer cell layers and not at the endodermis level. Therefore, these plant species have evolved flood-adapted root tissues that are central to flood tolerance, such as the aerenchyma. The *Pseudomonas*-induced suberin deposition in roots shown to help plants cope with high salinity might also contribute to flood tolerance. Such a hypothesis would have to be tested.

6. *Pseudomonas* spp. reduce oxidative stress in plants and also for their own survival

Under stress, plants accumulate reactive oxygen species (ROS), which can damage proteins, lipids, and nucleic acids, leading to impaired cellular processes and slower plant growth (Zhang et al., 2016; Smirnoff and Arnaud, 2019). Plants are however able to decrease ROS levels to some extent, through the production of antioxidant metabolites such as proline, ascorbate, and glutathione, or via the synthesis of antioxidant enzymes including superoxide dismutase, peroxidase, and catalase (Mittler, 2002; Zandi and Schnug, 2022). Several *Pseudomonas* strains have been shown to modulate the plant antioxidant activity under various stress conditions, leading to decreased oxidative stress. For instance, under high salinity conditions, *P. frederiksbergensis* OS261 was able to improve catalase activity and reduce superoxide dismutase activity in red pepper, while reducing ROS concentration overall (Chatterjee et al., 2017). Another strain, *P. putida* GAP-P45, decreased antioxidant enzyme activity and ROS levels in *A. thaliana* under osmotic stress, and increased proline turnover (Ghosh et al., 2017, 2018). The mechanisms mediating this modulation of the plant antioxidant machinery remain unknown (Rajkumar et al., 2017). However, another *Pseudomonas* strain, *P. guariconensis* MTCC5279 (formerly called *P. putida* MTCC5279), was shown to affect the regulation of antioxidant enzyme genes in chickpea during and following osmotic stress (Tiwari et al., 2016). This

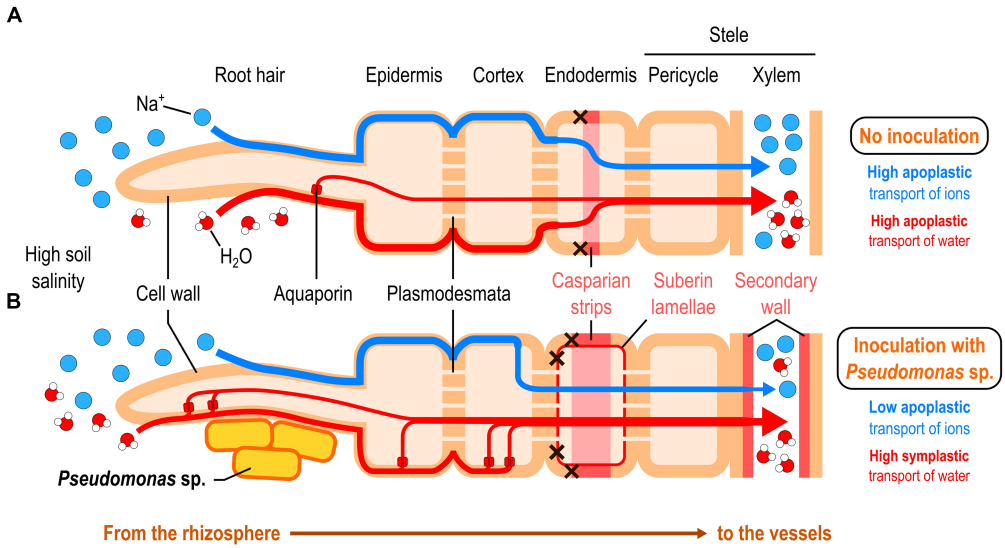

FIGURE 4
Pseudomonas-mediated reinforcement of the root apoplastic barriers under high soil salinity conditions. **(A)** Non-inoculated plants. **(B)** Plants inoculated with a *Pseudomonas* strain.

suggests an indirect effect of the *Pseudomonas* strain, potentially through phytohormone signaling pathways.

Plant-beneficial *Pseudomonas* must also produce their own antioxidant enzymes, such as peroxidases and superoxide dismutases, to colonize and survive in the plant vicinity (Kim and Park, 2014; Guan et al., 2017; Nikel et al., 2021). Plants generate ROS to regulate several basic processes including growth, development, and immunity (Liszkay et al., 2004; Baxter et al., 2014). Consequently, bacteria living close to plants, for example in the rhizosphere or the phyllosphere, are exposed to these reactive compounds, even more under climate-related stresses, which drive ROS accumulation in plants. The well-studied plant-beneficial strain *P. putida* KT2440 has recently been shown to display greatly improved root colonization abilities in alfalfa when overexpressing a specific peroxidase (Santamaría-Hernando et al., 2022). Its colonization levels were about 30 times higher in the roots and 900 higher when considering the root tip only, compared to the parent strain. Interestingly, this strain was also shown to improve tolerance to high salinity in soybean and maize, and to drought in tomato (Costa-Gutierrez et al., 2020a,b; Saglam et al., 2022). Strains with such enhanced antioxidant activity may then be more effective to improve plant tolerance to climate-related stresses. This remains to be assessed but antioxidant activity could be a relevant criterion when screening for plant-beneficial *Pseudomonas* strains.

7. Exopolysaccharide production by *Pseudomonas* spp. improves rhizosphere soil structure and water content under climate-related stresses

Many plant-beneficial *Pseudomonas* spp. form biofilms, which are aggregates of bacteria attached to a surface and/or to each other, embedded in a matrix of highly hydrated polymeric compounds that they secrete (Flemming et al., 2016; Zboralski and Filion, 2020). These compounds include proteins, lipids, extracellular DNA, and exopolysaccharides (Flemming et al., 2016). Biofilms improve the tolerance of their producing bacteria to various stresses, such as osmotic and oxidative stress, and enable them to reach cell densities needed to produce plant-beneficial secondary metabolites (Danhorn and Fuqua, 2007; Flemming et al., 2016; Svenningsen et al., 2018). The role played by biofilm formation, and especially by exopolysaccharides, in the alleviation of climate-related stresses, has been investigated in several *Pseudomonas* strains. In this regard, strain *P. putida* GAP-P45 has notably been studied (Sandhya et al., 2009, 2010a,b; Sandhya and Ali, 2015). This strain produces relatively high amounts of exopolysaccharides under drought and high salinity conditions (Sandhya et al., 2010b; Sandhya and Ali, 2015). It was shown to alleviate osmotic stress in sunflower and maize and to increase the amount of exopolysaccharides in the rhizosphere, along with improving soil aggregate stability and leaf relative water content (Sandhya et al., 2009, 2010a). Another team investigated the effects of high salinity in soybean and maize in three mutants of *P. putida* KT2440. These mutants were impaired in *lapA*, *lapF*, or both genes, which are necessary for cell-surface and cell–cell attachment, respectively (Costa-Gutierrez et al., 2020b). They were not able to produce mature biofilms but were shown to overproduce exopolysaccharides and to display exopolysaccharide profiles distinct from the wild type, potentially to compensate for biofilm defects (Martínez-Gil et al., 2013). Inoculation with the double *lapA-lapF* mutant better increased the fresh and dry weight of maize plants than inoculation with the wild type, which already improved plant growth. Exopolysaccharides may then help the bacteria cope with osmotic and ionic stress in soil, but may also directly help the plant by improving water retention in the rhizosphere and by binding to cations, which limit their penetration into the plant (Costa-Gutierrez et al., 2020b). Nonetheless, characterization of the exopolysaccharides produced by rhizosphere *Pseudomonas* strains is lacking (Zboralski and Filion,

2020). This information could contribute to a better understanding their role in the alleviation of abiotic stresses in plants.

8. Conclusion and research perspectives

Plants are facing increasing pressure from climate change globally, notably through drought, high salinity, heat, and flooding. The effects of these stressful conditions on plants are diverse and can combine and amplify each other. However, many bacterial strains belonging to the genus *Pseudomonas* have been shown to improve plant growth under such stresses, by producing plant hormones, emitting volatile compounds, reinforcing the root apoplast barriers, mitigating oxidative stress, and excreting exopolysaccharides (Figure 5). These mechanisms can decrease the effects of climate-related stresses on plants and directly promote growth at the same time, improving yields overall under such abiotic stresses. The great diversity and versatility of plant-beneficial *Pseudomonas* strains could thus offer many opportunities to successfully develop *Pseudomonas*-based bioinoculants able to counter the effects of climate-related stresses. However, many gaps in knowledge must be addressed to achieve this goal. Some perspectives and recommendations are presented hereinafter to overcome these obstacles.

8.1. Sequence the genome of promising strains

Many *Pseudomonas* strains of interest for abiotic stress alleviation, often studied for many years, do not have their entire genome sequenced. Such information can be instrumental in understanding the molecular mechanisms of stress alleviation, while offering a reliable identification at the species level, much more reliable than 16S rRNA sequencing (Mulet et al., 2010; Garrido-Sanz et al., 2016; Zboralski et al., 2023). It especially enables transcriptional studies and site-directed mutagenesis, allowing to efficiently suppress specific genes or clusters from a genome.

8.2. Generate single-gene mutants to elucidate stress-alleviating mechanisms

Many studies characterize their strains of interest in terms of production of phytohormones, biofilm, siderophores, antibiotics, or phosphate solubilization capacity, and then try to correlate these traits with the stress-alleviating effects observed in plants when inoculating these strains. While this approach is interesting to describe new promising strains and to suggest the mechanisms potentially at play in stress alleviation, it does not provide conclusive evidence on the exact mechanisms involved. Yet, this understanding is central to identify and characterize bacterial functions of interest to help crops face climate-related stresses. Generating mutants for each of the traits potentially involved in stress alleviation would certainly help.

8.3. Screen for stress-tolerant strains alone

To improve plant growth under climate-related stresses, *Pseudomonas* strains must be able to cope with osmotic, ionic, heat, or flooding stress before helping the plant alleviate them. For instance, Yasmin et al. screened 44 bacterial strains for halotolerance and found a single *Pseudomonas* strain, *P. pseudoalcaligenes* SRM-16, that was able to survive under 20% NaCl conditions (Yasmin et al., 2020). This strain was subsequently shown to improve soybean tolerance to high salinity conditions. It is therefore important to assess the ability of a strain of interest to grow under stress conditions before testing it *in planta*.

8.4. Assess promising strains *in vivo* under different types of stresses

Many *Pseudomonas* strains have been shown to be effective in alleviating a specific climate-related stress in plants. In this review, we highlighted that some of these stresses, such as drought and high salinity, can affect plants in similar ways. *Pseudomonas* strains effective against a given stress may then be effective against others as well. We suggest assessing the effects of promising strains on multiple stresses rather than solely focusing on one. This could also offer

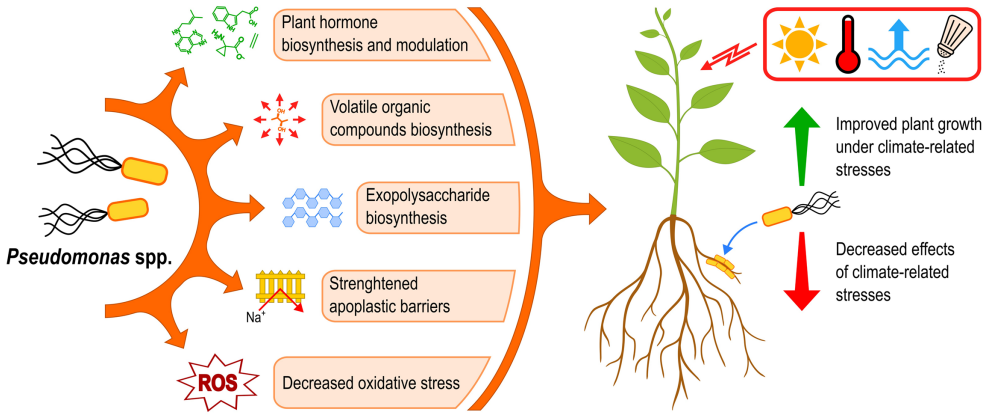

FIGURE 5
Plant-beneficial *Pseudomonas* strains use a variety of mechanisms to improve the plant tolerance to climate-related stresses. ROS, reactive oxygen species.

fruitful collaboration opportunities between research groups working on different stresses.

8.5. Investigate how beneficial bacteria can help plants recover from stress

Research on plant stress alleviation by *Pseudomonas* strains often focuses on direct stress-alleviating effects. However, in the field, stress conditions are likely to last only for a limited period during the growing cycle, leaving time for the plants to recover. It would then be interesting to investigate the effect of beneficial bacteria on this recovery phase as well (Jagadish et al., 2021).

Author contributions

AZ: conceptualization, investigation, writing—original draft, and writing—review and editing. MF: 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.

Publisher's note

Supplementary material

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Stenotrophomonas in diversified cropping systems: friend or foe?

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In the current scenario, the use of synthetic fertilizers is at its peak, which is an expensive affair, possesses harmful effects to the environment, negatively affecting soil fertility and beneficial soil microfauna as well as human health. Because of this, the demand for natural, chemical-free, and organic foods is increasing day by day. Therefore, in the present circumstances use of biofertilizers for plant growth-promotion and microbe-based biopesticides against biotic stresses are alternative options to reduce the risk of both synthetic fertilizers and pesticides. The plant growth promoting rhizobacteria (PGPR) and microbial biocontrol agents are ecologically safe and effective. Owning their beneficial properties on plant systems without harming the ecosystem, they are catching the widespread interest of researchers, agriculturists, and industrialists. In this context, the genus *Stenotrophomonas* is an emerging potential source of both biofertilizer and biopesticide. This genus is particularly known for producing osmoprotective substances which play a key role in cellular functions, i.e., DNA replication, DNA-protein interactions, and cellular metabolism to regulate the osmotic balance, and also acts as effective stabilizers of enzymes. Moreover, few species of this genus are disease causing agents in humans that is why; it has become an emerging field of research in the present scenario. In the past, many studies were conducted on exploring the different applications of *Stenotrophomonas* in various fields, however, further researches are required to explore the various functions of *Stenotrophomonas* in plant growth promotion and management of pests and diseases under diverse growth conditions and to demonstrate its interaction with plant and soil systems. The present review discusses various plant growth and biocontrol attributes of the genus *Stenotrophomonas* in various food crops along with knowledge gaps. Additionally, the potential risks and challenges associated with the use of *Stenotrophomonas* in agriculture systems have also been discussed along with a call for further research in this area.

KEYWORDS

crop protection, ecofriendly, ecosystem, organic, osmoprotective, PGPR

Introduction

In recent years, the harmful effects of pesticides and synthetic fertilizers on humans, animals as well as on the whole ecosystem have led to the expansion of novel beneficial microbes. There are likely many undiscovered microorganisms in unexplored plants and soils that may play a crucial role in promoting plant growth through their various activities. The bacterial genus *Stenotrophomonas* is referred to as a potential PGPR with advantageous effects because of its capacity to produce siderophores, the ability to solubilize phosphate, and the generation of phytohormones and spermidine (Ulrich et al., 2021). This genus belongs to the family Xanthomonadaceae as an emended description of the Lysobacteraceae family (Cutiño-Jiménez et al., 2020). The Lysobacteraceae (Xanthomonadaceae) family covers a diverse group of bacteria, which includes *Pseudoxanthomonas, Stenotrophomonas*, *Xanthomonas*, and *Xylella*, these are closely related bacterial genera that form a phylogroup referred to as XSXP (Bansal et al., 2021). The Xanthomonadaceae family also contains plant pathogenic bacteria namely *Xanthomonas* and *Xylella,* which are reported to cause economic losses in several crops (Parte, 2018). On the other hand, this family also included the PGPR including *Pseudomonas geniculata* and *S. rhizophilia* with medical, environmental, and biotechnological significance (Cutiño-Jiménez et al., 2020). The *Stenotrophomonas maltophilia* was earlier described as *Pseudomonas maltophilia* in the year 1961 (An and Berg, 2018; Wang et al., 2018a,b).

Stenotrophomonas species are Gram-negative and associated with wide a range of habitats, including animals as well as plant hosts (Hayward et al., 2010). Additionally, this bacterium is cosmopolitan and ubiquitous that found in an environmental habitat range, including extreme ones, although naturally it is associated with plant's rhizosphere and mainly contributed to the elemental cycling of sulphur and nitrogen, and also degrades complex compounds and pollutants, and promotes the growth of plants and their health (An and Berg, 2018; Pérez-Martínez et al., 2020). Moreover, the bacterium *S. maltophilia* is the first member of this genus which is a predominant species observed in plants, water, soil, animals, and humans (Wang et al., 2018b). *Stenotrophomonas maltophilia* has a sequenced genome with a genome size of approximately 4.8 Mbp with a G + C content of 66.7% (Crossman et al., 2008). The whole genome sequence analysis of *S. indicatrix* BOVIS40 yielded a 4.42 Mb genome size with ~66.4% G + C content (Adeleke et al., 2021). Bansal et al. (2021) suggested that, in light of deep phylotaxonomy genomics findings along with published polyphasic data, XSXP phylogroup warrants reunification and need to consider *Xylella, Stenotrophomonas,* and *Pseudoxanthomonas* as synonyms of *Xanthomonas*.

