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Microbial interaction with banana: Mechanisms, symbiosis, and integrated diseases control

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Microbial interaction with banana: Mechanisms, symbiosis, and integrated diseases control

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Editorial: Microbial interaction with banana: mechanisms, symbiosis, and integrated diseases control

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Editorial on the Research Topic
Microbial interaction with banana: mechanisms, symbiosis, and integrated diseases control

1 Introduction

Bananas serve as important fruits globally and as the main food source in developing countries. Because of their vegetative propagation, they are vulnerable and susceptible to disease. Therefore, new integrated protection technologies are urgently needed for sustainable production. The highly destructive soil-borne fungal pathogen that triggers Fusarium wilt of bananas (FWB) is *Fusarium oxysporum* f. sp. *cubense* (*Foc*), especially Tropical Race 4 (TR4). Effective detection and validation are important before implementing adequate management (Yang et al., 2023). Comprehensive approaches to the prevention and management of existing and emerging diseases are critical for maintaining a healthy banana industry (Xu et al., 2011).

Currently, climate change has dramatically resulted in yield loss caused by shifting to pests and diseases (Bebber, 2019; Varma and Bebber, 2019; Alkhalifah et al., 2023; Kilwenge et al., 2023) and emerging diseases, such as banana blood disease (Remenant et al., 2011; Ray et al., 2021; Prakoso et al., 2022; Rincón-Flórez et al., 2022). Biocontrol agents play important roles in addressing these challenges.

2 Content of Research Topic

This topic has arisen from increasing awareness that microbes play an important role in mitigating soil-borne diseases. Original research articles on six aspects were collected: cover plants in agro-systems, microbiome diversity, microbiome isolation and evaluation, microbiome genome annotation, novel mycoviruses in *Foc*, and microbiome product applications.

Cover plants have received considerable attention because of their advantages in increasing the diversity of agro-systems linked to production (Damour et al., 2015). Therefore, the application of cover plants together with resistant cultivars is the best method (Zheng, 2022). In this regard, Wang et al. found that a legume cover plant, Siratro, and natural weeds could change soil fungal diversity in several spans of banana production. They found that fungal diversity significantly decreased after planting bananas. However, both Shannon and Simpson diversity significantly increased after natural weed or legume cover compared to bare soil (Wang et al.). Therefore, the application of suitable cover plants can lead to sustainable banana production by manipulating soil microbial communities.

Soil microbial communities in banana plantations correspond to different ecological conditions. Birt et al. confirmed the core fungal microbiomes of bananas and plantains. There are differences in the diversity and composition of core fungal microbiomes between plant compartments and soils, irrespective of the host genotype (Birt et al.).

Banana-associated effective bacterial and fungal disease suppression is usually achieved through direct isolation from banana plantations. Fan et al. found that *B. velezensis* YN1910 had a significant control effect on FWB. Xiang et al. showed that another *B. velezensis* strain, EB1, also possesses significant antagonistic capability against *Foc* TR4. Yun et al. identified 647 metabolites from the extracts of *S. hygroscopicus* subsp. hygroscopicus 5-4 and found that hygromycin B inhibited the growth rate of *Foc* TR4 mycelia. Different beneficial microbes have specific biological functions. Further investigation into more beneficial microbes adapting to local banana plantations is warranted. Enhancing the biological activities of existing microbes through mutation via traditional physical mutagenesis such as UV and novel physical mutagenesis techniques such as ion beam, intense pulsed light and space mutagenesis could be another direction.

The full genome sequences of biocontrol agents will facilitate the exploitation of specific genes responsible for secondary metabolite production during the development of potential biocontrol agents. In this study, Cuellar-Gaviria et al. reported the complete genome annotation of *B. tequilensis* EA-CB0015. This strain has been shown to efficiently control the banana foliar disease black sigatoka (Cuellar-Gaviria et al.). In the future, more biocontrol agents will be sequenced, and detailed genome annotations will be available. Thus, the whole picture of the genome will shed light on the functional genes involved in the production of effective metabolites and their potential capability.

Mycoviruses from plant pathogens, which induce hypovirulence in host fungi, have received considerable attention as potential biocontrol agents (García-Pedrajas et al., 2019). To explore the presence of mycoviruses in *Foc*, Ye et al. discovered the diversity of *Foc* mycoviruses. They first demonstrated the presence of mycoviruses in *Foc* and discussed the possibility of using mycoviruses for the biocontrol of FWB (Ye et al.). Therefore, the exploration of mycoviruses could be another option for the biocontrol of FWB.

The effective translation of research findings from the laboratory to field applications is essential to confirm the success of biocontrol agents. Tian et al. found that the application of *B. amyloliquefaciens* QST713 significantly affected the diversity of bacteria and fungi in the resistant cultivar Yunjiao No.1 but not in the susceptible cultivar Brazilian. Du et al. designed a compound microbial agent that achieved the most durable control effect in the *Foc* TR4 heavily infected field. The disease control effect reached 57.14% against FWB after the application of the four-strain combination (Du et al.). Damodaran et al. developed an interesting bioimmune system and showed significantly higher weights of bunches, hand numbers per bunch, and finger numbers per hand in treated plants compared to control. Therefore, manipulating banana holobionts by inserting novel endophytes into plantlets to create a new type of "banana hybrid" with increased resilience could be a future direction for enhancing biocontrol agents.

3 Conclusion

In the last century, the transition from Gros Michel to Cavendish stands out as the most successful example of employing resistant cultivars for FWB management as a singular solution. However, breeding a new resistant cultivar or transitioning cultivars to meet market demands is not always straightforward. To date, numerous beneficial microbes have been isolated, identified, and evaluated in greenhouse studies; however, their effective application in the field remains limited. Hence, enhancing the colonizing efficiency of the microbe in its host for enduring antagonism against pathogens and unraveling its intricate interaction mechanisms will be imperative for further research. Understanding host-pathogen-endophyte biocontrol interactions is crucial for maintaining sustainable and healthy development in the banana industry, even with *Foc* TR4 present in the long term.

Author contributions

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Methodology, Investigation, Conceptualization. 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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Construction of a compound microbial agent for biocontrol against Fusarium wilt of banana

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Banana wilt caused by *Fusarium oxysporum* f. sp. *cubense* has devastated a large number of banana plantations worldwide. Biological control is a possible method to conquer this disease. However, the control effect was often low and unstable while a single biocontrol strain had been applied in the field. Therefore, this study aimed to construct an effective compound microbial agent to control Fusarium wilt of banana (FWB) in the field. In addition to it, the compounding strategy of combining single strains for improving the control effect was investigated. Based on the compatibility test, five representative biocontrol strains were selected for the combination of all possible permutations. The pot experiment indicated that every biocontrol strain and their 26 combinations could control FWB to varying degrees. The control effect of combinations on FWB was higher than that of a single strain. In terms of the number of combinatorial biocontrol strains, the control effect of the four-strain combinations was the highest. According to the taxonomic differences of the five biocontrol strains, 26 biocontrol strain combinations could be divided into four groups. Among the strains in the combination, the larger the taxonomic differences the more easily it was to obtain a higher control effect. To obtain stable and efficient combinations, eight combinations were selected out and evaluated for their effectiveness in controlling FWB in different type soil. Compared with the other seven combinations, the four-strain combination T28 (Pt05 + Bc11 + Ba62 + gz-2) got the highest and stablest control effect in the four types of soil in greenhouse. And then the control effect of combination T28 was evaluated in field conditions, compared with commercially agents *Bacillus subtilis*, *Trichoderma harzianum*, and carbendazim. After four consecutive applications in the field, the control effect of T28 against FWB was the highest, reaching 57.14%. The results showed that combination T28 had a good application prospect, and the finding provided a reference for the construction of compound microbial agents.

KEYWORDS

Fusarium oxysporum* f. sp. *cubense*, combination agents, *Bacillus* sp., *Paenibacillus terrae*, *Trichoderma harzianum

1 Introduction

Banana (*Musa* spp.) is one of the most important fruit and food crops in tropical and subtropical regions worldwide (Dale et al., 2017). In 2018, the global banana planting area was about 5.73 million hectares, with an annual output of more than 115 million tons (FAO, 2020). Fusarium wilt of banana (FWB) caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) has led to huge economic losses and seriously threatened the healthy development and safety of the global banana industry (Ploetz, 2006; Lin et al., 2008; Butler, 2013; Ghag et al., 2015). For a soil-borne disease, the control of FWB mainly depended on non-host crop rotation (Huang et al., 2012), chemical fungicide control (Nel et al., 2007), and breeding of disease-resistant varieties (Wang et al., 2022). However, FWB is particularly difficult to control. The reasons are as follows: (1) *Foc* can survive in the soil for more than 20 years, even in the absence of plant hosts (Stover, 1962; Buddenhagen, 2009); (2) chemical fungicides have difficulty killing *Foc* after soil application (Ploetz, 2015; Pegg et al., 2019); and (3) no commercial varieties are available with high *Foc* resistance and good agronomic traits (Bubici et al., 2019). Thus, it is urgent to explore new methods to control this disease (Ploetz, 2015; Pegg et al., 2019). Biocontrol microbes have been successfully applied in controlling soil-borne diseases, including peanut root rot (Sharma et al., 2012), Fusarium root rot in wheat (Wang et al., 2015), cumin wilt (Kumar et al., 2016), cucumber Fusarium wilt (Luo et al., 2019), and pepper root rot (Zhang et al., 2021). That provides new opportunities for FWB control.

Numerous biocontrol microbes, including *Pseudomonas* sp., *Xanthomonas* sp., *Bacillus* sp., *Burkholderia* sp., *Streptomyces* sp., *Trichoderma* sp., and *Rhizobium* sp., have been screened to prevent and control FWB (Raza et al., 2017; Duan et al., 2020; Wang et al., 2022). Although many biocontrol microbes were effective against *Foc in vitro* or in the greenhouse, their control efficiencies were often lower and unstable in the field, due to the single strain cannot cope with the variable environmental factors, such as soil pH, osmotic pressure, organic matter, and salt concentration (Denoth et al., 2002).

Compound microbial agents, which are composed of multiple biocontrol strains, have been shown to improve their control effect and stability against soil-borne diseases. For example, a compound agent composed of two arbuscular mycorrhizal fungi, *Trichoderma harzianum*, and *Pseudomonas fluorescens* was significantly effective than that of a single strain against tomato Fusarium wilt in the field (Srivastava et al., 2010). In another study, a combination of eight strains of *P. fluorescens* showed a higher control effect than a single strain in controlling tomato bacterial wilt caused by *Ralstonia solanacearum* (Hu et al., 2016). A combination consisting of seven bacteria strains had been used to control maize seedling blight caused by *Fusarium verticillioides*. The result indicated that the inhibited effects of each strain were not as strong as those of the combination (Niu et al., 2017). As we have seen, compound microbial agent may improve disease control effect compared to a single strain (Pandey and Maheshwari, 2007). However, the construction of compound microbial agents was often blind and random. There was a lack of systematic research on the compound strategy of biocontrol strains for improving control effect.

For the reasons above, this research aimed to: (1) construct an effective microbial agent to control FWB and (2) to investigate the combination strategy of synergy in biocontrol strains. Based on the compatibility test, representative strains were selected for the combination of all possible permutations, and their control effects on FWB were determined using a pot experiment. Moreover, to obtain combinations of biocontrol stains with stable and efficient control effects, the compound microbial agents were evaluated for their effectiveness in controlling FWB in various banana plantation soil. The control effect of the optimal combination was determined under field conditions. The results provide a reference for the effective prevention and control of FWB and the construction method of compound microbial agents.