Many researchers reported the benefits of the genus *Stenotrophomonas* for plant systems (Figure 1). The genus *Stenotrophomonas* colonizes extreme manmade niches in space shuttles, hospitals, and clean rooms (An and Berg, 2018). *Stenotrophomonas* bacteria are becoming more researchable because of their potential use as effective bioinoculants for promoting plant growth and managing several diseases of food crops. This aspect is of growing biotechnological interest (Ulrich et al., 2021). Nowadays, this genus has become a research opportunity as a multidrug-resistant human pathogen, which does not commonly infect healthy humans but it may be associated with high morbidity and mortality in severely immunocompromised and debilitated patients by causing several infectious diseases (Flores-Treviño et al., 2019). These bacteria can also be recovered from polymicrobial infections, most especially from the respiratory tract (lungs) of cystic fibrosis patients (Brooke, 2014). Additionally, it has biodefense capacity against plant pathogenic fungi and bacteria as well as resistance to biotic and abiotic stress, anti-quorum sensing, and anti-biofilm bioactivities (Ulrich et al., 2021). The closely related *S. rhizophilia* provides a substitute for biotechnological applications without any harm to human health (An and Berg, 2018).

The role of *Stenotrophomonas* in human infectious diseases has been discussed previously (Berg and Martinez, 2015; Anđelković et al., 2019; Gil-Gil et al., 2020; Dadashi et al., 2023), but to date, there have been no reviews on its significance of plant disease management and plant growth promotion. In this review, the growth and biocontrol traits of the genus *Stenotrophomonas* in several food crops, as well as the probable risks and challenges associated with the use of *Stenotrophomonas* in agriculture systems and potential future research, have been discussed.

Biocontrol potential of the genus *Stenotrophomonas*

Efficiency against plant pathogens

In recent years, the use of antagonistic microorganisms to manage plant diseases has been regarded as a viable alternative to synthetic fungicides. The effects of beneficial microorganisms on growth improvement and higher disease tolerance have been studied in many cropping systems. Microbial biocontrol agents have been shown to have a high possibility of enhancing the yield of food crops by increasing phosphate solubilization, fixing nitrogen, or restricting diseases (Parnell et al., 2016). The genus *Stenotrophomonas* is used as a potential microbial biocontrol agent against many plant pathogens hampering commercial crops (Mahaffee and Kloepper, 1997; Germida and Siciliano, 2001; Mehnaz et al., 2001; Berg et al., 2002). In addition to their other plant growth-promoting characteristics, *Stenotrophomonas* spp. exhibited antagonistic patterns against pathogenic oomycetes, fungi, bacteria, and insect pests. There are several *Stenotrophomonas* spp. that may interact well with plants, especially *S. maltophilia* and *S. rhizophilia* (Ryan et al., 2009). However, still, no report is available on the plant pathogenic nature of *Stenotrophomonas* and its related species.

According to de Oliveira-Garcia et al. (2003), *S. maltophilia* produces various kinds of pili that are involved in the development of complex biofilms as well as adherence to surfaces. The potential of *S. maltophilia* to interact with other microbes on the plant's surface may be influenced by both adhesion and biofilm formation (Elvers et al., 2001). *S. maltophilia* strains has *S. maltophilia* fimbriae 1 (SMF1) fimbriae, which is made up of fimbrin subunits that are significantly similar to numerous pathogenic *Escherichia coli* and the *Pseudomonas aeruginosa* fimbria in terms of amino-terminal of amino acid sequence. The genome of *S. maltophilia* strains includes genes that encode type I and type IV pili, type I pili involved in adhesion and the early phases of biofilm formation. Type IV pili are also involved in adherence, auto aggregation, twitching motility, and biofilm formation. This might imply that invasion strategies of *Stenotrophomonas* spp. in plants form biofilms and exhibit twitching

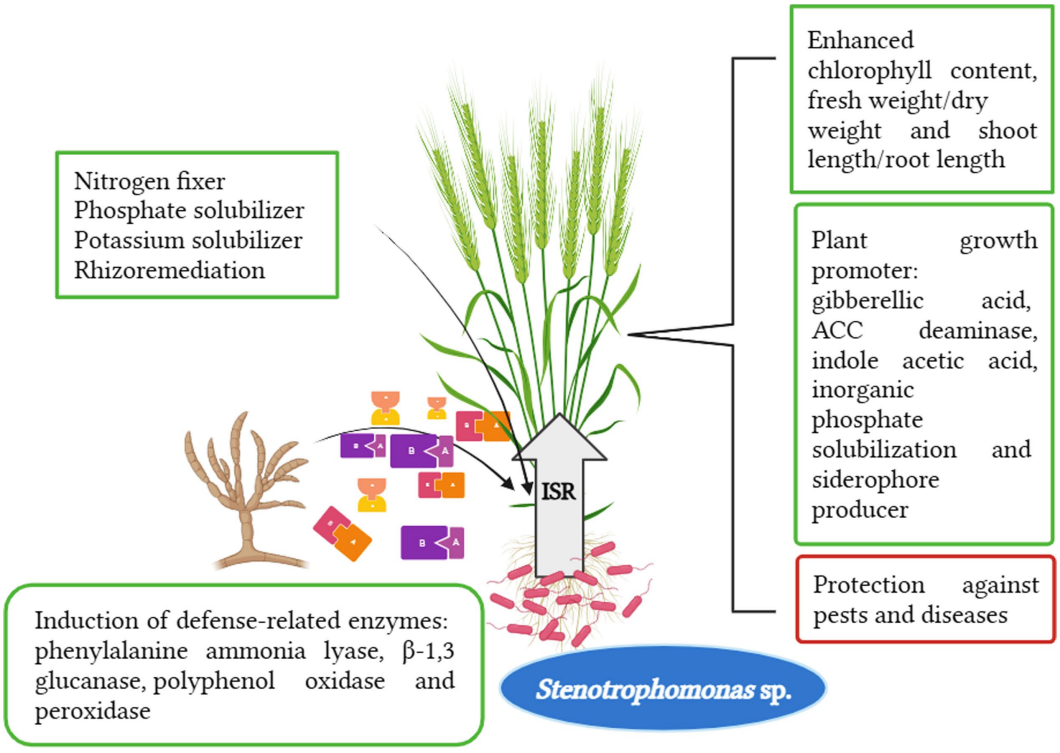

FIGURE 1
Multi-fold action of *Stenotrophomonas* spp. in plant system.

motility. *S. maltophilia* has the mannose-6-phosphate *(Man)* gene and lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes (*rmlA*, *rmlC*, and *xanB*), which encode enzymes involved in the synthesis of lipopolysaccharides (LPS) and exopolysaccharides (Huang et al., 2006).

The biocontrol ability of *Stenotrophomonas* spp. has been proved in numerous crops. Through several mechanisms, these bacteria suppress the growth of phytopathogens. The novel antifungal substances maltophilin (Jakobi et al., 1996) and xanthobaccin (Nakayama et al., 1999) have been identified, and the majority of *S. maltophilia* isolates exhibit antifungal activity against various pathogens *in vitro* (Minkwitz and Berg, 2001). The strains of *S. maltophilia* generate a wide range of proteases, chitinases, glucanases, RNases, DNases, lipases, and laccases, and they have an incredibly high hydrolytic potential (Tandavanitj et al., 1989; Galai et al., 2009). The biocontrol capability of *S. maltophilia* includes both chitinolytic and proteolytic activities. Through the breakage of fungal cell walls, chitinases may protect plants against phytopathogens, but they may also play a part in activating induced defense systems (Mastretta et al., 2006). It is necessary to determine the precise roles played by the several exoenzymes involved in the antagonistic activity of *Stenotrophomonas* spp., thus making it possible that additional bacterial products may similarly control plant disease by inducing plant defenses.

The competition for iron is another essential factor in preventing pathogens. Siderophores produced by various pathogens such as ferrichrome, which is produced by several fungi, including the phytopathogenic *Ustilago maydis* can be efficiently captured by *Stenotrophomonas* (Ardon et al., 1997). The sequenced *Stenotrophomonas* spp. genomes feature large amounts of volatile organic compounds (VOCs), outer membrane proteins, and TonB-dependent receptors (TBDRs). Moreover, *Stenotrophomonas* spp. may release VOCs that act as inter- and intracellular communication signals and adversely influence the development of several pathogens (Wheatley, 2002). Several distinct VOCs produced by *S. maltophilia* and *S. rhizophila* inhibited the development of *Rhizoctonia solani*, which causes severe damage to economically significant crops and trees worldwide. Phenylethanol and dodecanal are the VOCs that have been identified in this study (Kai et al., 2007). However, the specific mechanism by which these secondary metabolites affect the pathogen is unknown and needs further research.

Further, *Stenotrophomonas* spp. release many hydrolytic enzymes that are important in the inhibition of plant diseases, including lipases, chitinases, RNAses, DNAses, and proteases (Berg et al., 2010). *S. maltophilia* MB9, a marine-isolated strain, was successful in preventing large numbers of phytopathogens including *R. solani, F. oxysporum,* and *Curvularia* sp. by producing dodecanoic acid, a broad-spectrum antifungal antibiotic (John and Thangavel, 2017). In a dual culture laboratory study, *Stenotrophomonas* spp. displayed significant zones of inhibition against *R. solani, Verticillium dahlia,* and *Sclerotinia sclerotiorum* (Wolf et al., 2002). *S. maltophilia* strains obtained from the rhizosphere of the brinjal crop in Egypt demonstrated inhibitory effects against *Ralstonia solanacearum*, a potato brown rot pathogen. The isolates were useful for decreasing symptoms in potatoes grown in soil in addition to being beneficial *in vitro* (Messiha et al., 2007).

According to Schmidt et al. (2012), a *S. rhizophila* strain (DSM14405T) promoted plant development by changing the rhizosphere microbiome. It was observed that the strain was successful

in colonizing both the root and the shoots of cotton and sweet pepper. Based on molecular profiling using single-strand conformation polymorphism (SSCP), the rhizosphere fungal microbiome appears to be affected by *S. rhizophila* DSM14405T. In another study, *S. maltophilia* C3-derived chitinase inhibited conidial development and germ tube extension in *Bipolaris sorokiniana* (Zhang et al., 2001). Amino acid sequencing, polyacrylamide gel electrophoresis, and purification indicated that the isolate produced at least two distinct chitinases with potential antifungal activity (Zhang et al., 2001). Kobayashi et al. (2002) isolated a protein and the responsible gene from *S. maltophilia* 34S1 having chitinolytic and antifungal activities. The release of elicitor molecules by hydrolytic enzymes, in particular, contributes to the activation of plant defense systems in addition to the degradation of pathogenic cell structures. In the other study treatment of wheat plants with *S. maltophilia* SPB-9 during the *in vivo* experiment led to a rise in defense enzymes (Singh and Jha, 2017).

There have also been reports of *Stenotrophomonas* species developing increased disease resistance to plant pathogenic viruses. *S. maltophilia* HW2, which was isolated from cucumber, improved the plant's resistance to the cucumber green mottled mosaic virus. HW2 application on cucumber may slow down virus replication and prevent the expression of viral protein genes. It also induced the expression of antioxidant enzyme genes and defense-related genes (Li et al., 2016). The most recent research on the biocontrol effectiveness of *Stenotrophomonas* species used against different plant diseases is summarized in Table 1. However, further research is needed to fully understand the mechanisms of action and to optimize (toxicity, formulation, consortium development) the use of this bacterium in agriculture.

Entomopathogenic effect against insect pests

Plant defense against attack by insect pests is associated with a number of phytohormones. Jasmonic acid (JA) is the main phytohormone that supports plant defense against insect pests (War et al., 2012). According to research by Pangesti et al. (2015), the JA signaling pathway is critical for *Arabidopsis thaliana* rhizobacteria-triggered ISR against *Mamestra brassicae* (cabbage moth). It is well known that plants need proteinase inhibitors (PIs) in order to protect themselves against insect pests. Studies revealed that PGPR-mediated ISR is associated with elevated expression of plant responsive genes that encode for PIs and elevated activity of defense-related enzymes (Harun-Or-Rashid and Chung, 2017). In plants that have received the PGPR treatment, defense responses may be generated more rapidly in the case of a pest attack. Inoculating tobacco plants with *S. rhizophilia* may induce JA accumulation and increase the transcript level of JA sensitive genes, resulting in the induction of systemic resistance in tobacco plants against *Spodoptera litura* (tobacco cutworm) (Ling et al., 2022). The biocontrol ability of *Stenotrophomonas* spp. against insect pests is less recorded and further research on integrated management of serious insect pests is required. However, the summary of recent research on *Stenotrophomonas* spp. biocontrol efficiency applied against insect and nematode pests is provided in Table 2. Further research on *Stenotrophomonas* spp. against insects is needed to fully understand its capabilities and limitations. However, the promising results of recent studies suggest that *Stenotrophomonas* may be a useful lever in integrated pest management for a variety of crops (Table 3).

Plant growth promoting activity of *Stenotrophomonas* spp.

Biofertilizers are a reliable source to promote plant growth without harming the soil, plants and environment (Reddy et al., 2020). The plant growth promoting rhizobacteria could be a viable substitute to overcome the load of synthetic fertilizers which are used indiscriminately in the agricultural sector as well as to enhance farmers income because they are cost-effective and durable (Pérez-Martínez et al., 2020). In this context, the genus *Stenotrophomonas is* characterized as promising plant growth promoting bacterium, which are reported as inducers and protectors against biotic and abiotic stresses (Table 3).

In addition to that, the three isolates of the genus *Stenotrophomonas* isolated from healthy tomato plants were able to produce indole-3-acetic acid; two of these strains had phosphate solubilization ability (Ben Abdallah et al., 2018). *S. maltophilia* P9 was isolated from the algal biomass and identified as a potential pectinase producing with biotechnological significance (Sharma and Sharma, 2018). In a study, Woźniak et al. (2019) reported that *Stenotrophomonas* strain ES2 promotes growth under *in vitro* conditions. Seed treated wheat plants with rhizospheric *S. maltophilia* SBP-9 originated under salt stress conditions, showed improved plant growth, such as increased shoot and root length, along with balanced chlorophyll content compared to controls (Singh and Jha, 2017). Similar results were reported with another strain of *S. maltophilia* BJ01 by Alexander et al. (2020), they found that peanut crop showed improved growth and enhanced photosynthetic pigments and growth hormones under salt stress conditions. In a recent study, Nigam et al. (2022) reported that *Stenotrophomonas* sp. were effective in increased growth, protein accumulation, osmotic adjustment, and Ascorbate peroxidase (PAX) activity in soybean and spinach cultivars under salt stress conditions.

In another study, Bashandy et al. (2020) isolated *S. maltophilia*-SR1 from oil free soils and applied it to soil contaminated by oily wastewater, reporting that the strain successfully used several aromatic hydrocarbons, including benzene, toluene, and xylene, as its sole carbon source and showed plant growth promoting (PGP) properties (indoleacetic acid (IAA), and phosphate solubilization). The mode of action of the arsenic-resistant *S. maltophilia* S255 isolated by Huda et al. (2022) appears to involve several mechanisms such as auxin and hydrogen cyanide production, phosphate solubilization, and nitrogen fixation. These mechanisms may enhance plant growth and improve nutrient uptake, which could be beneficial in agriculture. However, it is important to note that these findings were obtained from a glasshouse study, and further research is needed to confirm the efficacy of this bacterium in large multilocated field trials. It is possible that the efficacy of the arsenic-resistant *S. maltophilia* S255 could vary depending on the origin of the isolates and that native isolates should be considered for recommendation. Therefore, future research should focus on investigating the efficacy of this bacterium in different regions and under various environmental conditions. It is also important to evaluate the safety and potential risks associated with the use of this bacterium as a growth promoter in agriculture.

Stenotrophomonas in nitrogen fixation

Nitrogen-fixing microorganisms use a complex enzyme system called nitrogenase to convert atmospheric elemental nitrogen into

TABLE 1 Biocontrol efficiency of *Stenotrophomonas* spp. against plant pathogens.

Stenotrophomonas spp.	*Pathogen*	Hosts	Reference
S. rhizophila	*Colletotrichum gloeosporioides*	Mango	Reyes-Perez et al. (2019)
S. maltophilia E38	*Ralstonia solanacearum*	Tobacco	Li et al. (2023)
S. maltophilia CR71	*Botrytis cinerea*	Tomato	Rojas-Solís et al. (2018)
S. maltophilia B8	*Fusarium oxysporum* f.sp. *cepae*	Garlic	Dewi et al. (2023)
S. maltophilia UN1512	*Colletotrichum nymphaeae*	Strawberry	Alijani et al. (2020)
S. rhizophila	*Fusarium proliferatum*	Muskmelon	Rivas-Garcia et al. (2018)
S. maltophilia PPB3	*Sclerotium rolfsii*	Tomato	Sultana and Motaher Hossain (2022)
Stenotrophomonas sp. BHU-S7 (AgNPs)	*S. rolfsii*	chickpea	Mishra et al. (2017)
S. rhizophila	*Leptosphaeria maculans*, *Leptosphaeria biglobosa*	Rapeseed	Schmidt et al. (2021)
S. maltophilia UPMKH2	*Pyricularia oryzae*	Rice	Badri Fariman et al. (2022)
S. maltophilia	*Magnaporthe grisea*	Rice	Etesami and Alikhani (2016)
S. rhizophila	*Fusarium oxysporum*	Cucumber	Wang et al. (2022)
Stenotrophomonas sp. TRM2	*Bipolaris sorokiniana*	Wheat	Villa-Rodríguez et al. (2019)
S. rhizophila KM01, KM02	*Pythium ultimum*	Chilli	Lara-Capistran et al. (2020)
S. maltophilia	*Colletotrichum musae*	Banana	Damasceno et al. (2019)
S. rhizophila 88bfp	*Athelia rolfsii*	Turfgrass	Ünal et al. (2019)
S. chelatiphaga	*Pseudomonas tolaasii*, *Ewingella americana*	Button mushroom	Aslani et al. (2018)
S. maltophilia S23, S24, S26 and S28	*Fusarium oxysporum* f. sp. *lycopersici*	Tomato	Aydi-Ben-Abdallah et al. (2020)
Stenotrophomonas sp. S81-1	*Ganoderma boninense*	Oil palm	Fadly et al. (2021)
S. maltophilia JVB5	Various pathogens	Sunflower	Adeleke et al. (2022)
Stenotrophomonas sp. P7T6–4	*Rhizoctonia solani*	Tomato	Hadi et al. (2020)
S. rhizophila 88bfp	*Fusarium cerealis*	Turfgrass	Senocak et al. (2020)
S. maltophilia TD 1	*Fusarium solani*	Citrus	Ezrari et al. (2021)
Stenotrophomonas sp. AG3	*Macrophomina phaseolina*	soybean	Santos et al. (2021)
S. rhizophila	*Xylella fastidiosa*	Olive	Mourou et al. (2022)
S. maltophilia strain A1w2	*Xanthomonas oryzae* pv. *oryzae*	Rice	Rahma et al. (2022)
S. maltophilia Sg3	*Cucumber Mosaic Virus*	Tobacco	Khalimi et al. (2020)

TABLE 2 Entomopathogenic effect of *Stenotrophomonas* spp. against insect pests.

Stenotrophomonas spp.	Insect	*Scientific name*	Reference
S. tumulicola T5916-2-1b	Aphids	*Aphis punicae*, *Aphis illinoisensis*	Baazeem et al. (2022)
S. maltophilia	Termites	*Coptotermes heimi*, *Heterotermes indicola*	Jabeen et al. (2018)
S. maltophilia W 2–7	Root-Knot Nematode	*Meloidogyne* spp.	Vishnu and Nisha (2020)
S. rhizophila	Tobacco cutworm	*Spodoptera litura*	Ling et al. (2022)
S. maltophilia	Potato beetle	*Leptinotarsa decemlineata*	Aktas et al. (2022)

plant-usable forms (Masson-Boivin and Sachs, 2018). Nonsymbiotic nitrogen fixation occurs among various genera, including *Acetobacter, Arthrobacter*, *Azotobacter, Bacillus, Clostridium, Diazotrophicus, Pseudomonas,* and *Stenotrophomonas* (Fitton et al., 2019), while symbiotic nitrogen fixation occurs among the members of Rhizobiaceae family along with leguminous plants (Dinnage et al., 2019). Beneficial soil microorganisms, including PGPR, are responsible for fixing a large portion of the elemental nitrogen that enters the soil under natural conditions (Tang et al., 2020). Thus, biological nitrogen fixation via plant-microbe interactions is a major factor in the manufacturing of organic fertilizers (Singh et al., 2023).

Recent research has shown that the *S. maltophilia* strain UPMKH2 can increase rice yield and productivity by providing the plant with a steady supply of nitrogen (Badri Fariman et al., 2022). As a nitrogen source, these bacteria can produce plant growth regulators like IAA, and ACC deaminase (precursor of ethylene), which aid plants in

TABLE 3 The effect of *Stenotrophomonas* spp. on host plant.

Host/ Source	Strain	Homology to the reference strain	Accession no.	Colonization status	Action	Reference
Rhizosphere of tomato plants	Two oxalotrophic strains (OxA and OxB)	*S. maltophilia*	-	Endophytically	Strain OxA and OxB protected host from the damage caused by high doses of oxalic acid Protected host from *S. sclerotiorum* and *B. cinerea* infections. Moreover, Callose deposition induced by OxA and OxB was required for protection against phytopathogens Inoculation of theses bacteria induced the production of phenolic compounds and the expression of PR-1 Both isolates exerted a protective effect against fungal pathogens in *Arabidopsis* mutants affected in the synthesis pathway of salicylic acid (sid2-2) and jasmonate perception (coi1) Moreover, *B. cinerea* and *S. sclerotiorum* mycelial growth was reduced in culture media containing cell wall polysaccharides from leaves inoculated with each bacterial strain	Marina et al. (2019)
Equisetum arvense	ES2	*S. maltophilia*	KY486848	Endophytically	Greatest ability to synthesize indole-3-acetic acid (IAA)-like compounds, Highest metabolic activity based on the Biolog GEN III test.	Woźniak et al. (2019)
Zea mays	ZR5	*Stenotrophomonas* sp.	KY486808			
Arctium lappa	AR4	*S. maltophilia*	KY486847			
Pistacia atlantica L.	Sm25	*S. maltophilia* IAM12423^T	KU693275	Endophytically	Siderophore production Plant growth promotion	Etminani and Harighi (2018)
	Sm97	*S. maltophilia* IAM12423^T	KU693276			
Nodule-associated bacteria (NAB) were isolated from wild legume nodules	LXo13	*Stenotrophomonas* sp.	–	–	The most frequent PGP activity identified among the strains isolated from wild legumes was IAA synthesis Two bacteria, *Stenotrophomonas* sp.	Tapia-García et al. (2020)
	LX010a					
Rhizospheric isolates	NGB-15	*Stenotrophomonas* sp. strain KG-16-3	LC322228	Rhizospheric	Phosphate solubilization Plant growth promoting activity over the control (increased shoot and root fresh and dry biomass)	Youseif (2018)
	NGB-18	*S. maltophilia* strain E136	LC322229			
Corn Rhizosphere	L,o.INCA-FRr1	*S. rhizophila*	–	Rhizospheric	Ability to perform the FBN, Solubilization of phosphorus, potassium and antagonistic activity against *Fusarium oxysporum*	Pérez-Martínez et al. (2020)
	INCA-FRc24	*S. pavanii*	–			
Aerial parts of poplar	*Stenotrophomonas* strain 169	*Stenotrophomonas* strain Fa6 (AY131216)	CP061204	Endophytically	The root weight of the inoculated plants was three times higher than that of the untreated plants, Showing a significant increase in both root and shoot length of the inoculated *in vitro* plants Tolerance of plants to abiotic stresses Production of siderophores and the ability to mobilize phosphates, as well as the production of the plant growth-promoting substances IAA and polyamines	Ulrich et al. (2021)

(Continued)

TABLE 3 (Continued)

Host/ Source	Strain	Homology to the reference strain	Accession no.	Colonization status	Action	Reference
11 wild plants (rhizosphere and phyllosphere)	*Stenotrophomonas* sp. *TmP43c*	*S. rhizophila*	–	rhizosphere	Highest number of psychrotolerant strains, Higher phosphate solubilization activity	Vega-Celedón et al. (2021)
Sugarcane rhizosphere	COA2	*S. maltophilia*	MN527324	Rhizospheric	Phosphate solubilizing, Siderophore Production IAA production Ammonia Production ACC production Antagonistic activity Increased the activity of SOD, CAT, PAL, CHI, and GLU enzyme Nitrogen fixative	Singh et al. (2020)
Sunflower root endosphere	SAMN18138830	*S. indicatrix* BOVIS40	JAGENA000000000	Endospheric	Plant growth promoter IAA production Siderophore production Phosphate solubilizing Exopolysaccharide Amylase, Cellulase, Xylanase, Mannanase and Protease enzymes enhancer	Adeleke et al. (2021)
Strawberry leaves	UN1512	*S. maltophilia*	MT448956	Endophytically	Antagonistic effect against *C. nymphaeae* in dual culture Secreted protease, chitinase, pectinase, siderophore, IAA, and gibberellin Produced volatile compounds (Benzothiazole, Cyclooctatetraene-1-carboxaldehyde, Carbonic acid, octadecyl phenyl ester, Benzaldehyde, 2,5-bis, Estragole, Benzaldehyde) Growth promoter	Alijani et al. (2020)
Oil-free soil	SR1	*S. maltophilia*-SR1	MH634684	Rhizospheric	Highest plant growth promoter Upregulated nitrate, nitrate reductase, total nitrogen, and nonenzymatic and enzymatic antioxidants in plants Suppressed oxidative and nitrosative stress Produced indoleacetic acid and ammonia as well as phosphate solubilization	Bashandy et al. (2020)
Tomatillo (*Physalis ixocarpa*) roots	CR71	*S. maltophilia*	MF992168	Endophytically	Promoted the shoot and root length, chlorophyll content, and total fresh weight of tomato Biocontrol of *B. cinerea* through the production of potent volatiles such as dimethyl disulphide	Rojas-Solís et al. (2018)
Salt-resistant *Carex distans* (distant sedge) roots	SRS1	*S. rhizophila* DSM14405	–	Endophytically	Increased the induction of plant genes related to abscisic acid and auxin signaling Enhanced plant growth	Manh Tuong et al. (2022)
Mucuna utilis var. *capitata* L. (Safed Kaunch)	RMC6	*S. maltophilia*	HM480495	Endophytically	Antagonistic against *Fusarium udum* Plant growth promoter (IAA, phosphate solubilization and ACC deaminase, siderophore production)	Aeron et al. (2020)
Soil Contaminated by Industrial Effluent	S25	*S. maltophilia*	KY651248	rhizosphere	As-reducing capability Produced Hydrogen Cyanide, Nitrogen Fixation, and Auxin Plant growth promoter	Huda et al. (2022)

absorbing nutrients and expanding their tissues (Sarkar et al., 2018). In addition to maize, peanuts, rice, sugarcane, and wheat and have all been shown to benefit from *S. maltophilia's* nitrogen-fixing abilities (Wang et al., 2018a) as PGPR. Different PGP-traits and nitrogenase activities were confirmed with nitrogenase (*nifH*) gene amplification after strains of *S. maltophilia*-COA2 were selected and identified by sequencing their 16S rRNA gene.

To combat sugarcane diseases and cut down on nitrogen fertilizer use, researchers have looked into *S. maltophilia* -COA2 for the first time (Singh et al., 2020). Cerezer et al. (2014) evaluated the nitrogen-fixing capabilities of the atmosphere and symptomatic *Stenotrophomonas* spp. It was discovered that *Stenotrophomonas* isolates have the ability to fix atmospheric nitrogen in the soil, leading researchers to speculate that the reduction of *nifH* clusters of genes is a conservation of energy adaptation of *Stenotrophomonas* during its evolution from a free-living to an opportunistic pathogenic form. Inoculating foxtail millet with the nitrogen-fixing strain *S. rhizophila* EU-FEN-32 as part of a microbial consortium resulted in greater increases in growth and physiological parameters compared to both synthetic fertilizer and the untreated control (Kaur et al., 2023).

Endophytic association with *S. maltophilia* is beneficial to anti-fungal activity and plant growth (phytohormone induction, N2 fixation) (Rojas-Solís et al., 2018a). In addition, *S. pavanii*, a Gram-negative, non-motile, and spore-less species, fixes N_2 in sugarcane (Ramos et al., 2011). Recent research has shown that common nitrogen fixers, such as rhizobia, do not always colonize or infect the plant roots of leguminous plants but instead typically coexist with *Stenotrophomonas* in other plants. Synergistic processes for nodule formation and enhanced nitrogen fixation capabilities have been postulated when PGPR, like *Stenotrophomonas* species, interact with *Rhizobium* (Abd-Alla et al., 2019). The ability to form nodules in the roots of *Robinia pseudoacacia* has been attributed in part to the horizontal transfer of essential nodulation and nitrogen-fixation genes from rhizobia to other Gammaproteobacteria (*Stenotrophomonas*) and Betaproteobacteria (*Burkholderia*) (Abbott and Peleg, 2015). These nitrogen-fixing *Stenotrophomonas* species can be evaluated in other cropping systems for their potential role in nitrogen fixation and any other side effects on beneficial microbes found in the soil ecosystem.

Stenotrophomonas in phosphorous solubilization

One of the main macroelements that plants require for growth and development is phosphorous, but due to its poor solubility, most of the phosphorous in the soil is unreachable to plants. Phosphorous cannot be utilized by plants because it quickly precipitates in the soil as insoluble combinations with a variety of cations, including Mg, Ca, Al, and Fe. Because P ions are strong ligands, they frequently unite with metal ions to create complexes (Shen et al., 2011). Despite their numerous disadvantages, chemical phosphorous fertilizers are frequently recommended for agricultural soils with a phosphorous deficiency. Due to a confluence of environmental factors and the excessive use of synthetic fertilizers, soil fertility is declining (Ali et al., 2021).

Phosphorus-solubilizing microorganisms are being investigated as an alternative way to address these issues and meet the phosphorous requirements of crop plants. Although many phosphorous solubilizing microorganisms have been identified (Kishore et al., 2015), the majority of them are not well suited to the environmental factors that lead to the production of available phosphorous in the field. Similar to this, *Stenotrophomonas* is a potent phosphorus solubilizing bacterial genus that can release an adequate amount of phosphorus in solution from insoluble rock phosphates and calcium phosphate (CP) (Amri et al., 2023). Xiao et al. (2009) used the National Botanical Research Institute Phosphate (NBRIP) medium to isolate *S. maltophilia* YC from Chinese phosphate mines. The isolate efficiently produced 180.5 mg/L when Tricalcium phosphate (TCP) was the only source of soluble P. The medium's pH decreased from its initial value of 7.0 to its lowest value of 4.3 after 4 days of incubation. According to the high performance liquid chromatography (HPLC) findings, the isolates were gluconic acid producers during the P-solubilization procedure. The isolate was found to solubilize phosphorus most successfully when fed a diet of maltose and ammonium nitrogen, according to the researchers (Paul and Sinha, 2017).

Singh and Jha (2017) also discovered *S. maltophilia* with P-solubilizing potential in the rhizosphere soil of *Sorghum bicolor*. The organism only made a tiny amount of soluble P (10.73 2.34 mg/mL) using TCP. On the other hand, *S. maltophilia* MB9 was discovered to be effective at producing noticeable zones of solubilization on TCP-containing agar plates after being successfully isolated from a marine environment (John and Thangavel, 2017). *Stenotrophomonas maltophilia* AVP27, which was isolated from the chilli rhizosphere, produced significant zones of P solubilization (Kumar and Audipudi, 2015). TCP significantly increased the isolate's ability to produce soluble P, as determined by quantitative techniques. *Stenotrophomonas maltophilia* MTP 42 was found to produce 362 mg/mL of soluble P in the rhizosphere soil of *Coleus forskohlii* (Patel and Saraf, 2017). It has been found that *Stenotrophomonas* sp. RC5 was discovered in the rhizosphere of ray grass (*Lolium perenne*), where it synthesizes carboxyl and hydroxyl ions to chelate cations or lower the pH to release P. In the periplasm, the direct oxidation path produces the organic acids. P ions are released due to the substitution of H^+ for Ca^{2+} that occurs during the excretion of these organic acids, which acidifies the microbial cells and the surrounding environment (Barra et al., 2019). These *Stenotrophomonas* species can be used in various crops for phosphate solubilization and plant growth improvement, but further research is needed to determine any side effects.

Stenotrophomonas in potassium solubilization

Although nitrogen and phosphorus have been extensively studied in relation to the success of exotic species invasions (Mudau et al., 2007; Sinha and Tandon, 2020), the role of potassium in such invasions has received much less attention. However, K is the second most abundant nutrient in leaves, after nitrogen, and the most abundant cation in plant cells. Soil is home to a wide range of potassium-solubilizing bacteria (KSB), as has been demonstrated by numerous studies (Han and Lee, 2005; Kumar et al., 2015; Meena et al., 2015; Sun et al., 2020). *Bacillus, Pantoea, Paenibacillus, Pseudomonas, Rahnella,* and *Stenotrophomonas*, are some of the KSB genera that have been the subject of research in the past (Bahadur et al., 2019; Adeleke and Babalola, 2022). *Stenotrophomonas maltophilia* MB1, MB5, MB6, and

MB9 potassium solubilization and biocontrol activities like production of ACC deaminase, siderophore, and yield enhancing strains are isolated from marine environments, in which MB9 is the most potent and dominant strain after application of *S. maltophilia* MB1, MB5, MB6, and MB9 in crops (John and Thangavel, 2017).

In another study, *S. maltophilia* RSD6 has been successfully isolated from the rhizospheric soil of *Oryza sativa*, and can be used as an alternative to agrochemicals (Nevita et al., 2018). *Bacillus* spp., *Burkholderia* spp., *Pseudomonas* spp., and *Stenotrophomonas* spp., were all isolated from tea rhizosphere soil and proven to be effective KSB strains (Gopi et al., 2020). Only *Streptomyces alboviridis, S. rhizophila,* and *Nocardiopsis alba* out of a panel of *Actinobacteria* strains studied were able to dissolve potassium from mica. By Pérez-Martínez et al. (2020) 15 *Stenotrophomonas* strains were isolated from the maize rhizosphere, and two of these were able to soluble potassium sources, while another six showed antagonisms against the pathogen. Acidic and neutral pH usually led to more K release, whereas alkalinity conditions only made *Stenotrophomonas* sp. INCA-FRr1 release more K (Verma et al., 2016). These *Stenotrophomonas* species can be utilized in food crops to enhance crop growth after multilocation and large-scale field trials, however, more potential KSB strains of *Stenotrophomonas* that have both disease control and plant growth promotion activity should be identified in future research so that they could be used as multi-fold agents.

Stenotrophomonas in phytohormones production

Apical dominance, cell elongation, cell division, tissue differentiation, and intracellular communication are just some of the physiological processes that are influenced by phytohormones, also known as plant growth regulators, which are substances synthesized by plants and act as signaling molecules (Tshikhudo et al., 2023). There are five broad categories based on their structural make-up and how they interact with plants' physiological processes. Auxins, gibberellins, cytokinins, ethylene, and abscisic acid make up the big five. In order to combat the harmful effects of environmental stress, plants will often keep their levels of endogenous hormones constant (Kumar et al., 2012). Phytohormones are produced by a diverse group of bacteria found in plants and soil. Plants' responses to hormones are crucial to their development and growth. Phytohormones play a significant role in mitigating both biotic and abiotic stress. Plant growth is controlled by a variety of hormones, including gibberellins, auxins, and cytokinins, and these hormones have been linked to developmental processes in plants (Wani et al., 2016). Here we describe how *Stenotrophomonas* affects plant growth and development. Gibberellic acid (GA), ethylene, and indole acetic acid (IAA) were the plant growth regulator traits found in *S. maltophilia* (Singh and Jha, 2017). Deconjugation of gibberellin-glucosyl conjugates secreted from the roots stimulates plant growth (Jędrzejuk et al., 2023). Roots contain inactive 3-deoxy gibberellins, which are converted by bacteria and fungi into their active forms, GA1, GA3, and GA4 (Salazar-Cerezo et al., 2018).