2 Materials and methods

2.1 Fungal and bacterial strains

Fusarium oxysporum f. sp. *cubense* tropical race 4 (TR4, strain Foc1402) was isolated from banana plants infected with FWB from Wuming town, Nanning city, Guangxi province of China. Nine biocontrol strains were isolated from rhizosphere soil from bananas grown in Tanluo town, Nanning city, Guangxi province of China, including *Trichoderma harzianum* (strain gz-2), *Burkholderia cepacia* (strain Bc11), *Paenibacillus terrae* (strain Pt05), *Bacillus velezensis* (strain Blz02), *Bacillus amyloliquefaciens* (strain Ba02, Ba62, Ba63, Ba48, and Ba310). All fungi and bacteria were cultured on potato dextrose agar (PDA) and nutrient agar (NA), respectively, and maintained at the Plant Protection Research Institute, Guangxi Academy of Agricultural Sciences.

2.2 Biological agents and fungicides

Bacillus subtilis wettable powder (active ingredient content of 1×10^9 bacteria cells$^{\bullet}g^{-1}$) was purchased from the Redsun Group Co., Ltd. (Nanjing, China). *Trichoderma harzianum* water-soluble powder (active ingredient content of 1×10^9 spores$^{\bullet}g^{-1}$) was purchased from Moon (Guangzhou) Biotech Co., Ltd. (Guangzhou, China). Carbendazim wettable powder (active ingredient content of 50%) was purchased from Shanghai Yuelian Chemical Co., Ltd. (Shanghai, China).

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TABLE 1 Biocontrol strains and their combinations used to determine their biocontrol efficacy on Fusarium wilt of banana.

Number of strains	Treatments	Combination	Number of strains	Treatments	Combination
/	Control	N/A	Three	T16	Pt05 + Bc11 + Blz02
Single	T1	Pt05 alone		T17	Pt05 + Bc11 + Ba62
	T2	Bc11 alone		T18	Pt05 + Bc11 + gz-2
	T3	Blz02 alone		T19	Pt05 + Blz02 + Ba62
	T4	Ba62 alone		T20	Pt05 + Blz02 + gz-2
	T5	gz-2 alone		T21	Pt05 + Ba62 + gz-2
Two	T6	Pt05 + Bc11		T22	Bc11 + Blz02 + Ba62
	T7	Pt05 + Blz02		T23	Bc11 + Blz02 + gz-2
	T8	Pt05 + Ba62		T24	Bc11 + Ba62 + gz-2
	T9	Pt05 + gz-2		T25	Blz02 + Ba62 + gz-2
	T10	Bc11 + Blz02	Four	T26	Pt05 + Bc11 + Blz02 + Ba62
	T11	Bc11 + Ba62		T27	Pt05 + Bc11 + Blz02 + gz-2
	T12	Bc11 + gz-2		T28	Pt05 + Bc11 + Ba62 + gz-2
	T13	Blz02 + Ba62		T29	Pt05 + Blz02 + Ba62 + gz-2
	T14	Blz02 + gz-2		T30	Bc11 + Blz02 + Ba62 + gz-2
	T15	Ba62 + gz-2	Five	T31	Pt05 + Bc11 + Blz02 + Ba62 + gz-2

2.3 Preparation of inocula

To prepare the *Foc* inoculum, fresh mycelia of strain Foc1402 was harvested from 7-day-old cultures grown on PDA medium, suspended in potato dextrose broth (PDB), and incubated at 150 $r^{\bullet}min^{-1}$ and 28°C for 3 days. The spore suspension was then filtered to separate the mycelia and adjusted to a concentration of 1×10^6 spores$^{\bullet}mL^{-1}$ with sterile water.

To prepare a spore suspension of *T. harzianum* gz-2, fresh culture mycelia was scratched and suspended in PDB medium, then incubated at 150 $r^{\bullet}min^{-1}$ and 28°C for 5 days. Spores of strain gz-2 were collected after filtering out the mycelium and adjusted to a final concentration of 1×10^8 spores$^{\bullet}mL^{-1}$ with sterile water.

Bacterial inocula were prepared as previously described (Zaim et al., 2013). The individual bacteria culture was inoculated in a nutrient broth (NB) medium, incubated at 150 $r^{\bullet}min^{-1}$ and 28°C for 48 h. The suspension of each bacterium was adjusted with sterile water to a final concentration of 1×10^8 bacteria cells$^{\bullet}mL^{-1}$.

2.4 Compatibility test between bacterial strains and *Trichoderma harzianum*

To construct compound microbial agents, compatibility tests were conducted among nine biocontrol strains before they were combined, which were performed as described by Rajeela et al. (2018) and Mulaw et al. (2020), with a few modifications. In a sterile Petri dish (9 cm in diameter), three sterilized Oxford cups were vertically placed on the surface of 2% water agar (WA) medium. Each Oxford cup was arranged 2.5 cm apart from the other. One of the biocontrol strains was selected as the indicator strain. NA medium was inoculated with 1% (v/v) of the indicator strain suspension after cooling to 45°C. The mixture was rapidly mixed and immediately poured evenly onto WA plates containing Oxford cups. After the upper plate was completely solidified, the Oxford cups were removed with sterilized tweezers to form some small wells with a diameter of 6 mm. As the challenge strains, the remaining biocontrol strains were added to 20 μL of the previously prepared suspensions (1×10^8 bacteria cells$^{\bullet}mL^{-1}$ or 1×10^8 spores$^{\bullet}mL^{-1}$) in each well and incubated at 28°C for 48 h. For the blank control, the same amount of sterile water was added, and each treatment was repeated in triplicate. If a transparent inhibitory zone was observed around challenge strains, it was considered antagonism (+); otherwise, it was considered compatibility (–).