Many aspects of plant development, such as differentiation and cell division, organogenesis, tropic responses, and gene regulation, are controlled by plant growth regulators like IAA (Ryu and Patten, 2008). Many different rhizobacterial strains have been shown to significantly increase plant growth by producing IAA. Due to their consistent release of IAA at minimal concentrations, some strains are also considered to be particularly effective at accelerating plant growth (Tsavkelova et al., 2007). Plant-associated *Stenotrophomonas* species, like many others, were found to effectively produce IAA in the medium used for crop cultivation, with or without the addition of tryptophan. IAA production in 16 *Stenotrophomonas* isolates (both clinical and environmental) was studied by Suckstorff and Berg (2003) and every single isolate tested positive for IAA production.

A study conducted in Germany with *S. maltophilia* e-p19 had an IAA concentration of 5.2 mg/mL, while *S. maltophilia* e-a23 had a concentration of 0.7 mg/mL. Isolates found to be part of environmental clusters were also found to produce more IAA than clinical cluster isolates (Hassan and Bano, 2016). In another study, IAA was found to be produced by *S. maltophilia* BE-25, which was isolated from the root of a banana plant. With or without tryptophan, IAA production from the isolate was sufficient, according to a thin layer and high-performance liquid chromatography analysis (Ambawade and Pathade, 2013). *Stenotrophomonas maltophilia*, isolated from the forest soil, also produced IAA in higher amounts (50.4 ± 0.9 g/mL) (Amri et al., 2023). The isolate was also capable of producing gibberellic acid, another plant growth regulator. *Stenotrophomonas maltophilia,* obtained from the rhizosphere of *Cenchrus ciliaris*, was also studied for its ability to promote plant development in the presence of tryptophan under salt stress conditions (Hassan and Bano, 2016). Their findings suggest that *S. maltophilia* IAA production is crucial to the induction of salt tolerance. However, their actual efficacy on a large field scale requires further attention for their commercialization.

Stenotrophomonas in siderophore production

Due to its importance in respiration, DNA synthesis, heme formation, and other biochemical reactions, iron deficiency can stunt the development of plants (Shetty and Corson, 2020). Iron is present in high concentrations in the Earth's crust, but its bioavailability is limited due to the insoluble nature of the Fe^{3+} ion (Behnke and LaRoche, 2020). Common minerals' hydroxide and oxide phases tend to accumulate iron, rendering it unavailable to plants and other living things. *Stenotrophomonas* is one of the PGPR that can make a siderophore, which is utilized to remove iron from insoluble mineral phases. According to their chemical makeup and coordination site, these low molecular weight (500–1,000 Da) ferric ion chelating compounds can be grouped into three groups: the catecholate, the carboxylic type, and the hydroxamate type (Pan et al., 2022). Although many pathogenic organisms view the production of siderophores as a virulence factor, plant-associated organisms view it as a growth-promoting trait because it facilitates iron uptake and prevents the spread of plant pathogenic microorganisms.

In addition to PGPR strains, it has been reported that several plant-associated *Stenotrophomonas* strains also produce siderophores. Like many other strains of *S. maltophilia,* SPB-9 was found to produce an orange color zone on chrome azurol S (CAS) agar plates (Singh and Jha, 2017). Hydroxamate-type siderophores are produced by *S. maltophilia* MTP-42, which has been found in the rhizosphere of *Coleus forskohlii* (Patel and Saraf, 2017). Ghavami et al. (2017) studied the siderophore production by *S. chelatiphaga* LPM-5 T and reported

that in the CAS liquid medium, the isolate was found to produce 12.66% siderophore. In a spectrophotometric test performed by Wilson et al. (2016), positive results indicated that the isolate generated a carboxylic siderophore. Siderophores are essential for preventing the invasion of pathogenic fungi and also help plants absorb more iron. By limiting the amount of ferric ions available, the growth of pathogenic fungal strains is stifled (Kloepper et al., 1980). Several strains of *Stenotrophomonas* were found to have the ability to produce siderophores, which were then shown to inhibit the growth of pathogenic fungi. *Stenotrophomonas* can scavenge molecules of the siderophore class that are produced by other microorganisms, such as the phytopathogenic fungus *Ustilago* (Sun et al., 2022). TonB-dependent outer membrane protein receptors (TBDRs) have been reported to be present in the genome of *S. maltophilia*, and their primary function is the active transport of the iron-siderophore complex. This superior iron uptake capacity suggested that they could pose a threat to other organisms as they evolved into endophytes or rhizosphere residents.

Exopolysaccharides (EPS) production by *Stenotrophomonas* spp.

Bacterial EPS supplies protection from different environmental stresses, such as predation, desiccation, and the effects of antibiotics (Limoli et al., 2015). EPSs have an important role in the aggregation of bacterial cells and supply carbon when the substrate is in low concentration (Banerjee et al., 2021). The biosynthesis of EPS by bacterial cells depends upon environmental and nutritional conditions (Nouha et al., 2018). Different microorganisms utilize various sources of carbon and nitrogen and differ in their mineral requirements, pH, and temperature, which are important factors for maximum EPS production (Aeron et al., 2020). *Brevibacillus parabrevis* (V4) and *S. maltophilia* (c6) were the two nodule endophytic isolates with the highest EPS production capability (among C1-C13 and V1-V7) (Abd-Alla et al., 2018). This research suggests that *Brevibacillus parabrevis* (V4) and *S. maltophilia* (c6) can generate a high yield of EPS when fed a diet rich in sucrose. *Stenotrophomonas maltophilia* (c6) does not use date molasses, lactose, galactose, or glucose as carbon sources for EPS production. Potassium nitrate stimulated EPS production in *S. maltophilia* (c6) and glycine did the same for *B. parabrevis* (V4) (Castellane et al., 2015). It is worth noting that *S. maltophilia* (c6) EPS yield and growth are suppressed by Fe_3O_4 (25–200 g/mL) and Fe_2O_3 (20–100 g/mL) NPs at varying concentrations. *Stenotrophomonas maltophilia* strain WR-C isolated from a clogged septic tank system that consistently formed biofilms on sand grains produced EPS, and caused clogging in the sand column (Abd-Alla et al., 2018).

Stenotrophomonas in rhizoremediation

Plant enzymes initiate the degradation of substances during phytoremediation, while the local microbial population carries it out during natural attenuation or bioaugmentation. It has been reported in several studies that certain microbes in the rhizosphere of plants used for phytoremediation or of plants growing from surrounding vegetation on a contaminated site play a significant role in the degradation of pollutants. This process, known as rhizoremediation, involves the rhizobacterial community (Rane et al., 2022). Sometimes, bacteria in the rhizosphere are essential to the decomposition process. There is widespread agreement that microbial bioremediation processes are a useful method for cleaning up polluted areas. This catabolic plasticity plays a crucial role in the breakdown of xenobiotic compounds and the conversion or accumulation of environmental pollutants.

In order to understand metabolic and regulatory networks and to provide novel pathways and microorganisms that will be useful for future applications, genome-based global studies are on the rise. After analyzing their genomes, scientists discovered that different strains of *Stenotrophomonas* can produce enzymes that aid in the breakdown of polychlorinated hydrocarbons and metals. Significant roles for *Stenotrophomonas* spp. in the breakdown of geosmin, hexahydro-1, 3, 5-trinitro-1, 3, 5–triazine (RDX), keratin, macrocyclic hydrocarbon, nitrophenol, and phenanthrene (Gao et al., 2013). Species of *Stenotrophomonas* have been found effective for bioremediation of agricultural soil by removing various chemical pesticides and insecticides, in addition to a wide range of environmental pollutants. The species will be useful in reducing the need for harmful chemicals in farms. A strain of *S. acidaminiphila* isolated by (Uniyal et al., 2016) from the rhizosphere soil of *Zea mays* was found to degrade fipronil, a common insecticide. A novel fipronil degrading pathway for the isolate has been proposed, and it has been suggested that the isolate could be used for bioremediation of fipronil-contaminated soil. Pourbabaee et al. (2017) also reported that *S. maltophilia* degraded diazinon (a pesticide). They also provided an analysis of the pesticide's likely degradation pathway based on FTIR. A mineralization pathway involving dechlorination, hydroxylation, and carboxylation processes was proposed based on genome annotation of the DDT degradation gene and GC–MS analysis of metabolites (Pan et al., 2016).

Zaffar et al. (2018) reported that a *S. maltophilia* EM-1 strain degrades the organochlorinated pesticide endosulfan. Endosulfan was the only sulphur source used by the bacteria. According to gas chromatography mass spectrometry testing, the bacteria are capable of metabolizing endosulfan into safer compounds like endosulfan diol. Organophosphorus insecticides belonging to the groups O, O-dialkyl phosphate, and O, O-dialkyl phosphorothioate were degraded by *Stenotrophomonas* sp. G1, which was isolated from the sludge of a chlorpyrifos manufacturer plant (Deng et al., 2015). Two *Stenotrophomonas* isolates, *S. maltophilia* MHF ENV20 and *S. maltophilia* MHF ENV23, were found to degrade chlorpyrifos, cypermethrin, and fenvalerate (Fulekar, 2014). It has also been reported that *S. maltophilia* M1 degrades the nemato-pesticide methomyl (Oxime carbamates). The strain was discovered at a site where methomyl was used for irrigation. A 5 kb plasmid (PMb) containing the gene responsible for methomyl degradation was confirmed after the transformation of the plasmid DNA into *Escherichia coli* (Mohamed, 2009).

In a symbiotic association with strain *Stenotrophomonas* sp. W16, the rate at which fomesafen is degraded in the soil increases from 29.17 to 57.87%. To aid in the bioremediation of herbicide-contaminated farming soil, this study presents a novel fomesafen-degrading rhizobium that may be used in conjunction with legumes in symbiotic systems to break down the chemical (Chen et al., 2023). Pesticides often have residual concentrations that are above regulatory thresholds. Wherever this is a problem, getting chemical-free agricultural soils fit for growing eco-friendly crops is a major obstacle.

Since bioremediation is a greener, cheaper, and more effective method than physical and chemical methods, it can be used to exploit the microbial metabolism of native microorganisms for degradation.

Potential risks and challenges of using *Stenotrophomonas* spp. in agriculture

Stenotrophomonas is a novel, multi-antibiotic resistant, opportunistic plant-associated bacterium on a global scale. It is reported that this bacterium remains associated with several plants. Moreover, it is used in bioremediation techniques and sustainable agricultural practices as a microbial biocontrol or anti-stress agent for crops. Several studies have demonstrated the immense potential of *Stenotrophomonas* spp. in agriculture; they may enhance plant growth and germination while suppressing plant diseases (Messiha et al., 2007).

On the other hand, *S. maltophilia* is also a novel human pathogen that can cause infectious diseases in humans. The only *Stenotrophomonas* species known to cause human illness is *S. maltophilia*; however, isolates of this species vary widely in terms of phylogeny and phenotype (Roscetto et al., 2008). This is probably related to the plethora of environmental niches occupied by this bacterium; the vast majority of infections are likely due to contact with distinct environmental sources. In fact, *S. maltophilia* epidemics are uncommon and are induced by contaminated origin, such as water sources (Park et al., 2008). *S. maltophilia* strains are undoubtedly equally capable of infecting people but only affect those with underlying illnesses, not the general population (Lira et al., 2017). The innate resistance of *S. maltophilia* to many first-line antimicrobials, including beta-lactams, macrolides, tetracycline, chloramphenicol, and quinolones, is the primary cause of the increase in *S. maltophilia* infections (Crossman et al., 2008). Additionally, *S. maltophilia* isolates may quickly evolve resistance to newer antibiotics via mutation; the underlying processes are unknown but are likely to be the consequence of excess production of intrinsic efflux transporters (Gould and Avison, 2006). Insufficient information exists regarding the pathogenicity of these organisms to humans. It is further demonstrated by the phenotypic and phylogenetic diversity analysis that there is a difference between pathogenic and other strains, but it is unquestionably a PGPR. Field tests and other direct applications cannot be conducted with opportunistic pathogens.

However, since *S. rhizophila* is not recognized as a human pathogen, these strains are interesting choices for biological control and stress resistance in plants because they often live endophytically. *Stenotrophomonas pavanii* is a recently discovered bacterium that can fix nitrogen from the atmosphere. The *S. pavanii* strain obtained from sugar cane is widely used in organic agriculture (Ramos et al., 2011). *S. pavanii* is a unique species of PGPR, and it is thought to focus on improving development and yield in many crops.

The diversity of *Stenotrophomonas* strains makes it difficult to distinguish characteristics because of their advantageous interactions with plants and their facultatively harmful infections with humans (Berg and Martinez, 2015). Differentiation of characteristics is crucial for future agricultural applications as well as for our understanding of infection risks and associated epidemiological issues. Predicting beneficial pathogen threats to human health is one of the current difficulties for agricultural biotechnology. Therefore, there is room to develop a new aspect of *Stenotrophomonas* spp. uses and importance in agriculture.

Future remarks

The genus *Stenotrophomonas* has been shown to possess a number of functional properties, and the research performed so far has demonstrated that it can be used to manage pests and pathogens as well as promote plant growth. However, there are still gaps in research that must be filled. For example, few species of *Stenotrophomonas* are human pathogens that can cause serious diseases in humans; however, further research is needed to discriminate between human pathogenic and non-pathogenic strains of *Stenotrophomonas* spp. and their deployment in crop improvement and *ex-situ* conservation. Using the novel molecular tools, the human pathogenic strains of *Stenotrophomonas* can be engineered into non-pathogenic strains so that they could be used in food crops for growth enhancement as well as for management of pests and diseases.

Although many studies were conducted in the past on exploring the applications of *Stenotrophomonas* in crop protection and crop improvement, further investigations are required to explore the various functions of *Stenotrophomonas* in plant growth promotion under diverse growth conditions and to demonstrate their interaction with the plant and soil systems under various environmental conditions. Further, advanced molecular techniques should be developed to identify and discriminate between human pathogenic and non-pathogenic strains of the genus *Stenotrophomonas,* so that non-pathogenic strains can be deployed in diversified cropping systems. Besides, molecular mechanisms underlying *Stenotrophomonas*-plant interactions are poorly understood and need to be carried out using advanced omics approaches such as transcriptomics, proteomics, and metabolomics. Further, as an opportunistic mycoparasite, research into its induction and regulation of enzyme expression is needed in order to improve its biocontrol abilities and to come up with potential commercial bio-fungicides. Besides, identifying various physiological traits to enhance industrial application of *Stenotrophomonas* as an alternative strategy for producing antibiotics and enzymes could be other areas for future research.

Conclusion

In conclusion, the use of beneficial microorganisms for crop health management has been studied in many cultivated crops. In this regard, *Stenotrophomonas* species have been proven to be efficient microbial biocontrol agents and have exhibited both antagonistic activity against phytopathogens and entomopathogenic activity against insect pests. The biocontrol capability of *Stenotrophomonas* spp. includes both chitinolytic and proteolytic activities, which may protect plants against phytopathogens but also play an important role in activating plant defense systems. In particular, research conducted worldwide demonstrates that various *Stenotrophomonas* species are effective in managing many pests and diseases of food crops, as well as promoting plant growth. The potential strains of *Stenotrophomonas* spp. for crop health improvement identified by the researchers can be commercialized among farmers after their validation under field conditions in larger

scale/multilocation trials. However, more work is needed to determine the cost–benefit ratio for economically commercializing bio-fertilizers and biopesticides based on *Stenotrophomonas* species. Furthermore, *Stenotrophomonas* species-based bio-products must be evaluated for regulatory risk parameters before they can be given to the farmers.

Author contributions

AK, LR, and VK: conceptualization and writing—original draft preparation. AKP and NR: writing—review and editing. KC and A: 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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Recent advances in PGPR-mediated resilience toward interactive effects of drought and salt stress in plants

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The present crisis at hand revolves around the need to enhance plant resilience to various environmental stresses, including abiotic and biotic stresses, to ensure sustainable agriculture and mitigate the impact of climate change on crop production. One such promising approach is the utilization of plant growth-promoting rhizobacteria (PGPR) to mediate plant resilience to these stresses. Plants are constantly exposed to various stress factors, such as drought, salinity, pathogens, and nutrient deficiencies, which can significantly reduce crop yield and quality. The PGPR are beneficial microbes that reside in the rhizosphere of plants and have been shown to positively influence plant growth and stress tolerance through various mechanisms, including nutrient solubilization, phytohormone production, and induction of systemic resistance. The review comprehensively examines the various mechanisms through which PGPR promotes plant resilience, including nutrient acquisition, hormonal regulation, and defense induction, focusing on recent research findings. The advancements made in the field of PGPR-mediated resilience through multi-omics approaches (*viz.*, genomics, transcriptomics, proteomics, and metabolomics) to unravel the intricate interactions between PGPR and plants have been discussed including their molecular pathways involved in stress tolerance. Besides, the review also emphasizes the importance of continued research and implementation of PGPR-based strategies to address the pressing challenges facing global food security including commercialization of PGPR-based bio-formulations for sustainable agricultural.

KEYWORDS

antioxidants, drought stress, glycine-betaine, osmolyte, oxidative stress, proline, PGPR, salt stress

1. Introduction

Besides environmental pressures that plants encounter (biotic or abiotic), they also suffer significant consequences due to their ability to travel from one location to another compared to other living things. As it is well documented, plants often suffer from numerous stressors (biotic and abiotic) during their life cycle. Each one of them can considerably impede plants' growth

and development. The main factor contributing to the global decline in agricultural crop yield is biotic stress, which is brought on by harmful microbes like bacteria, fungi, viruses, insects, and nematodes (Murali et al., 2021a; Gowtham et al., 2022; Hamidian et al., 2023; Karimian et al., 2023). In addition, the abiotic stressors that are detrimental to plants include flooding, drought, soil salinity, extreme temperature, extremely high or low light conditions, contamination with organic pollutants and heavy metals, and excessive radiation (Sarker et al., 2021; Murali et al., 2021a; Ahmad et al., 2022). The abiotic stresses adversely affect the physiological, biochemical, and molecular responses of plants in a multitude of manners, which lowers productivity (Murali et al., 2021a; Munir et al., 2022). The plants grow poorly when exposed to these stresses due to osmotic stress, oxidative stress, reactive oxygen species (ROS) production, hormonal imbalance, ionic toxicity, and reduced nutrient mobilization (Ma et al., 2020).

The abiotic stresses impact plant responses, including the alteration of genes involved in the central metabolic pathways and a change in the growth rate leading to a significant loss in the yield of the crops (Ullah et al., 2021). Most stressed plants point to environmental changes; their roots are where they first respond to such challenging circumstances. Out of the available arable lands, 90% are vulnerable to these stressors, and it is noted that the crop output is reduced and limited to up to 70% when exposed to these abiotic stressors continuously (Waqas et al., 2019). Due to global climate change, drought and soil salinity are two major environmental factors that reduce plant growth and productivity in many plant species, especially in arid and semi-arid regions of the world. The world will face a significant challenge of 70% more food production to adequately sustain the projected 2.3 billion more people by 2050. Therefore, it is imperative to induce stress resilience in crops against drought and salinity stress to meet future generations' food demands. The plant growth-promoting rhizobacteria (PGPR) have emerged as promising allies in sustainable agriculture, offering the potential to enhance plant tolerance to abiotic stresses, such as drought and salinity. From the literature it has been well noted that these PGPR have been found as effective biological agents in the management of crop plants against drought and salt stress through multi-omic strategies thereby by improving plant growth and production (Kim et al., 2014; Singh et al., 2017; Khan et al., 2021a,b; Mellidou et al., 2021; Vafa et al., 2021; Nishu et al., 2022; Zhao et al., 2022; Patel et al., 2023). The review aims to delve into the multifaceted realm of PGPR-mediated plant resilience, with three primary objectives: (i) to assess the impact of PGPR on enhancing plant tolerance to abiotic stresses such as drought and salt; (ii) to highlight recent advancements in understanding the mechanisms by which PGPR mediate plant resilience; and (iii) to critically evaluate the potential applications of PGPR-based strategies in sustainable agriculture. Previous salient reviews have primarily focused on either general PGPR-plant interactions or specific stress responses but often lack a comprehensive analysis of recent advancements and their implications for sustainable agriculture. Hence, the current study bridges this gap by amalgamating recent evidence to provide a holistic understanding of the impact of PGPR on plant tolerance to drought and salinity, elucidating the latest mechanistic insights, and critically evaluating their potential for sustainable agriculture. Consequently, it aims to offer a comprehensive reference for researchers, agronomists, and policymakers seeking innovative solutions to enhance crop resilience in a changing world.

2. Interactive effects of drought and salt stress

The repercussions of climate change have been growing, which has resulted in a sharp rise in drought in recent years. In addition to being an issue, soil salinity is also a result of global warming, especially for crops that require sufficient irrigation (Khan, 2022). Some farmlands are under-irrigated, resulting in salt accumulation inside the soil due to insufficient water supply in many areas. In this situation, the irrigation water either benefits the plants by being utilized or evaporates, leaving salt in the soil. Furthermore, the most salt-affected grounds are found in the arid and semi-arid regions of the world. The main barrier to plant growth is the high salt levels brought on by irrigation, which worsens drought impacts (Ullah et al., 2021). Soil salinity is measured by its electrical conductivity (EC), expressed in dS/m. At the same time, drought is described in the percent (%) field capacity of soil moisture content which can be defined as the amount of water available in the soil. Excessive salt levels are currently thought to have a detrimental impact on 20% of the world's agricultural land used to cultivate irrigated crops (Khan, 2022). Drought and salinity stress significantly impact future agricultural production, which frequently co-occurs due to changes in climate and the struggle for water, land, and energy (Morari et al., 2015; Mitra et al., 2021; Khan, 2022). Drought and salt stress severely inhibit food crop growth and physiochemical activity. For instance, plants under these stressors have the same morphological and physiochemical traits. It is observed that the drought stress progress in plants is facilitated via greater salt concentrations because salt-related solutes prevent water uptake impacting the leaf water content (Ahluwalia et al., 2021).

The plants explore common and distinct responses to modify plant growth and adaptation under drought and salinity stress. Most plants initially react similarly to drought and salinity, primarily caused by water deficit within the plant which results in a decreased growth rate. After the plants absorb salt, the sodium ions (Na^+) are transported to the plant's shoots via the xylem, eventually accumulating in the leaves and shoots (Ullah et al., 2021). The consensus is that sodium ion buildup in plants is harmful because it competes for binding sites with potassium ions (K^+) essential for cellular activity (Desoky et al., 2020; Hasanuzzaman et al., 2022). Similarly, ROS are developed within the plants that can subsequently induce lipid peroxidation in plant cell membranes, lead to electrolyte leakage from plant cells, and either augment photorespiration or reduce the transpiration ensuing in a slower photosynthesis rate, thereby having a detrimental effect on the production and quality of the plants (Khan, 2022).

It is necessary to advance agricultural production in terms of productivity and food safety due to the predicted population growth and rising living standards. Innovative agricultural technologies and production methods are urgently needed to simultaneously achieve sustainable agricultural productivity improvement and environmental and economic sustainability. To overcome salinity stress, plants employ various mechanisms (Figure 1) like (i) control of sodium ion absorption by roots and successive transfer of these ions to leaves, (ii) selective accumulation of sodium ions (Na^+) in vacuoles or exclusion of these ions, (iii) vacuolar compartmentalization of Na^+ at the plant cellular level, (iv) modification of plant cell membranes, (v) modulation of the level of plant hormones, and (vi) synthesis of antioxidant enzymes and compatible solutes (Ullah et al., 2021; Khan, 2022). The salt stress tolerance also depends on the cultivars' growth

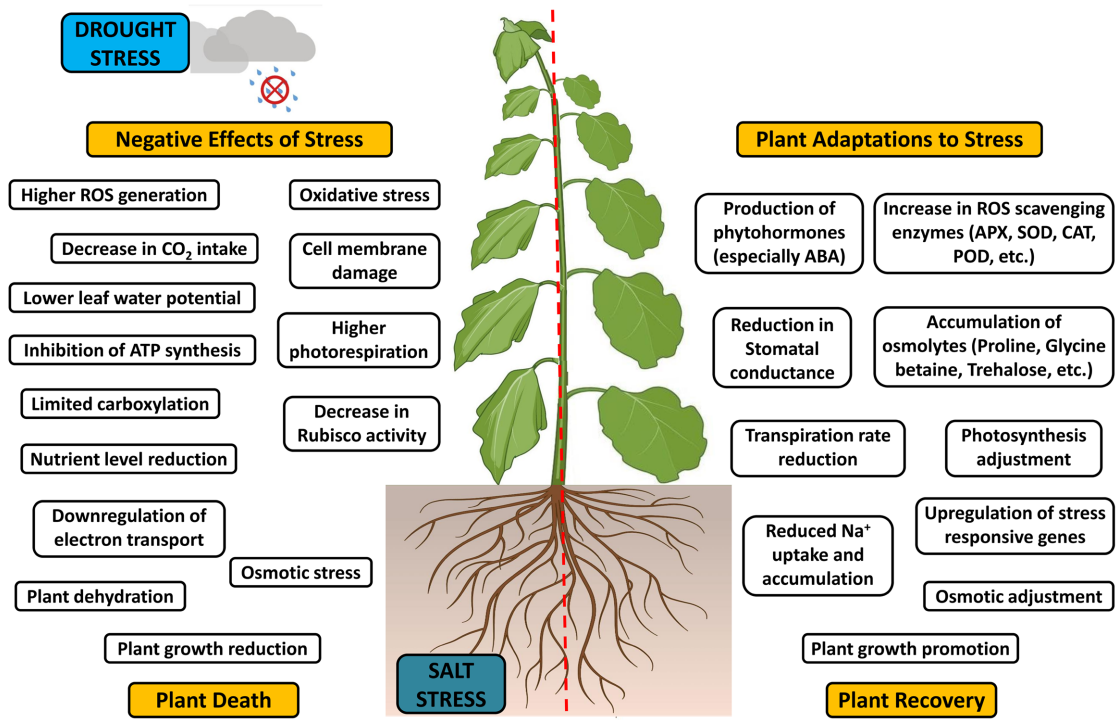

FIGURE 1
Interactive effects and adaptive mechanism to drought and salt stress in plants.

stage and health, soil composition, microbe association, etc. (Ma et al., 2020). Developing crops that can withstand salt and drought using transgenic technologies and conventional breeding techniques is often expensive, time-consuming, and challenging. Hence, investigating the potent PGPR as a viable replacement for toxic chemicals will help in the improvement of plants' productivity and soil sustainability even under unfavorable environmental conditions, thereby providing a better perspective for agriculture (Brijesh Singh et al., 2019; Murali et al., 2021a; Hoseini et al., 2022; Munir et al., 2022; Mawar et al., 2023). Additionally, it will endeavor to comprehend the biochemical, physiological, and genetic pathways that the PGPR mediate, as they are crucial for enhancing plant tolerance to these environmental challenges.

3. Plant growth-promoting rhizobacteria

The rhizosphere-resident bacteria, commonly termed PGPR, can induce plant growth through several means, which might be either direct or indirect processes. These rhizobacteria are known as effective disease-fighters, helping agricultural productivity and sustainability (Bhat et al., 2022; Gamalero and Glick, 2022; Gowtham et al., 2022). The PGPR that flourishes in the rhizosphere improves plant growth by various mechanisms, such as nitrogen fixation, production of phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Sagar et al., 2020), exopolysaccharides (EPS; Sayyed et al., 2015, 2019; Ilyas et al., 2020), siderophores (Nithyapriya et al., 2021; Srivastava et al., 2022), antioxidants (Gowtham et al., 2022), osmoprotectants (Ilyas et al., 2020), nutrient uptake (Jabborova et al., 2020, 2022; Deepranjan et al., 2021; Kapadia et al., 2021; Sarkar et al., 2021), and induced systemic resistance (ISR; Reshma et al., 2018; Ali B. et al., 2022; Desai et al., 2023) in stressful conditions. These PGPR can also influence plant metabolism and gene expression directly, as well as the expression of root proteins, root morphology, and root growth (Vacheron et al., 2013; Kalam et al., 2020; Basu et al., 2021; Hamid et al., 2021; Ahmad et al., 2022; Lobhi et al., 2022). Besides, PGPR application exogenously can alter the plant rhizosphere microbial communities in soil, which modulates the host's capacity for nutrient adsorption and pathogen interaction apart from modifying the ability of the plant to tolerate both biotic and abiotic stressors (Hariprasad et al., 2021; Munir et al., 2022). Therefore, they are possible options for chemical fertilizers in agriculture production, which are mentioned below depending on the host and stress factors.

4. Mechanisms induced by PGPR during amelioration of drought and salt stress

The stress-tolerant bacteria can survive better in severe drought and salt stress conditions and overcome their effect by different mechanisms (Fazeli-Nasab and Sayyed, 2019), such as changing plant root system morphology and structure, balancing osmotic stress and oxidative stress, and regulating ion homeostasis (Tables 1, 2). These plausible mechanisms involved during the plant-PGPR interaction to prevail over drought (Figure 2) and salt stress (Figure 3) are discussed below in detail.

TABLE 1 Overview of PGPR-mediated enhancement of plant tolerance against drought stress.

Plants	PGPR Strains/ Consortia	Beneficial traits produced by PGPR	Experimental details	References
Abelmoschus esculentus	*Bacillus subtilis*	–	Field experiment	Puthiyottil and Akkara (2021)
Arabidopsis thaliana	*Pseudomonas chlororaphis*	Volatiles: 2R,3R-butanediol	Laboratory and pot experiments	Cho et al. (2008)
	B. endophyticus and *P. aeruginosa*	IAA, cytokinin, gibberellic aci and EPS	Laboratory experiment	Ghosh et al. (2019)
	Pseudomonas sp.	IAA, ABA, gibberellic acid, EPS, and ACC deaminase	Pot experiment	Yasmin et al. (2022)
Brassica juncea	*B. marisflavi*	ABA analog/Xanthoxin	Laboratory experiment	Gowtham et al. (2021)
Cicer arietinum	*P. putida*	Phosphate solubilization, IAA, and ACC deaminase	Pot experiment	Tiwari et al. (2016)
Eleusine coracana	*Variovorax paradoxus, P. palleroniana, P. fluorescens,* and *Ochrobactrum anthropi*	ACC deaminase		Chandra et al. (2020)
H. annuus	*P. putida*	EPS		Sandhya et al. (2009)
	B. subtilis and *B. thuringiensis*	ACC deaminase		Singh et al. (2019)
Lactuca sativa	*B. subtilis*	Cytokinin		Arkhipova et al. (2007)
Lolium perenne	*Pseudomonas* sp. and *Bacillus* sp.	ACC deaminase, IAA and EPS	Laboratory and pot experiments	He et al. (2021)
Medicago sativa	*B. amyloliquefaciens*	IAA, EPS and siderophores	Pot experiment	Han et al. (2022)
Mentha piperita	*P. fluorescens* and *B. amyloliquefaciens*	ACC deaminase and IAA production		Chiappero et al. (2019)
Mucuna pruriens	*Enterobacter* sp. and *Bacillus* sp.	IAA and ACC deaminase		Saleem et al. (2018)
		ACC deaminase		Brunetti et al. (2021)
Ocimum basilicum	*Azospirillum baldaniorum*	Induced immune response (ISR)	Greenhouse experiment	Mariotti et al. (2021)
Oryza sativa	*B. haynesii, B. paralicheniformis* and *B. licheniformis*	ACC deaminase	Pot experiment	Joshi et al. (2020)
	B. altitudinis and *B. methylotrophicus*	ABA		Narayanasamy et al. (2020)
	Gluconacetobacter diazotrophicus	Nitrogen fixation and IAA	Field experiment	Silva et al. (2020)
Pennisetum glaucum	*B. amyloliquefaciens*	ACC deaminase	Pot experiment	Murali et al. (2021a,b)
Pisum sativum	Consortia of *Pseudomonas* sp., *O. pseudogrignonense* and *B. subtilis*	ACC deaminase		Saikia et al. (2018)
Setaria italica	*P. migulae, P. fluorescens*, and *E. hormaechei*	EPS and ACC deaminase		Niu et al. (2018)
Solanum lycopersicum	*Enterobacter* spp.	IAA and gibberellic acid	Laboratory experiment	Bhatt et al. (2015)
	B. amyloliquefaciens	EPS	Pot experiment	Wang et al. (2019)
	Streptomyces spp.	ACC deaminase and IAA		Abbasi et al. (2020)
	B. subtilis	ACC deaminase		Gowtham et al. (2020)
	Bacillus megaterium	Extracellular arginine		Morcillo et al. (2021)
Solanum tuberosum	*V. paradoxus, P. oryzihabitans* and *Achromobacter xylosoxidans*	IAA and ACC deaminase	Pot and field experiments	Belimov et al. (2015)
	B. subtilis	–	Pot experiment	Batool et al. (2020)
Sorghum bicolor	*Pseudomonas* sp.	ACC deaminase		Carlson et al. (2019)
	Streptomyces sp. and *Nocardiopsis* sp.	Phosphate solubilization, IAA, siderophore, and ACC deaminase		Silambarasan et al. (2022)

(Continued)

TABLE 1 (Continued)

Plants	PGPR Strains/ Consortia	Beneficial traits produced by PGPR	Experimental details	References
Spinacia oleracea	*B. amyloliquefaciens* and *Bacillus* sp.	Phosphate solubilization, IAA, and siderophore	Laboratory experiment	Petrillo et al. (2022)
Trifolium repens	*B. megaterium and P. putida*	IAA	Pot experiment	Marulanda et al. (2009)
Trigonella foenum-graecum	*B. subtilis*	ACC deaminase		Barnawal et al. (2013)
Triticum aestivum	*B. thuringiensis*	Reduction in volatile emissions		Timmusk et al. (2014)
	Klebsiella sp., *E. ludwigii* and *Flavobacterium* sp.	Phosphate solubilization, EPS, IAA, siderophore, and ACC deaminase		Gontia-Mishra et al. (2016)
	P. palleroniana and *P. fluorescens*	ACC deaminase		Chandra et al. (2018)
	P. stutzeri, Moraxella pluranimalium, E. aerogenes, B. thuringiensis, B. simplex, B. pumilus, B. muralis, and *B. amyloliquefaciens*	IAA		Raheem et al. (2018)
	O. anthropi, P. palleroniana, P. fluorescens, and *V. paradoxus*	ACC deaminase		Chandra et al. (2019)
	Bacillus sp. and *Enterobacter* sp.	IAA and salicylic acid		Jochum et al. (2019)
	B. cereus and *Planomicrobium chinense*	EPS	Field experiment	Khan and Bano (2019)
	B. subtilis and *A. brasilense*	EPS, Sugar and Proline	Pot experiment	Ilyas et al. (2020)
	Streptomyces pactum	–	Laboratory experiment	Li et al. (2020)
	B. subtilis	ACC deaminase	Pot experiment	Sood et al. (2020)
	P. azotoformans	EPS		Ansari et al. (2021)
	P. helmanticensis and *P. baetica*	Phosphate solubilization, IAA and siderophore		Karimzadeh et al. (2021)
	Pseudomonas sp. and *Serratia marcescens*	Phosphate solubilization, ACC deaminase, IAA, siderophore and EPS		Khan and Singh (2021)
	Chryseobacterium sp., *Acinetobacter* sp. and *Klebsiella* sp.	IAA and EPS	Jar experiment	Latif et al. (2022)
	B. megaterium and *B. licheniformis*	ACC deaminase and IAA	Pot experiment	Rashid et al. (2022)
Vigna mungo	Consortia of *Pseudomonas* sp., *O. pseudogrignonense* and *B. subtilis*	ACC deaminase		Saikia et al. (2018)
Vigna radiata	*P. aeruginosa*	IAA production	Lab, pot, and field experiments	Uzma et al. (2022)
Vitis vinifera	*B. licheniformis* and *P. fluorescens*	ABA	Laboratory experiment	Salomon et al. (2014)
	P. corrugata and *E. soli*	ACC deaminase	Pot experiment	Duan et al. (2021)
	Enterobacter sp. and *Bacillus* sp.	IAA and salicylic acid		Jochum et al. (2019)
	B. velezensis	ACC deaminase and EPS	Laboratory experiment	Nadeem et al. (2020)
	P. fluorescens	ACC deaminase	Field experiment	Zarei et al. (2020)
	Bacillus spp.	Nitrogen fixation, phosphate solubilization, and IAA and EPS	Pot experiment	Azeem et al. (2022)
Ziziphus jujuba	*P. lini* and *S. plymuthica*	ACC deaminase		Zhang et al. (2020)

TABLE 2 Overview of PGPR-mediated enhancement of plant tolerance against salt stress.