2.5 Control effect of biocontrol strains and their combinations on Fusarium wilt of banana

Banana seedlings (*Musa acuminata* L., AAA group, Cavendish subgroup, cv. "Williams") susceptible to *Foc* TR4, which were purchased from Nanning Xiangjie Agricultural Technology Co., Ltd., in Guangxi, China, were used to determine the control effect of biocontrol strains and their

combinations on FWB under greenhouse conditions. This study was performed as described by Saravanan et al. (2003) with a few modifications and was conducted from 24 June to 26 July 2020 in the greenhouse of the Plant Protection Institute of Guangxi Academy of Agricultural Sciences. Based on the results of the compatibility test, five compatible biocontrol strains were selected as representative strains, and combinations of all possible permutations were designed. Inocula of biocontrol strain combinations were prepared as follows: each of the biocontrol strains was cultured individually referring to section "2.3 Preparation of inocula" and mixed in an equal volume according to the combination design at a final concentration of 1×10^8 CFU•mL^{-1}. Two-month-old banana seedlings were transplanted to plastic pots (20 cm diameter, 22 cm height) containing sterilized vermiculite. One seedling was transplanted to each pot and watered every second day. Ten days after transplanting, each seedling was inoculated with 100 mL of biocontrol strain inoculum. Twenty-four hours later, the seedlings were inoculated with 50 mL of *Foc* suspension (concentration 1×10^6 spores•mL^{-1}) after wounding. These seedlings continued to grow for 6 days and were again inoculated with the same volume of biocontrol strain inoculum. The biocontrol strains were applied twice during the experiment. As a control, 100 mL of sterilized NB medium was used instead of suspensions of biocontrol strains, and a total of 32 treatments were designed (**Table 1**). All treatments were repeated three times, and nine plants were used for each treatment. After being inoculated twice with biocontrol strains for 15 days, the corms of the banana seedlings were cut to detect the infection degree of *Foc*. The disease index was carried out based on the extent of corm discoloration on 0–7 grade: Grade 0 = No symptoms; Grade 1 = 1–25% initial corm discoloration; Grade 3 = 26–50% slight discoloration of the corm; Grade 5 = 51–75% discoloration of the corm; Grade 7 = over 76% complete discoloration of the corm (Orjeda, 1998). The disease index and control effect were calculated using the following formulas:

$$\text{Disease index} = \frac{\Sigma(\text{Number of diseased plants in each grade} \times \text{value of relative grade})}{\text{total number of plants observed} \times \text{maximum disease grade}} \times 100$$

$$\text{Control effect (\%)} = \frac{\text{Disease index of control treatment} - \text{disease index of biocontrol agent treatment}}{\text{disease index of control treatment}} \times 100\%$$

2.6 Stability of the control effect of biocontrol strain combinations

To obtain combinations of biocontrol stains with stable and efficient control effects, eight combinations with high biocontrol efficiency on FWB were selected to determine their control effect in different types of banana plantation soil. The experiment was conducted from 21 September to 23 October 2020 in the greenhouse of the Plant Protection Institute of Guangxi Academy of Agricultural Sciences. Soil was sampled from a 0–30 cm tillage layer in banana plantations of different regions in Guangxi Province: Type I (Luoxu town, Nanning city, N23°11′52″, E108°01′17″), Type II (Tanluo town, Nanning city, N22°57′17″, E107°54′22″), Type III (Natong town, Nanning city, N23°02′56″, E107°54′30″), and Type IV (Linfeng town, Baise city, N23°35′18″, E107°12′4″). The physicochemical properties of the soil are shown in **Table 2**. The soil sample was air-dried and sieved through a 2 mm sieve, then filled into fresh plastic pots (20 cm diameter, 22 cm height). Each type of soil sample was treated as follows: T9, T11, T14, T20, T25, T27, T28, and T29. As a control, the same volume of sterilized NB medium was used instead of biocontrol strain suspensions. All treatments were repeated three times, and nine plants were used for each treatment. This assay was conducted by referring to the method in section "2.5 Control effect of biocontrol strains and their combinations on Fusarium wilt of banana."

2.7 Field trial

The field trial was carried out on a banana plantation in Tanluo town, Nanning city, Guangxi province, China (N22°57′17″, E107°54′22″) from 27 May to 30 October 2021. The study site suffered severe serious FWB in the last year, with an incidence rate of 20–30%, and had been under continuous banana cropping for 5 years since 2016. The soil was clay loam in texture, with a pH of 4.2. The plot size was 0.24 hectares by 17 rows, and the plant spacing was 2.5 m between rows and 2 m within rows. There were 30 plants in each row. Banana plantlet (*Musa acuminata* L., AAA group, Cavendish subgroup, cv. Williams) was the perennial banana with 9–12 true leaves, and it was in the vegetative growth period during the treatments of biocontrol strain inoculum. There were four treatments as follows: (1) a biocontrol strain combination of T28 at a concentration of 1×10^9 CFU•mL^{-1}, applied at a rate of 62.5 L•ha^{-1}. (2) *B. subtilis* wettable powder at a concentration of 1×10^9 bacteria cells•g^{-1} (BD), applied at a rate of 62.5 Kg•ha^{-1}; (3) *T. harzianum* water-soluble powder at a concentration of 1×10^9 spores•g^{-1} (TD), applied at a rate of 62.5 Kg•ha^{-1}; and (4) carbendazim wettable powder at a concentration of 50% (FD), applied at a rate of 420 g•ha^{-1}. The same volume of water was used as the control treatment.

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TABLE 2 Physicochemical properties of soil samples from different banana plantations.

Soil type	Available *P* (mg/kg)	Available K (mg/kg)	Organic matter (g/kg)	pH	Effective boron (mg/kg)	Exchangeable Ca (cmol/kg)	Exchangeable Mg (cmol/kg)	Hydrolysable N (mg/kg)
Type I	34.6	901	23.4	4.1	0.42	2.2	0.4	817
Type II	45.0	167	29.3	4.2	0.29	0.8	0.1	151
Type III	49.9	434	28.2	4.5	0.52	4.0	0.4	136
Type IV	19.4	495	24.5	6.9	0.60	14.9	1.9	125