Plants	PGPR strains/Consortia	Beneficial traits produced by PGPR	Experimental details	References
Arabidopsis thaliana	*B. amyloliquefaciens*	Spermidine	Laboratory experiment	Chen et al. (2017)
	P. putida	–		Chu et al. (2019)
	Stenotrophomonas maltophilia	Nitrogen fixation	Laboratory experiment	Alexander et al. (2020)
Avena sativa	*Klebsiella* sp.	IAA and ACC deaminase		Sapre et al. (2018)
Capsicum annuum	*B. licheniformis, Brevibacterium iodinum* and *Zhihengliuela alba*	ACC deaminase	Pot experiment	Siddikee et al. (2011)
Cicer arietinum	*Pantoea dispersa*	ACC deaminase and IAA		Panwar et al. (2016)
Cucumis sativus	*B. megaterium, P. fluorescens* and *V. paradoxus*	ACC deaminase, IAA, and siderophore	Laboratory experiment	Nadeem et al. (2016)
Glycine max	*Bacillus* sp. and *Pseudomonas* sp.	ACC deaminase, IAA, and EPS		Kumari et al. (2015)
Lactuca sativa	*Lactobacillus* sp., *P. putida* and *Azotobacter chroococcum*	IAA	Laboratory experiment	Hussein and Joo (2018)
Medicago sativa	*Halomonas maura* and *Ensifer meliloti*	EPS	Greenhouse and field experiments	Martínez et al. (2015)
Mentha arvensis	*B. pumilus, Exiguobacterium oxidotolerans* and *Halomonas desiderata*	Phosphate solubilization, siderophore, EPS, and ACC deaminase	Pot and glass house experiments	Bharti et al. (2014)
Oryza sativa	*Enterobacter* sp.	ACC deaminase, IAA, siderophore, and phosphate solubilization	Laboratory experiment	Sarkar et al. (2018)
	Glutamicibacter sp.	IAA and ACC deaminase	Pot experiment	Ji et al. (2020)
	B. aryabhattai and *B. tequilensis*	EPS	Glasshouse experiment	Shultana et al. (2020)
	B. aryabhattai, A. denitrificans and *O. intermedium*	IAA, phosphate solubilization, and Nitrogen fixation	Pot experiment	Sultana et al. (2020)
	B. pumilus	Phosphate solubilization, ACC deaminase, IAA, and EPS		Kumar et al. (2021)
Pistacia vera	*Arthrobacter endophyticus, Staphylococcus sciuri* and *Zobellella denitrificans*	ACC deaminase, auxin, siderophore, EPS, and phosphate solubilization		Khalilpour et al. (2021)
Pisum sativum	*A. protophormiae*	ACC deaminase		Barnawal et al. (2014)
	V. paradoxus	ACC deaminase		Wang et al. (2016)
Raphanus sativus	*A. chroococcum, Lactobacillus* sp., and *P. putida*	IAA	Laboratory experiment	Hussein and Joo (2018)
Solanum lycopersicum	*Pseudomonas* sp., *Pantoea* sp., *Leifsonia* sp., *Bacillus* sp. and *Arthrobacter* sp.	IAA, siderophore, and phosphate solubilization	Pot experiment	Cordero et al. (2018)
	Leclercia adecarboxylata	ACC deaminase and IAA		Kang et al. (2019)
	Pseudomonas sp.	ACC deaminase and trehalose		Orozco-Mosqueda et al. (2019)
Triticum aestivum	*Bacillus* sp., *B. insolitus* and *Aeromonas hydrophila/caviae*	EPS		Ashraf et al. (2004)
	Streptomyces sp.	IAA and siderophore	Greenhouse experiment	Sadeghi et al. (2012)
	E. cloacae, P. putida, P. fluorescens and *S. ficaria*	ACC deaminase	Field experiment	Nadeem et al. (2013)
	Klebsiella sp.	ACC deaminase	Pot experiment	Singh et al. (2015)
	B. subtilis and *Marinobacter lipolyticus*	EPS		Talebi Atouei et al. (2019)
	P. fluorescence, E. aurantiacum and *B. pumilus*	Phosphate solubilization, ACC deaminase, and IAA		Nawaz et al. (2020)
	B. amyloliquefaciens	Spermidine	Laboratory experiment	Chen et al. (2017)
	B. aquimaris	IAA		Li and Jiang (2017)
	B. subtilis and *B. safensis*	ACC deaminase	Pot experiment	Misra and Chauhan (2020)
	E. cloacae	Siderophore, IAA, and EPS ACC deaminase		Ali S. A. M. et al. (2022)

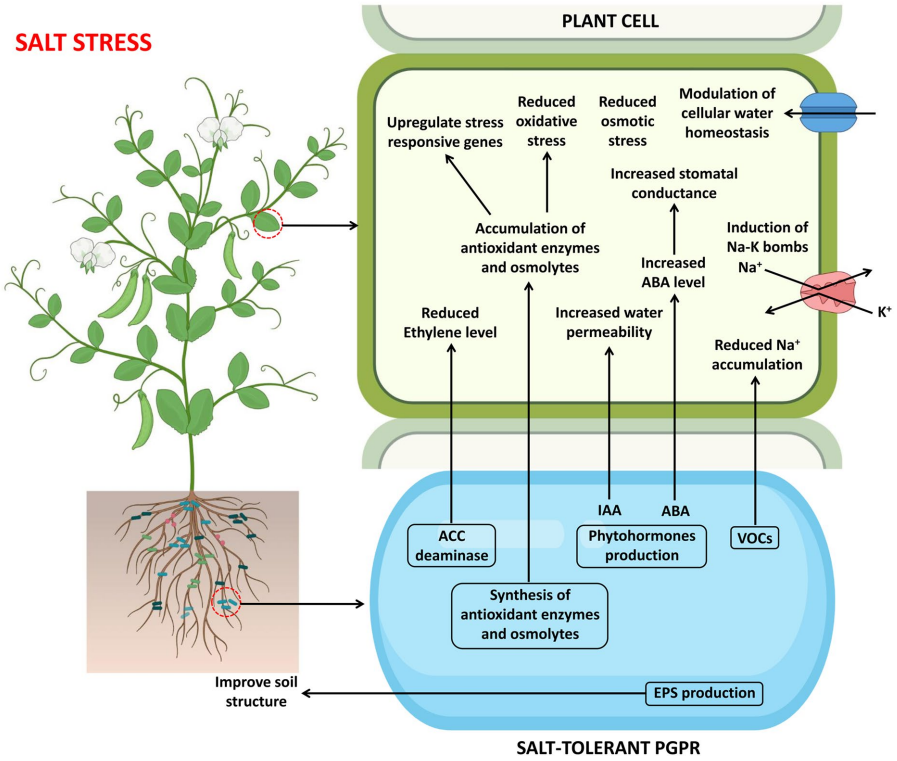

FIGURE 2
A pictorial overview of the functioning of PGPR in support of plant growth and stress tolerance against drought stress.

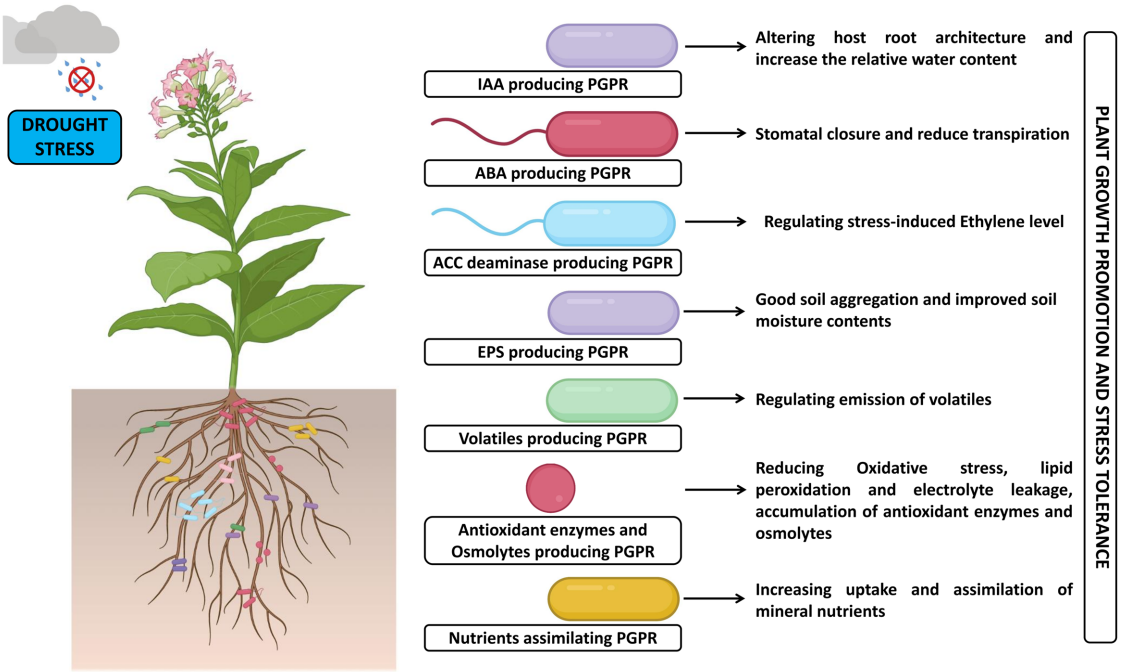

FIGURE 3
A pictorial overview of the functioning of PGPR in support of plant growth and stress tolerance against salt stress.

4.1. Changes in plant root system architecture

The root system architecture describes the entire spatial configuration and covers the root's density, angle, surface area, volume, and biomass. Plants with a larger root architecture help in higher water absorption from the soil, thereby leading to the modification of root morphology that allows them to cope under drought stress and variations in root morphology are found to be species-specific (Paez-Garcia et al., 2015). Inoculating agricultural crop plants with PGPR can lead to significant changes in the root system architecture, wherein the changes are observed through stimulation of root elongation and density which helps in nutrient and water uptake. Besides, PGPR is known to induce the formation of lateral roots and root hairs that assist in nutrient absorption, stabilizing the plant in the soil, promote deeper root growth, enhance the formation of mycorrhizal associations for symbiotic relationships with plant roots, fix atmospheric nitrogen, and support stress tolerance. The changes in root system architecture induced by PGPR ultimately contribute to improved plant growth, increased crop yield, and better crop health (Grover et al., 2021; Mohanty et al., 2021; Gowtham et al., 2022).

The rhizosphere microbial communities significantly influence host plant health and phenotypic traits by changing the soil processes in stressful conditions (Grover et al., 2021). During water stress conditions, some bacteria alter the cell membrane elasticity in root cells; these modifications are considered the first step in improving drought tolerance (Vacheron et al., 2013). Mishra et al. (2020) have noted that inoculating *Zea mays* with *Ochrobactrum* sp. under drought conditions improved the development of root hairs, root length, and dry weight. The combination and concentration of mineral nutrients in the soil substantially influence plant growth and development. The plants can retain enough nutrient content despite shifting soil environments through changes to root architecture, the emergence of root-based transport systems, and symbiotic relationships with helpful soil bacteria (Naylor and Coleman-Derr, 2017). It is important to note that the specific changes in root system architecture can vary depending on the crop species, the strain of PGPR used, and environmental conditions (Brijesh Singh et al., 2019; Murali et al., 2021a; Gowtham et al., 2022). Therefore, selecting appropriate PGPR strains and implementing proper agronomic practices are essential for maximizing the benefits of PGPR inoculation in agriculture. As a result, it was discovered that PGPR strains may enhance soil fertility, control pH, safeguard crops from phytopathogens, and lessen the effects of abiotic stress on various crops.

4.2. Balancing osmotic stress

Osmotic stress is the first immediate effect caused by drought and salt stress, which disrupts leaf water potential (Ψ) and causes stomatal closure, generates ROS, membrane lipid peroxidation, and increases antioxidant enzymatic activities and accumulation of osmolytes in plants (Brijesh Singh et al., 2019; Gowtham et al., 2022). In contrast, the stomatal limitations reduced the efficiency of photosystem II and limited CO_2 assimilating enzyme activities, which are the significant challenges posed by the plants that lead to reduced photosynthetic rates under extreme drought conditions (Batool et al., 2020). Due to the imbalanced gas exchange and decreased leaf area, photosynthesis slows down, and therefore, to mitigate the effect of these stressors on plants, they should sustain water homeostasis and maintain their photosynthetic structures unharmed. Further, the osmotic stress brought on by salt and drought directly impacts numerous soil processes, including stressing out the microorganisms (Hasanuzzaman et al., 2022). When under stressful conditions, the soil bacteria adjust their osmotic conditions and sustain themselves hydrated by cellular compatible solute accumulation that assists in maintaining the right amount of water in their cells (Desoky et al., 2020).

Meenakshi et al. (2019) and Abbasi et al. (2020) have illustrated that *Solanum lycopersicum* and *Triticum aestivum* plants' vulnerability to the adverse effect of drought stress decreased the bacterial inoculation that assisted in the increased water usage effectiveness, maintaining cell membrane integrity and RWC in the infected plants' shoot and root tissues. In addition, *Setaria italica* plants showed better growth under drought conditions due to treatment with *Pseudomonas fluorescens*, which increased soil moisture by colonizing both the root surface and the soil adhering to them (Niu et al., 2018). Besides, using *Bacillus subtilis,* a PGPR strain, enhanced the RWC in tomato plants and improved plant growth compared to untreated plants (Gowtham et al., 2020).

The plant-rhizobacterial interactions are researched to date to understand better the mechanisms implicated in PGPR-mediated osmotic stress tolerance. These bacterial associations significantly impact the formation of extracellular chemicals, increased food availability in the rhizosphere, and protection against abiotic challenges affecting plant growth. Accordingly, exopolysaccharides (EPS), volatile organic compounds (VOCs), and suitable osmolytes, which are produced by bacteria outside of their cells, operate as signal molecules for plant growth in challenging environments (Ma et al., 2020; Kapadia et al., 2022; Khumairah et al., 2022; Sagar et al., 2022a). The EPS are highly hydrated organic polymers with fundamental defensive roles against abiotic stress during plant-microbe interactions (Sayyed et al., 2015; Ahmad et al., 2022). The EPS production is assumed to be directly responsible for the regulation of water potential, aggregation of soil particles, ensures requisite communication between rhizobacteria and plant roots, resulting in the sustainability of the host under initial osmatic stress (Naseem et al., 2018; Ilyas et al., 2020).

Similarly, PGPR has been employed to successfully combat the severe impact of drought through uplifting the EPS production and also through rhizome-sheaths formation around the roots to guard against dehydration, thereby denoting the importance of EPS-producing PGPR in reducing the scarcity of water and improving global food security (Ahmad et al., 2022). The EPS-producing PGPR, including *Agrobacterium* spp., *A. vinelandii*, *Rhizobium* sp., *R. leguminosaru*, *Bacillus drentensis*, and *Xanthomonas* sp., are synergistically essential for nourishing the soil and supporting crop production under salinity stress (Mahmood et al., 2016). Likewise, Ma et al. (2020) have confirmed that the EPS-producing PGPR maintained soil aggregation, dehydration, and water potential, which are critical in improving nutrient uptake that directly correlates to enhancing plant growth. Nevertheless, relief of osmatic stress in plants mainly relies on the growth stage, intensity, and period of the stress and the efficiency of PGPR application to relieve osmotic stress. Therefore, it is important to identify the rhizobacteria that produce EPS to get over the adverse effect of drought and salt stress in crop plants.

The VOCs are a mixture of low molecular weight volatile compounds produced by bacteria that possess antibacterial properties and other cross-talk interactions with the plant pathogens and their host (Tilocca et al., 2020). These bacterial volatiles can alter the formation or dispersal of biofilms and alter bacterial motility, including ammonia, nitric oxide, hydrogen sulfide, trimethylamine, and 2-amino-acetophenone (Schulz-Bohm et al., 2017). Some bacteria produce VOCs, which can change how other bacteria behave, control the level of antibiotic resistance, and have antagonistic effects on other nearby bacteria in the rhizosphere. The emission of VOCs plays an intriguing signaling function during the association of microbes, and it is well observed that certain rhizobacterial species can secrete or emit VOCs as extracellular molecules, which can alleviate the osmotic stress originating due to drought and salt stress (Russo et al., 2022). Similarly, Vaishnav et al. (2015) have noted an improvement in the production of VOCs upon inoculation with the PGPR *Pseudomonas simiae* strain, which in turn improved the growth, enhanced proline and chlorophyll content in *Glycine max* seedlings and elicited the induced systemic tolerance against osmotic stress caused by salt stress condition. The microbial VOCs (acetoin being the main one) produced by *Bacillus amyloliquefaciens* were able to significantly alter the morphological characters that helped in the higher accumulation of total chlorophyll and also helped in the reduction in the ABA levels in *Mentha piperita* under salt stress when compared to uninoculated plants (Cappellari and Banchio, 2020). The VOCs produced from PGPR strains are the factors for triggering induced systemic tolerance in the plant against stressors. Similarly, the better exploitation of VOCs emitted from these rhizobacteria has been noted as the prime plant defense mechanism for regulating their growth and enhancing stress tolerance through interactions with the phytohormones (Sudha et al., 2022).

To lessen competition for nutrient resources and niche spaces, various bacteria excrete antimicrobial compounds (such as bacteriocins; Subramanian and Smith, 2015). The most numerous and varied class of the bacteria's defense systems are the bacteriocins, which are antimicrobial peptides produced by ribosomal enzymes. The development of fruitful plant-microbe partnerships is controlled by a large number of bacteriocin-producing PGPR strains, which create a variety of bacteriocins and exchange them in the rhizosphere (Nazari and Smith, 2020; Shah et al., 2021). In the rhizosphere, the bacteriocins function as signaling molecules between microbes or between microbes and plants (Figure 4). When exposed to abiotic stress, it not only prevents rival microorganisms from occupying its niche but also physically widens the niche by enhancing plant growth, thereby acting as a biostimulant agent for the sustainable agricultural industry.

The maintenance of osmotic equilibrium and to have a better response to drought and salt stress, plants lose intracellular water, which leads them to amass appropriate osmolytes in the cytoplasm which include trehalose, proline, etc. (Gowtham et al., 2022). The PGPR increases the amassing of osmolytes in the host plant as a defense mechanism against osmotic stress in environmental stressors. Besides, these microbes manufacture osmolytes faster than the plants linked with them. The defense mechanism the PGPR uses to counteract the osmotic stress in environmental stresses involves the enhanced accumulation of osmolytes in the host plant. Moreover, osmolytes are more quickly produced by the PGPR than their associated plants as their inoculation in plants improves the

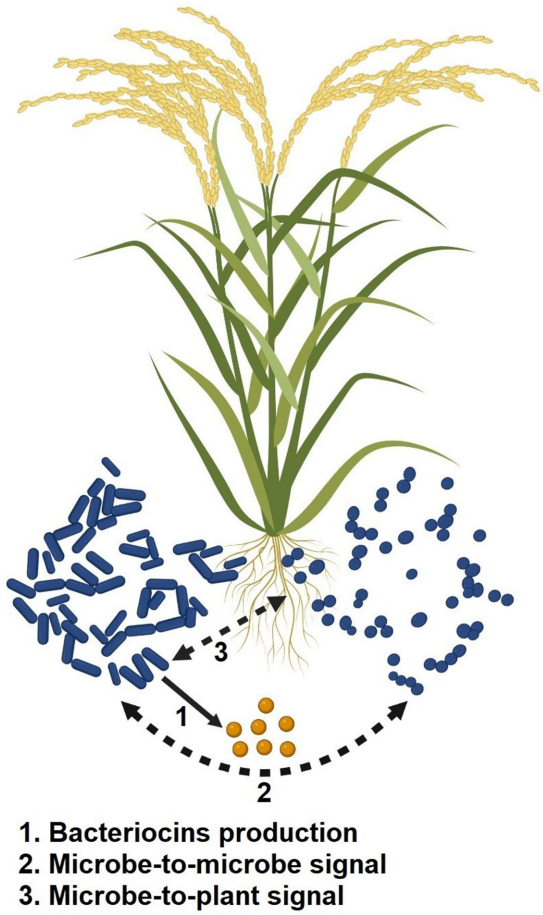

FIGURE 4
Mechanism of action of bacteriocins excreted by PGPR in abiotic stress resilience.

production of osmolytes, which may be related to the roots absorbing bacterial solutes or *de novo* synthesis in plants (Ma et al., 2020). When exposed to salinity, osmolytes produced by PGPR can improve root hydraulic conductivity and water potential, which benefits the plant's stomatal opening and transpiration rate (Ilangumaran and Smith, 2017). Therefore, the accumulation of certain osmolytes has facilitated various plants to resist drought and salt stress in beneficial PGPR.

One of the compatible solutes a plant makes when its water supply is cut off is proline. It decreases the cells' water potential and helps sustain turgor pressure, assuring the plant's development, metabolism, and yield rate. Proline content is closely correlated with drought stress, and proline concentration is highly linked with stress intensity. Due to water constraints, cells with a high proline concentration maintain their water balance and membrane stability (Khan et al., 2021a,b). Therefore, evaluating the proline content is essential to determining how plants are sensitive and resistant to abiotic stressors. Among the several osmolytes, the increase of proline content within the bacteria amid osmotic stress has drawn much attention and the PGPR can also control how proline is expressed in plants. Glycine betaine is one of the well-known compatible osmolytes which help increase resistance to abiotic stresses (specifically drought and salt) by improving water status and protecting cell membranes from ROS (Khan et al., 2020; Sagar et al., 2020; Kusale et al., 2021a). In addition, glycine betaine has

been well documented to augment the plant defense mechanisms, including the osmotic balance, enzyme activities, and genes associated with the tolerance. The plants exposed to PGPR inoculation under drought and salinity conditions exhibited a more significant accumulation of glycine betaine content as a major factor in reduced water loss. The higher glycine betaine content was noticed in drought-stressed *Z. mays* plants inoculated with *Pseudomonas* spp. than in untreated plants (Sandhya et al., 2010). *Pseudomonas putida* and other rhizobia have been reported to mitigate drought stress in wheat (Najafi et al., 2021; Tanvere et al., 2023). Choline is another crucial osmolyte produced from glycine betaine accumulation to cope with drought stress. Studies have demonstrated that the microbial populations in the soil contribute significantly to the buildup of choline, a precursor to the metabolism of glycine betaine (Ghosh et al., 2021).

Trehalose is a non-reducing sugar that protects against extreme abiotic stress, like salt and drought, by stabilizing sugar glasses, vitrifying sugar molecules, and acting as a xeroprotectant in plant and bacterial cells (Ahmad et al., 2023). Trehalose helps plants to communicate because it can keep the osmotic balance in their cells and protect the biological structures from damage during desiccation. The stress tolerance signal pathway can be established by applying even a small quantity of trehalose to the plant roots. Trehalose promotes the survivability of PGPR and other biocontrol agents when they are commercially formulated and stored for longer durations, besides boosting the competence of these microbes in the root regions and rendering a hand in the resistance against abiotic stresses. *Azospirillum brasilense* inoculation of *Z. mays* plants results in the conferment of drought resistance and a significant enhancement of root and leaf biomass (Rodríguez-Salazar et al., 2009).

Polyamines are the low molecular weight aliphatic amines, which play the complex role as an osmolyte associated with plant growth promotion which is subjected to abiotic stress response by accelerating the function of enzymes and genes involved in the antioxidant defensive system and ROS homeostasis (Chen et al., 2019). The three primary polyamines found in plants like, spermine, spermidine, and putrescine, are also crucial in how they react to biotic and abiotic stressors. Exogenous polyamines have been shown to increase drought and salinity tolerance, but further study is needed to comprehend how polyamines released by the PGPR ultimately affect plants. Under osmotic stress, *Oryza sativa* seedlings accumulated polyamines due to the inoculation of *A. brasilense* (Cassán et al., 2009). The spermidine secreted by the beneficial rhizobacterium *Bacillus amyloliquefaciens* reduced the impact of oxidative damage, decreased the toxicity of Na^+, and ABA accumulation in *Z. mays* was inhibited, thereby resulting in the improvement of plant salt sensitivity (Chen et al., 2017). The regulation of endogenous free polyamines in plants by the PGPR under water and salt stress was found to elevate the antioxidant defense capacity and encourage the expression of genes linked to antioxidants.

4.3. Balancing oxidative stress

Oxidative stress is critical for plants due to the large amounts of ROS produced in the membranes, which, under abiotic stress, can result in severe denaturation of protein, DNA mutation, and membrane lipid peroxidation (Chaves and Oliveira, 2004). PGPR employment can suppress the oxidative stress level by balancing the level of phytohormones, maximizing the activities of antioxidants and production of osmoprotectants, and correcting ion imbalance in plants grown under water deficit and salinity (Batool et al., 2020). The phytohormones are endogenous substances with a lower molecular weight that efficiently trigger the immune system's response to biotic and abiotic stresses. Due to these abiotic stressors, the plants produce many hormones that include indoleacetic acid (IAA), ethylene, and abscisic acid (ABA), which aid the plant's defense system (Ma et al., 2020). These hormones alter the metabolism, morphology, and other systems of plants. Bacterial hormone production and its ability to activate endogenous hormones are essential for increasing drought tolerance (Singh and Jha, 2017; Jochum et al., 2019). Furthermore, soil bacteria could directly impact plants' hormonal equilibrium by generating exogenous phytohormones. As a result, it is believed that alterations in hormone signaling, mediated by interactions between plants and microbes, are a likely mechanism for causing plants to tolerate drought and soil salinity (Hariprasad et al., 2021).

One of the main auxins, the IAA is physiologically crucial for the growth and development of plants as it is involved in cell division, structure of xylematic vessels, root branching, root elongation, differentiation of vascular tissues, phototropism, gravitropism, and plant tolerance to adverse environmental conditions. The IAA may have positive benefits when present in the optimum concentrations, but too much of this auxin can harm the plants in adverse environmental conditions. Multiple studies have shown that exogenous IAA typically applied to plants decreased drought and salt stress by controlling the photosynthetic rate, the effectiveness of water usage, and Na^+ buildup (Desoky et al., 2020). The higher auxin level may help maintain plant growth, especially roots and leaves, as these are the significant elements that serve in their better resistance under an abiotic stress environment. The IAA production from soil bacteria is widespread and originates from many taxonomic groups (Gowtham et al., 2017).

Some PGPR produces IAA by converting L-tryptophan using several biosynthetic pathways (Ahmad et al., 2020). These pathways involve intermediates such as indole-3-acetamide (IAM), indole-3-acetaldoxime (IAOx), indole-3-pyruvic acid (IPyA), and tryptamine (TAM). The four biosynthetic pathways leading to IAA production from L-tryptophan in bacteria include (i) IAM pathway wherein L-tryptophan is first converted into indole-3-acetamide (IAM) through the action of the enzyme tryptophan decarboxylase which gets transformed into IAA by the action of amidase enzymes; (ii) IAOx pathway in which L-tryptophan is first converted into IAOx through the action of tryptophan-2-monooxygenase and subsequently into IAA by the action of the enzyme indole-3-acetaldoxime hydrolase; (iii) IPyA pathway where L-tryptophan is first converted into IPyA through the action of tryptophan aminotransferase and then converted into IAA by the action of the enzyme indole-3-pyruvate decarboxylase and (iv) TAM pathway that includes conversion of L-tryptophan into TAM primarily through the action of tryptophan decarboxylase and subsequently into IAA by the action of the enzyme tryptamine 5-hydroxylase. The pathways mentioned above may be found in various bacteria that can produce IAA from L-tryptophan to impact plant growth promotion. However, the tryptophan-independent pathway for IAA production has been described in *Azospirillum brasilense,* wherein the bacterium could produce IAA without relying on an exogenous tryptophan supply (Prinsen et al., 1993). In addition, the enzymes that are participatory in the

tryptophan-independent pathway have not been identified yet. It is important to note that not all bacteria can synthesize IAA, irrespective of the pathways identified. Plant roots may uptake IAA synthesized by bacteria, thereby increasing the inherent plant pool. Enhanced IAA generally stimulates plant growth, suppressed when their accumulation is elevated. The PGPR-producing IAA improves root system architecture, increases water permeability into cells, increases leaf uptake, and regulates metabolic homeostasis to mediate abiotic stress tolerance. Moreover, IAA-producing PGPR is found to alleviate the agricultural production losses that result due to drought and salinity stress.

Commonly referred to as a "plant stress hormone" ethylene plays a part in several biological processes in plants, including the ripening of fruits, flowering, seed germination, leaf abscission, and tissue differentiation, and also manages elongation and branching of roots (Shekhawat et al., 2023). However, under biotic and abiotic stressors, a substantial amount of ethylene is produced within the plants, which limits the growth of the root, shoot, and leaf, resulting in plant growth restriction (Brijesh Singh et al., 2019; Murali et al., 2021a). The precursor molecule of ethylene, 1-aminocyclopropane-1-carboxylate (ACC), is produced by involving ACC synthase as the first step of its synthesis, and ACC oxidase then transforms ACC into ethylene. The rhizobacteria can considerably produce ACC deaminase, which converts ACC into α-ketobutyrate and ammonia. By preventing the production of ACC and ethylene, the PGPR impacts the plant's ethylene cycle (Gowtham et al., 2020; Murali et al., 2021b). Thus, the levels of ethylene inside plants did not rise to the levels detrimental to plant growth. The scientific literature showed the effectiveness of PGPR in producing ACC deaminase enzyme, which can significantly promote plant development and enhance plants' ability to tolerate ethylene generated under salt or drought stress by lowering the ACC produced by the plants and maintaining the same at appropriate levels. It is well known that the bacterial-produced ACC deaminase is linked to the ability of many crops to withstand salt and drought stress (Chandra et al., 2019; Danish et al., 2020).

By reducing levels of stress-induced ethylene and minimizing related growth inhibition, the PGPR containing ACC deaminase may be able to lessen the impacts of stress and increase plant growth under these conditions. Similarly, *S. lycopersicum* and *Z. mays* treated with ACC deaminase-producing *B. subtilis* and *Achromobacter xylosoxidans*, respectively, were able to protect the plants from drought-induced oxidative damage by regulating plant ethylene levels (Danish et al., 2020; Gowtham et al., 2020; Sagar et al., 2022b). Moreover, some PGPR are known for their mutual production of IAA and ACC deaminase under adverse environmental conditions by promotion of cell division and root growth (by IAA) and hydrolyzation of excess amount of ACC and ethylene (by ACC deaminase) apart from improving the plant growth. The synergistic effects of bacterial IAA and ACC deaminase will help the plant to withstand adverse environmental conditions. However, the additional PGPR plant-beneficial characteristics, including the production of IAA and osmoprotectant molecules, are closely attributed to bacterial ACC deaminase activity (Sagar et al., 2020).

The ABA is a stress-related phytohormone primarily produced to defend plants against drought and salt stress. Under stress, the ABA can be transported from the roots to the leaves. Numerous PGPR act as plant ABA content modulators and can alter ABA levels in plants, allowing for the regulation of salt and drought stress. According to studies, PGPR treatments raised the levels of ABA in plants. According to Cohen et al. (2009), ABA-producing *Azospirillum lipoferum* strains can still protect *Z. mays* plants from the osmotic damage caused by drought stress. According to Salomon et al. (2014), ABA-producing *Pseudomonas* and *Bacillus* strains operate as stress relievers and aid *Vitis vinifera* plants in dealing with drought stress by promoting ABA production and thereby reducing the rate of plant water loss. In this regard, the evidence generally suggests that the PGPR capable of producing ABA is considerably utilized for abiotic stress management in plants.

The PGPR not only produce phytohormones but also fix nitrogen, sequester iron-chelators (siderophores), and solubilize phosphate for the plants, enhancing their capacity to absorb soil nutrients and reduce the adverse effects of salt stress and drought (Raheem et al., 2018; Gontia-Mishra et al., 2020). The Dissimilatory Nitrate Reduction to Ammonium (DNRA) is a microbial process (anaerobic respiration) that involves the reduction of nitrate (NO_3^-) to ammonium (NH_4^+), and the process occurs in certain bacteria, including some saprophytic bacteria like *Bacillus* and others (Sun et al., 2016; Liu et al., 2021). The saprophytic bacterium takes nitrate (a common form of nitrogen available) from their environment and is enzymatically reduced to nitrite (NO_2^-) inside the bacterial cells. Further reduction of nitrite occurs, leading to the formation of intermediates like nitric oxide (NO) and eventually nitrous oxide (N_2O) is an essential step in the DNRA process. The nitric oxide (NO) and/ or nitrous oxide (N_2O) are further enzymatically reduced to ammonium (NH_4^+) which can be used as a nitrogen source for the growth and metabolism of the bacteria (Sun et al., 2016). Overall, the DNRA process helps certain saprophytic bacteria to obtain energy by using nitrate as a terminal electron acceptor under anaerobic conditions and plays a significant role in the cycling of nitrogen compounds and affects the availability of nitrogen to the host plant. It is important to note that not all bacteria can perform DNRA, as it depends on their specific metabolic pathways and the presence of relevant enzymes. The DNRA activities are prevalent in many bacteria, such as *Shewanella loihica*, *S. oneidensis,* and *S. putrefaciens,* which harbor competing dissimilatory nitrate reduction pathway with the periplasmic nitrate reductase (Nap) genes (*nrfA, nirS,* and *nirK*) required for the first step of nitrate reduction (Nojiri et al., 2020; Liu et al., 2021). The rhizobacterial strains which accomplish the dissimilatory nitrate ammonification exhibit the sequential reduction of nitrate to nitrite and subsequently to ammonium under anaerobic conditions. Therefore, the relative contribution of DNRA activities of rhizobacteria plays an important role in plant growth, nitrogen balance, and even climate change.