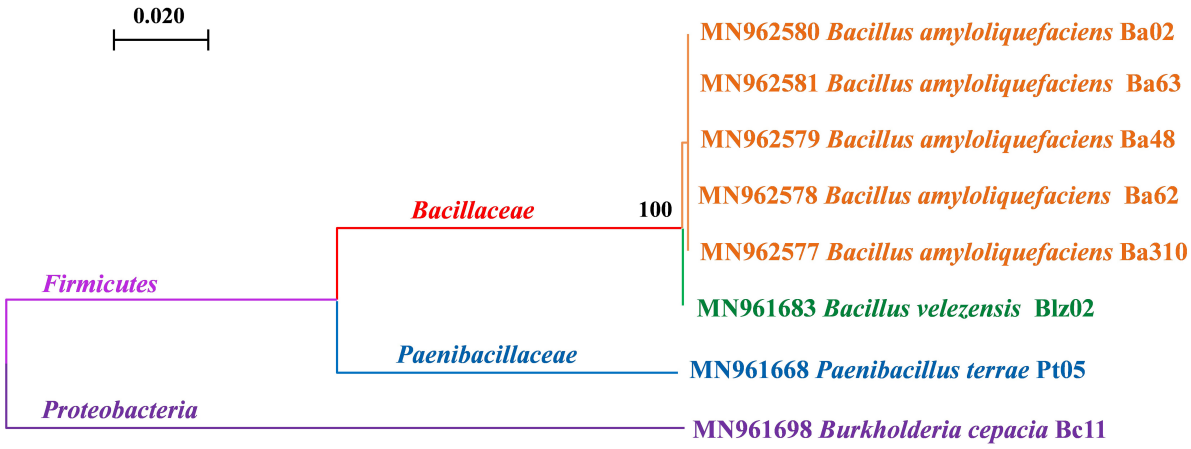

FIGURE 1
Evolutionary relationships of biocontrol bacteria. Phylogenetic analyses were conducted by maximum likelihood using the 16s rDNA sequences, which showed the genetic relationship between *Bacillus amyloliquefaciens*, *Bacillus velezensis*, *Paenibacillus terrae*, and *Burkholderia cepacia*. The numbers represent the accession numbers in GenBank. The confidence probability of each node was estimated using a bootstrap test (1,000 replicates). The scale bars represent a genetic distance of 0.02 substitutions per nucleotide position. All strains were labeled with different colors: orange was *B. amyloliquefaciens*, green was *B. velezensis*, blue was *P. terrae*, and purple was *B. cepacia*.

Experiments were performed using a completely randomized design, and the two rows at the edge served as protective rows.

To prepare the biocontrol strain inoculum for field application, each member strain of T28 was activated according to the method described in section "2.3 Preparation of inocula." The culture was inoculated into a 50-L fermenter (biotech-50JS, Shanghai Baoxing Biological Equipment Engineering Co., Ltd., Shanghai, China) containing a 10 L fermentation medium with 1% (*v*/*v*) inoculum for monoculture fermentation. The fermentation medium was the same as the activation medium for each strain. Strains Blz02 and Ba62 were fermented for 2 days at 150 rpm under aerobic conditions at 30°C, while strains Pt05 and gz-2 were fermented for 3 and 5 days, respectively. After fermentation was completed, the fermentation broth of each biocontrol strain was adjusted to a final concentration of 1×10^9 CFU•mL^{-1} with sterile water and mixed with an equal volume before being applied in the field.

The field experiment was performed as described by Saravanan et al. (2003) and Damodaran et al. (2020), with a few modifications. The first inoculation was performed by spraying inoculum in the banana rhizosphere on 27 May 2021, with a volume of 0.33 L per plant used for field inoculation. Each treatment was repeated with three replicates, and 30 banana plants per replicate. Inoculation was performed four times in total at intervals of 1 month. Banana plants were managed by normal field practices during the period of the experiment without chemical fungicide or fertilizer. The number of infected plants in each treatment was counted on 30 October 2021, when the banana plants were in fruit-set periods, and the control effect (%) was calculated ($n = 30$) according to the following formula:

$$\text{Control effect (\%)} = \frac{(\text{R1} - \text{R2})}{\text{R1}} \times 100\%,$$

Where R1 and R2 indicate the number of infected plants in the control and treatment areas, respectively.

2.8 Statistical analysis

Statistical analyses of all data were carried out using SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA). Differences were subject to one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at $P = 0.05$. Differences at $P < 0.05$ were considered statistically significant. Each test

was replicated three times. Charts were drawn using Origin 8.0 (Origin Lab, Massachusetts, USA).

3 Results

3.1 Compatibility among the biocontrol strains

Nine biocontrol strains from two microbial kingdoms were used for the compatibility test, including one fungus (*T. harzianum* gz-2) and eight bacteria. A genetic kinship tree of the eight bacteria is shown in **Figure 1**. At the phylum level, these bacteria belonged to *Proteobacteria* (*B. cepacia* Bc11) and *Firmicutes*. Bacteria belonging to *Firmicutes* were divided into two families: *Paenibacillaceae* (*P. terrae* Pt05) and *Bacillaceae*. Among the six strains belonging to *Bacillus* sp., strain Blz02 was *B. velezensis* (strain Blz02), whereas the other five were *B. amyloliquefaciens* (strain Ba02, Ba62, Ba63, Ba48, and Ba310).

The results of the compatibility testing are shown in **Table 3**. Regardless of the indicator or challenge strain, *T. harzianum* gz-2 was compatible with the eight bacteria since there was no inhibition zone formed between them on the NA medium. Co-cultures of six *Bacillus* strains (*B. amyloliquefaciens* Ba02, Ba62, Ba63, Ba48, and Ba310 and *B. velezensis* Blz02) showed no inhibition zones against one another when they were used as the challenge strain, indicating that they were compatible. As a challenge strain, *P. terrae* Pt05 was compatible with all tested strains, but as an indicator strain, it was antagonistic to *B. amyloliquefaciens* Ba02, Ba63, Ba48, Ba310, and *B. cepacia* Bc11. Strain PT05 did not inhibit the growth of these bacteria but was inhibited by them when they were cultured together. Therefore, strains Pt05, Bc11, Blz02, Ba62, and gz-2, which were compatible with each other, were selected to construct a compound microbial agent.