The availability of essential nutrients, including nitrogen, is necessary for plant growth and productivity. The nitrogen-fixing bacteria are crucial for biological nitrogen fixation under abiotic stresses (Jabborova et al., 2021). An enzyme nitrogenase complex present in bacteria is responsible for the nitrogen-fixation mechanism. These bacteria have the regulation of nitrogenase genes, which are necessary for nitrogen fixation as well as the synthesis and regulation of enzymes. The halo-tolerant PGPR possessing the potential to absorb the nitrogen will considerably improve the K^+/ Na^+ ratio by inhibiting Na^+ uptake and elevating K^+ and Ca^{2+} in salt-sensitive plants such as *Glycine max* and *Triticum aestivum* (Desoky et al., 2020; Hasanuzzaman et al., 2022). The PGPR controls the exchange of micro- and macro-nutrients, which decreases the buildup of Na^+ and

Cl^- ions. The use of specific bacteria is to enhance nutrient availability and improve the nutrient content of plants, particularly in terms of zinc uptake and accumulation in the rhizosphere. In this context, certain bacteria, especially zinc-solubilizing and zinc-accumulating bacteria, play a crucial role in increasing zinc availability and uptake by plants (Yadav et al., 2022). Iron deficiency is the primary constraint inducing plant chlorosis, eventually impacting crop quality and productivity (Han et al., 2022). Siderophores are the small organic compounds produced by some gramineous plants and microbes in iron-deficient environments, allowing plants to absorb iron from their surroundings even when there is less iron (Saha et al., 2016). Under unfavorable conditions, the siderophores are crucial for phytostabilization, offer metal coalescence, enhance plant development, and lower soil metal bioavailability. The pathogen is depleted of essential iron due to the formation of siderophores that firmly attach to soil Fe^{3+}. By increasing the amount of iron in the environment, PGPR like *Azotobacter vinelandii*, *Bacillus* spp., and *Pseudomonas* sp. use siderophores produced to fulfill their requisite iron requirements in the rhizosphere (Ferreira et al., 2019). It was observed that the siderophores produced by PGPR are gaining more attention due to their function as iron chelators and their advantage over the application of synthetic chelating agents in terms of biodegradability. The usage of siderophores is practically limited in agriculture due to their complex structure and difficulty in production with low yield.

Under abiotic conditions like drought, salt, etc., the plants typically experience a nutritional shortfall due to a lack of phosphorus, which is primarily found in the soil in both organic and inorganic forms (Bechtaoui et al., 2021). Plants' insoluble phosphorus contributes to the phosphorous deficit, yet plants can only take up phosphorous as monobasic and dibasic ions. Plants may benefit from the phosphorus-solubilizing bacteria that can assist them with water shortages and overcome the limited phosphorus availability to plant systems in rhizospheric soil (Kour et al., 2020). Phosphate-solubilizing bacteria like *Serratia, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavbacterium, Microbacterium,* and *Rhizobium* can be employed as biofertilizers. Rhizobacteria can solubilize the inorganic phosphorus that the plants cannot absorb, aiding plant development (Munir et al., 2022). The improvement of agricultural production is achieved by PGPR-mediated phosphate-dependent regulation under abiotic stress conditions, while the phosphorus solubilization might be attributed to the synthesis of organic acids by the PGPR at the rhizosphere (Kour et al., 2019).

The overproduction of ROS typically brought on by drought and salt stress damages normal cell metabolism by causing oxidative damage to DNA, lipids, and membrane proteins (Hasanuzzaman et al., 2022). Malondialdehyde (MDA), a lipid peroxidation marker that also serves as an oxidative stress marker, is frequently referred to as MDA. According to earlier research, beneficial bacteria can help plants develop under drought stress by lowering MDA levels, avoiding ROS buildup, and stimulating antioxidant enzyme activities (Abdelaal et al., 2021; Murali et al., 2021b). Plants use the principal enzymatic ROS scavenging system to reduce high ROS levels to protect themselves from oxidative stress. Salt-stressed *Glycine max* plants have higher levels of antioxidant enzymes (such as GSH and SOD) after being inoculated with halotolerant rhizobacterial strains (Khan A. et al., 2019; Hasanuzzaman et al., 2022). Soil bacteria activate the antioxidant system to improve cell membrane stability, increasing drought resistance. The PGPR regulates the antioxidant enzyme activity to prevent oxidative damage due to drought stress. The expression of antioxidant genes was increased after PGPR application, which enhanced the activities of antioxidant enzymes. Superoxide was reduced by the increased enzyme activity, which also shielded the chloroplast from ROS impact. According to Zhang et al. (2020), ACC-deaminase-producing bacterial-treated *Ziziphus jujuba* plants significantly reduced the MDA level by enhancing the activity of antioxidant enzymes (POD and SOD) compared to non-inoculated plants with increased water stress. ACC deaminase-producing *B. subtilis*-treated plants can boost APX and SOD activity by lowering MDA and H_2O_2 contents compared to control plants cultivated in extreme drought conditions (Gowtham et al., 2020). Accordingly, it can be deduced from the literature that PGPR treatment boosted enzyme activities and decreased the MDA level under water stress by increasing the plant's capacity for scavenging and controlling the expression of antioxidant genes.

Gene expression research can be used to compare and comprehend how an organism responds to its surroundings. Recent studies using molecular methods have examined how genes are expressed under drought stress in relation to PGPR-induced tolerance (Ghosh et al., 2019; Gowtham et al., 2022). Each PGPR has a unique gene set that enables it to respond in various protective ways to the damaging impacts of abiotic stressors like salinity and drought. A number of PGPR can change a plant's gene expression, increasing the output of stress-protective substances such ROS detoxifying enzymes and osmolytes. IAA secretion (*iaaM*), nitrogen fixation (*nifU*), phenazine (*phzCEF*), siderophore (*sbnA*), and spermidine (*speB*) production are among the functional genes found in the PGPR that have been linked to plant growth promotion as well as stress tolerance (Xiong et al., 2019). Khan A. et al. (2019) have shown that the expression of soybean salt tolerance 1 (*GmST1*) and IAA-mediating (*GmLAX3*) genes was found to be upregulated upon the inoculation with the halo tolerant rhizobacterial strains in salt-stressed *Glycine max* plants. The PGPR inoculation can also cause the up-regulation of proteins associated with phosphatase activity related to phosphate solubilization.

4.4. Regulation of ion homeostasis

When the influx of ions exceeds the exclusion rate, salinity accumulates hazardous Na + and Cl − concentrations within leaves. Initially, the plants compartmentalize the excess salts in vacuoles to prevent their buildup in the cytosol and intracellular spaces, impeding respiration and photosynthesis. The ability of the soil bacteria to sustain ion homeostasis must be advantageous for plant development and salinity tolerance. By ensuring a high K^+/Na^+ ratio, the PGPR can control the homeostasis of hazardous ions. It reduces the buildup of Na^+ and Cl^-in leaves, boosts ion exclusion from root cells, activates the development of ion transporters, and controls the exchange of micro- and macro-nutrients (Kusale et al., 2021b). The plasma membrane-bound proteins known as high-affinity K^+ transporters (HKTs) mediate Na^+ transportation in plants and prevent excessive Na^+ ion concentrations from building up in the shoots by preventing them from reaching the roots. The inoculation of rhizobacteria

influences the expression of several ion affinity transporters that help maintain cellular ion homeostasis in salt-stressed plants, which requires tissue-specific regulation of the HKT genes during plant-microbe interactions. It has been reported that rhizobacterial inoculation controls the expression of various ion affinity transporters. The tissue-specific regulation of HKT genes is essential in plant-microbe interactions for maintaining cellular ion homeostasis in salt-stressed plants. Salt overload sensitive (SOS) genes and other enzymes that play a role as sodium antiporters can help plants adapt to salt stress. By decreasing the concentration of the ion Na^+ and increasing the absorption of K^+ion in salt-stressed *Glycine max* plants, the introduction of halotolerant rhizobacterial strains preserved the osmotic equilibrium (Khan M. A. et al., 2019). Microorganisms produced during EPS synthesis protect plants from harmful ion absorption. They act as a physical barrier that guards against ion toxicity and safeguards the root system. The EPS can attach to cations like Na^+, making it impossible for plants to absorb in saline surroundings.

5. Cry for help

Filed research in plant-microbe interactions has shown that the composition and diversity of the microbial community in the rhizosphere play a crucial role in the plant's ability to withstand stress. Indeed, the "Cry for Help" concept is a significant aspect of microbial recruitment in the plants' rhizosphere under abiotic and biotic stresses. During their lifecycle, plants encounter various stress, such as drought, salinity, pathogen attack, or nutrient deficiency, wherein plants release specific chemical signals, known as VOCs, root exudates, or other chemical signals, into the rhizosphere. These exudates serve as a salt overly sensitive signal to attract beneficial microbes that can aid the plant in mitigating the stress and improving overall growth and health. The process of attraction through these chemical signals is called microbial recruitment. Several functions are attributed to the recruited microbes in the rhizosphere, including enhanced nutrient acquisition, pathogen suppression, plant growth promotion, and abiotic stress tolerance. Several key points are essential in understanding the concept of Cry for Help in microbial recruitment in the rhizosphere, like chemical signaling, microbial diversity, mutualistic relationships, enhanced stress tolerance, and induced systemic resistance (ISR). The study of Cry for Help and microbial recruitment in the rhizosphere is essential for understanding the complex interactions between plants and microbes. It has implications for sustainable agriculture and environmental management. By harnessing the power of beneficial microbes, it may be possible to develop strategies to enhance plant resilience to various stresses and reduce the reliance on chemical inputs in agriculture.

6. Key challenges and multi-omics approach

Several key challenges need to be addressed before widespread adoption and successful commercialization can be achieved through PGPR by ensuring consistent and reliable results across different crops and environments. Successful integration of PGPR into existing conventional agricultural practices is essential as it is prone to specific challenges, which include (i) the maintenance of viability and activity of the bacteria during storage to achieve desired results in the field; (ii) compatibility with other agricultural inputs, such as fertilizers and pesticides to avoid any negative interactions; (iii) obtaining regulatory approval for the commercial use of PGPR products can be time-consuming and costly as they need to ensure the efficacy of these products for environmental safety, non-toxic to humans and animals and deliver the claimed benefits (Table 3). Raising awareness for the farming community, providing training, and offering technical support are essential for successfully adopting these products. To be widely adopted, these products must demonstrate clear economic benefits that outweigh their costs. While PGPR can be environmentally friendly compared to certain chemical fertilizers and pesticides, it is crucial to assess the long-term effects of PGPR application on soil health and microbial communities. Scaling up the production of PGPR products to meet the demand of commercial agriculture can be challenging. Establishing an efficient distribution network to reach farmers globally is crucial for successful commercialization. Therefore, addressing these challenges will require continued research, collaboration between researchers and industry, and efforts to create awareness and understanding among farmers and stakeholders.

Enhancing the application of PGPR under stressful conditions is a promising avenue for sustainable agriculture, and tailoring PGPR formulations and applications to specific stress types requires a deeper understanding of stress-specific mechanisms. Transcriptomics, proteomics, and metabolomics can be employed to analyze the plant-PGPR interaction under particular stress conditions. Integrating these omics data can provide insights into the stress-responsive genes, proteins, and metabolites involved, thereby facilitating the development of stress-tailored PGPR products. Not all PGPR strains exhibit the same level of stress tolerance, limiting their effectiveness under harsh conditions, and the application of genomics and metagenomics can help identify stress-tolerant PGPR strains from diverse microbial communities in the rhizosphere. In addition, metatranscriptomics and metaproteomics can assess changes in the gene expression and protein profiles of PGPR under stress. The above knowledge can guide the development of stress-adaptive PGPR formulations that maintain activity and viability during adverse conditions. The signaling pathways between PGPR and plants are complex and can be altered under stress, affecting communication and beneficial outcomes.

Similarly, phosphoproteomics and epigenomics can help unravel the changes in signaling pathways between PGPR and plants under stress. Understanding these modifications can enable the fine-tuning of PGPR formulations to enhance crop stress tolerance and growth promotion. Further, stress conditions will alter the soil microbial community, potentially influencing the interaction between PGPR and other microorganisms, and these changes can be assessed through metagenomics and 16S rRNA sequencing. Translating lab-scale findings to field conditions can be challenging, and monitoring PGPR performance in large-scale agricultural practice is essential for their practical applications. Besides, remote sensing technologies, combined with transcriptomics and metabolomics analyses of plant samples from various field sites, can enable real-time monitoring of

TABLE 3 Multi-omics approaches associated in combating abiotic stress in plants upon application of PGPR.

PGPR strain	Multi-omics approach	Advancements and findings	References
Pseudomonas spp.	Genomics	Identification of stress-responsive genes in *P. fluorescens* for drought and salt tolerance	Cho et al. (2015); Saakre et al. (2017)
	Transcriptomics	Characterization of plant gene expression changes in response to the PGPR under drought and salt stress	Mellidou et al. (2021); Nishu et al. (2022)
	Metabolomics	Profiling of metabolites involved in salt stress mitigation by the PGPR	Mellidou et al. (2021)
	Functional validation	CRISPR-Cas9 knockout of candidate genes to validate the role of PGPR in salt stress tolerance	Chauhan et al. (2022)
Bacillus spp.	Proteomics	Identification of salt stress-related proteins produced by *Bacillus* sp.	Zhao et al. (2022)
	Transcriptomics	Examination of plant gene expression changes in response to the PGPR under salt stress	Akbar et al. (2022)
	Metagenomics	Analysis of the rhizosphere microbiome and its interaction with *B. subtilis* for drought tolerance	No et al. (2022)
	Metabolomics	Elucidation of metabolic pathways influenced by *Bacillus* sp. in salt stress condition	Zhao et al. (2022)
Azospirillum spp.	Epigenomics	Study of DNA methylation patterns in the presence of *A. brasilense* in response to abiotic stresses	Lephatsi et al. (2021)
	Metagenomics	Studying metagenomics to explain plant growth promoting mechanisms of *A. lipoferum* in drought stress	No et al. (2022)
	Comparative genomics	Comparison of *A. brasilense* genomes to identify stress-related genes	Wiggins et al. (2022)
	Integrative analysis	Systems biology modeling of *A. brasilense*-plant interactions under salt stress	Zuluaga et al. (2022)
Enterobacter spp.	Proteomics	Investigation of differential expressed proteins in inducing plant tolerance to salt stress upon *E. cloacae* inoculation	Singh et al. (2017)
	Comparative genomics	Studying the alleviation of salt stress by *Enterobacter* sp.	Kim et al. (2014)
Rhizobium spp.	Transcriptomics	Differential drought stress-related gene expression in plants due to inoculation of *R. leguminosarum*	Jiménez-Guerrero et al. (2018); Barquero et al. (2022)
	Functional validation	RNAi-mediated silencing of specific genes to assess the role of *R. leguminosarum* in salt stress resistance	Dong et al. (2013)
Arthrobacter spp.	Metabolomics	Identification of metabolites produced by *Arthrobacter* under salt stress condition	Khan et al. (2021a,b)
	Metagenomics	Studying metagenomics to explain plant growth promoting mechanisms of *A. chlorophenolicus* in drought stress	No et al. (2022)
	Comparative genomics	Comparative analysis of *Arthrobacter* sp. genomes to find stress-related genes under drought stress	Chhetri et al. (2022)
	Transcriptomics	Studying to understand how *Arthrobacter* sp. adapts its metabolism in response to PEG-induced drought stress	Gabriel et al. (2022)

PGPR-mediated responses under diverse stressful conditions. This integrated approach can help optimize PGPR application strategies for different crops and environments. By addressing these key challenges through multi-omics approaches, we can enhance the commercial application of PGPR under stressful conditions and unlock their full potential in sustainable agriculture.

7. Conclusion

In an era marked by climate unpredictability and the ever-increasing demand for agricultural productivity, harnessing the power of beneficial microbes like PGPR is emerging as a pivotal approach to enhance plant stress resilience. The review highlights the salient strategies and recent advancements in manipulating PGPR to combat the detrimental effects of stress on crop plants. Recent research underscores the role of PGPR in modulating root architecture that enable plants to explore a larger soil volume, deeper resources and establish a more efficient nutrient and water absorption system. The synergy between PGPR and plants is increasingly recognized as a powerful mechanism for stress mitigation wherein the partnership enhances nutrient uptake and bolsters the plant's ability to withstand adverse conditions. Emerging technologies like the multi-omics approach and synthetic biology hold promise for tailoring PGPR strains and optimizing their performance in addition to integrating PGPR into

precision agriculture systems, leading to more targeted and efficient stress management. In conclusion, the symbiotic relationship between PGPR and plants offers an exciting avenue for agricultural sustainability in the face of mounting environmental challenges. By strategically selecting and applying PGPR strains alongside complementary stress management practices, we can empower crops to thrive in stressed environments. These strategies and innovations might be explored in plant-microbe interactions as we move closer to a future where resilient crops stand as a safeguard against the uncertainties of climate change and global food security.

Author contributions

MM and RS: conceptualization, supervision, and writing—original draft. AO, MR, and AA-T: data curation, formal analysis, writing—review and editing, and fund acquisition. 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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