3.2 Efficiency of biocontrol strains and their combinations in controlling Fusarium wilt of banana

Fifteen days after the second inoculation, obvious symptoms started to appear in the control plants, while there were no visible symptoms in the treatment groups (**Figure 2**). Based on the results (**Figure 3** and **Table 4**), the disease index of the control was the highest at 26.19, which was significantly higher than that of the biocontrol strains and ranged from 5.56 to 20.63. The results suggest that all biocontrol strains and their combinations significantly inhibited FWB (**Figure 3A**).

The control effect of a single control strain (T1–T5) on FWB ranged from 21.21 to 60.61%, with an average control effect of 47.27% (**Figures 3C,D**). Except for T6, T19, and T21, all control effects of the biocontrol strain combinations (T6–T31) were greater than 51.52% (**Figures 3C,D**). Of these, there were 11 combinations (T9, T11, T12, T14, T17, T20, T25, T26, T27, T28, and T29), with control effects exceeding 72.73%, and the highest was 84.85% (**Figure 3B**). Overall, the control effects of the compound microbial agents were better than those of a single strain.

Nevertheless, based on the number of strains in the combination, there was no positive relationship between the strain number and the control effect on FWB (**Figure 3C**). Four-strain combinations had the highest average control effect of 76.97%. The average control effects of the two-strain combinations and the three-strain combinations were comparable to those of the five-strain combination, which were 67.27, 57.27, and 57.58%, respectively (**Figure 3D**).

The 26 combinations of biocontrol strains were split into four groups based on their genetic relationships, with differences in species, family, phylum, and kingdom levels (**Figure 3E**). The average control effects of these four groups on FWB were 54.5, 57.58, 62.34, and 68.28%, respectively (**Figures 3F,G**). The more distantly related combinations tended to obtain higher control effects. Moreover, the combinations with the highest control effect appeared in distantly related groups at the phylum (T7, T11, and T26) and kingdom levels (T9, T12, T14, T20, T25, T27, T28, and T29) (**Figure 3G**). Eight combinations with a control effect exceeding 75% were selected to further evaluate their stability in controlling FWB (**Figure 3B**).

3.3 Effect of biocontrol strain combinations against Fusarium wilt of banana in different soil types

The control effects of eight biocontrol strain combinations against FWB were determined in four types of soil. The disease index of all compound microbial agents was significantly lower compared with the control ($P < 0.05$). The disease index of compound microbial agents ranged from 11.64 to 28.04, while that of the controls ranged from 49.21 to 57.67 (**Figure 4A**). In different types of soil, the control effects of the compound microbial agents differed, ranging from 43.01 to 79.82% (**Figure 4B**). Of these, T28 showed the highest biocontrol efficiency against FWB, exceeding 73.12% in different soil types.

Regarding the stability of the control effect, T27 and T28 of the four-strain combination displayed the smallest magnitude of change in the four types of soil, while their median values were the highest at 68.95 and 75.71%, respectively (**Figure 4C**). The median values of all two- and three-strain combinations were between 63.13 and 65.46%, which were lower than those of T27 and T28. Their control effects varied greatly among the four types of soil. For all two- and three-strain combinations, their control effects varied greatly in the four types of soil, and were lower than those of T27 and T28, with median values

TABLE 3 Compatibility assay among *Trichoderma* and bacterial strains.

Indicator strains	Challenge strains								
	gz-2	Bc11	Pt05	Blz02	Ba02	Ba62	Ba63	Ba48	Ba310
gz-2		–	–	–	–	–	–	–	–
Bc11	–		–	–	–	–	–	–	–
Pt05	–	+		–	+	–	+	+	+
Blz02	–	–	–		–	–	–	–	–
Ba02	–	–	–	–		–	–	–	–
Ba62	–	–	–	–	–		–	–	–
Ba63	–	–	–	–	–	–		–	–
Ba48	–	–	–	–	–	–	–		–
Ba310	–	–	–	–	–	–	–	–	

"+" indicates antagonism, and "−"indicates compatibility.

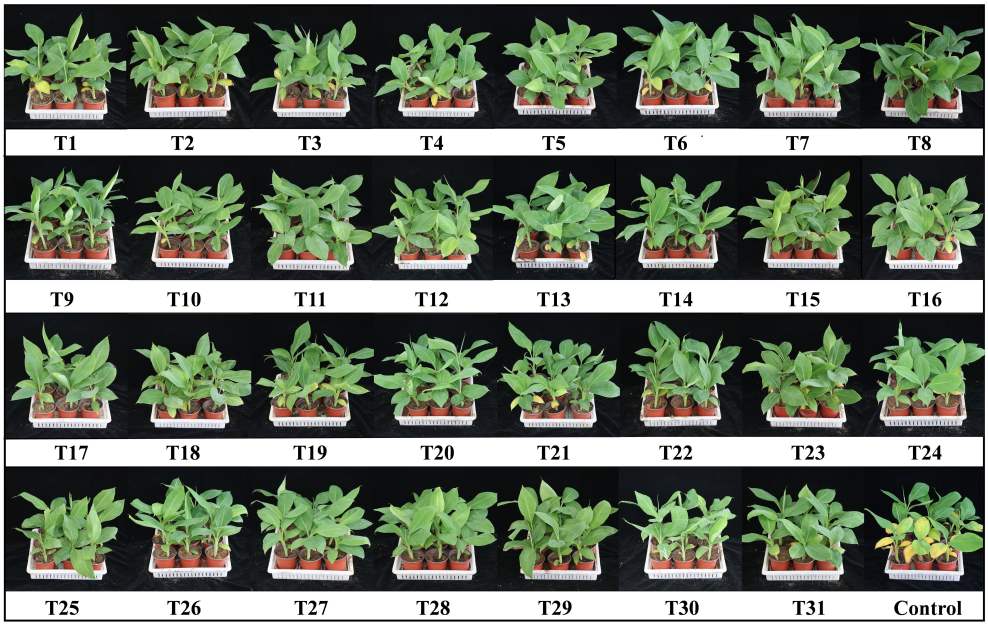

FIGURE 2
Symptoms of Fusarium wilt of banana observed on infected banana seedlings 15 days after inoculation.

ranging between 63.13 and 65.46%. Overall, T28 of the four-strain combination (Pt05 + Bc11 + Ba62 + gz-2) showed the best stability and control against FWB compared to the other 7 combinations in different types of soil.

3.4 Biocontrol efficiency of the T28 combination against Fusarium wilt of banana under field conditions

In comparison with commercial biological agents and fungicides, the control effect of the best combination T28 against FWB was determined under field conditions. After four consecutive applications in the field, significant differences were observed in the control effect between treatments (Figure 5). Compared to BD, TD, and FD treatments, T28 had a significantly higher control effect ($P < 0.05$), up to 57.14%. The control effect of TD was the lowest, at only 11.43%. In contrast, the control effects of BD were comparable to those of FD without any significant difference ($P < 0.05$).

4 Discussion

FWB is a devastating soil-borne disease in banana. Currently, no effective treatment is available for this disease.

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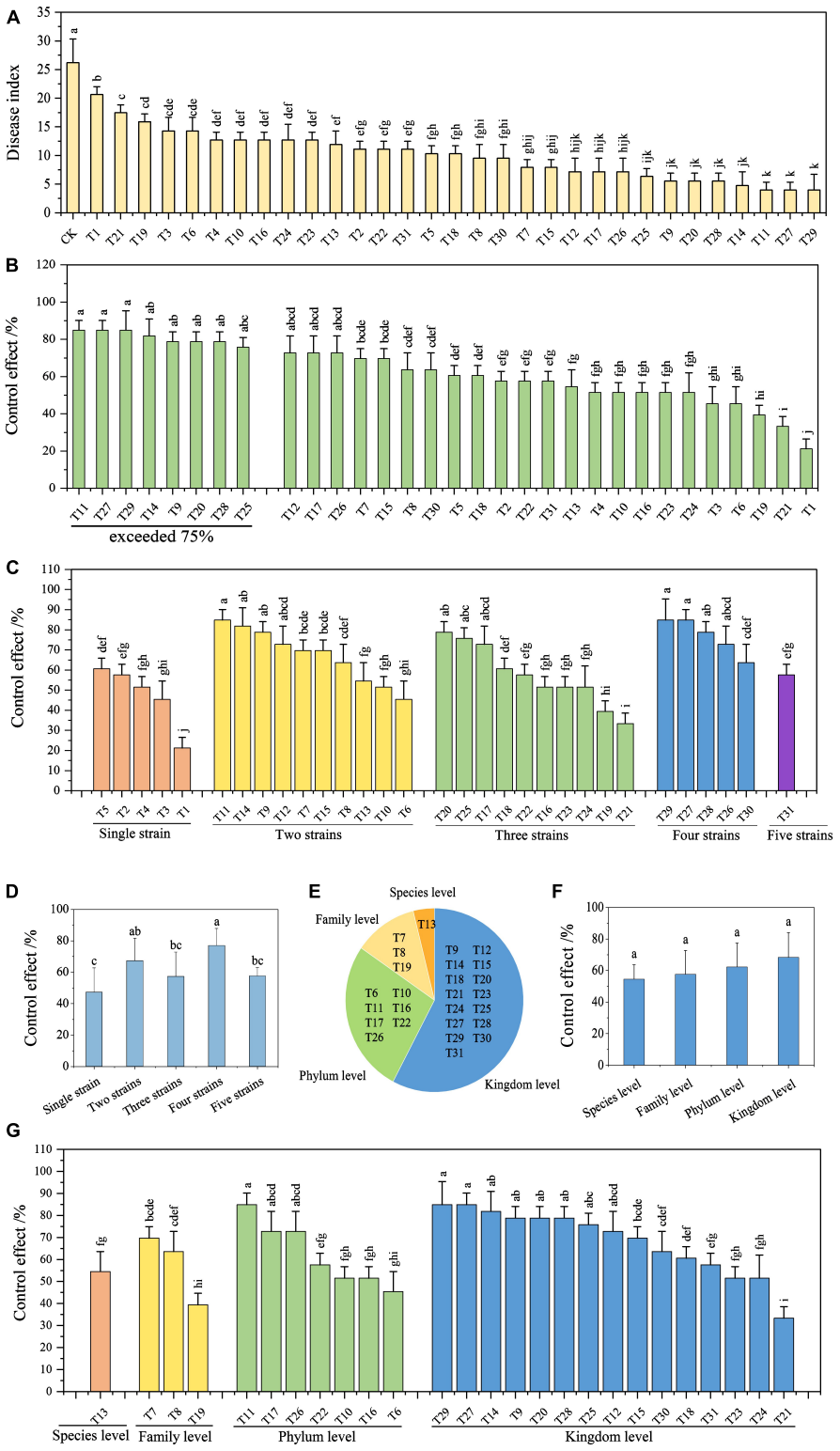

FIGURE 3
Efficiency of biocontrol strains and their combinations in controlling Fusarium wilt of banana. **(A)** Disease index of infected banana seedlings. **(B)** Control effect of each treatment on Fusarium wilt of banana. **(C)** Analysis of the control effect based on the strain number in the combinations. **(D)** Average control effect of combinations with a different number of strains. **(E)** Groups of biocontrol strain combinations based on genetic relationship differences. **(F)** Average control effect of combinations with genetic relationship differences. **(G)** Analysis of control effect based on genetic relationships in the combinations. Different lowercase letters above the bars denote significant differences among treatments ($P < 0.05$).

Biological control is considered an ideal way to control FWB. Many biocontrol microbes have been screened to control FWB, but their efficiency is unsatisfactory under field conditions (Getha et al., 2005). A single strain that is difficult to adapt to various soil environments might be one of the most important causes of poor efficiency. Therefore, many studies have attempted to use biocontrol strain combinations to control FWB. Different combinations of AM fungi, *T. harzianum* and *P. fluorescens*, were tested for controlling FWB, showing control effects of 40–80% in pot conditions and 42.85–64.29% in field conditions (Sukhada et al., 2010). Among the 11 combinations of *Trichoderma* sp. against FWB, Thangavelu and Gopi (2015) found that the combination of rhizosphere *Trichoderma* sp. nrcb3 and endophytic *T. asperellum* prr2 showed the best efficiency, with control effects of 100 and 54.90% in pot and field conditions, respectively. Gui et al. (2020) used a combination of non-pathogenic *F. oxysporum* sp., *Paecilomyce* sp., and *Trichoderma* sp. to control FWB under pot conditions and only found a control efficiency of 48%. In this study, we optimized the number and species of strains in the biocontrol strain combinations for controlling FWB. The highest combination efficiency was reached at 84.85% in the pot experiment, while the effect of the optimal combination T28 was 57.14% in the field.

Many studies have shown that the combination of biocontrol strains can significantly improve the effectiveness of disease prevention (Srivastava et al., 2010; Grosch et al., 2012; Alizadeh et al., 2013; Niu et al., 2017; Zaim et al., 2018; Izquierdo-García et al., 2020). Compared with similar results from these studies, most of the biocontrol strain combinations in this study were more effective in controlling FWB than single strains. Nevertheless, more strains in the combination do not always improve the disease control efficiency. In this study, the control effect of the five-strain combinations (T31) was not the highest among the 26 combinations tested, which was comparable to that of the single strain (T2, T3, T4, and T5). Four-strain combinations had the highest average control effect among all combinations. These results suggest that, in addition to the strain number and the defensive ability against the disease of each strain, the control effect of the combinations also depends on the interaction between the members (Whipps, 2001; Guetsky et al., 2002). The similar result was shown in the experiment that controlling FWB and root rot of papaya by combination of *Glomus mosseae*, *Trichoderma harzianum,* and *Pseudomonas fluorescens*. The best effects were both provided by *G. mosseae* + *T. harzianum*, but not the combination of all three strains (Sukhada et al., 2010, 2011). From the perspective of taxonomic differences between each strain in the combinations, among the 26 combinations tested, the combinations with the highest efficiency appeared at the phylum and kingdom levels, with an effect exceeding 72.73%. All combinations involving differences in species and family level showed control effects of less than 69.70%. Overall, the combinations containing fungi and bacteria had a higher control effect than the combinations containing only bacteria. This indicates that the further the genetic relationship in the taxonomy of the strains contained in the

TABLE 4 Disease index of banana seedlings and control effect of biocontrol strains against Fusarium wilt of banana.

Treatments	Combination	Disease index	Control effect (%)
T1	Pt05 alone	20.63 ± 1.37 b	21.21 ± 5.25 j
T2	Bc11 alone	11.11 ± 1.37 efg	57.58 ± 5.25 efg
T3	Blz02 alone	14.29 ± 2.38 cde	45.45 ± 9.09 ghi
T4	Ba62 alone	12.70 ± 1.37 def	51.52 ± 5.25 fgh
T5	gz-2 alone	10.32 ± 1.37 fgh	60.61 ± 5.25 def
T6	Pt05 + Bc11	14.29 ± 2.38 cde	45.45 ± 9.09 ghi
T7	Pt05 + Blz02	7.94 ± 1.37 ghij	69.70 ± 5.25 bcde
T8	Pt05 + Ba62	9.52 ± 2.38 fghi	63.64 ± 9.09 cdef
T9	Pt05 + gz-2	5.56 ± 1.37 jk	78.79 ± 5.25 ab
T10	Bc11 + Blz02	12.70 ± 1.37 def	51.52 ± 5.25 fgh
T11	Bc11 + Ba62	3.97 ± 1.37 k	84.85 ± 5.25 a
T12	Bc11 + gz-2	7.14 ± 2.38 hijk	72.73 ± 9.09 abcd
T13	Blz02 + Ba62	11.90 ± 2.38 ef	54.55 ± 9.09 fg
T14	Blz02 + gz-2	4.76 ± 2.38 jk	81.82 ± 9.09 ab
T15	Ba62 + gz-2	7.94 ± 1.37 ghij	69.70 ± 5.25 bcde
T16	Pt05 + Bc11 + Blz02	12.70 ± 1.37 def	51.52 ± 5.25 fgh
T17	Pt05 + Bc11 + Ba62	7.14 ± 2.38 hijk	72.73 ± 9.09 abcd
T18	Pt05 + Bc11 + gz-2	10.32 ± 1.37 fgh	60.61 ± 5.25 def
T19	Pt05 + Blz02 + Ba62	15.87 ± 1.37 cd	39.39 ± 5.25 hi
T20	Pt05 + Blz02 + gz-2	5.56 ± 1.37 jk	78.79 ± 5.25 ab
T21	Pt05 + Ba62 + gz-2	17.46 ± 1.37 c	33.33 ± 5.25 i
T22	Bc11 + Blz02 + Ba62	11.11 ± 1.37 efg	57.58 ± 5.25 efg
T23	Bc11 + Blz02 + gz-2	12.70 ± 1.37 def	51.52 ± 5.25 fgh
T24	Bc11 + Ba62 + gz-2	12.70 ± 2.75 def	51.52 ± 10.5 fgh
T25	Blz02 + Ba62 + gz-2	6.35 ± 1.37 ijk	75.76 ± 5.25 abc
T26	Pt05 + Bc11 + Blz02 + Ba62	7.14 ± 2.38 hijk	72.73 ± 9.09 abcd
T27	Pt05 + Bc11 + Blz02 + gz-2	3.97 ± 1.37 k	84.85 ± 5.25 a
T28	Pt05 + Bc11 + Ba62 + gz-2	5.56 ± 1.37 jk	78.79 ± 5.25 ab
T29	Pt05 + Blz02 + Ba62 + gz-2	3.97 ± 2.75 k	84.85 ± 10.5 a
T30	Bc11 + Blz02 + Ba62 + gz-2	9.52 ± 2.38 fghi	63.64 ± 9.09 cdef
T31	Pt05 + Bc11 + Blz02 + Ba62 + gz-2	11.11 ± 1.37 efg	57.58 ± 5.25 efg
T32	Control	26.19 ± 4.12 a	–

Values are the means (± SEM) of the three replications. Means in a column with similar letter(s) were not significantly different ($P < 0.05$).

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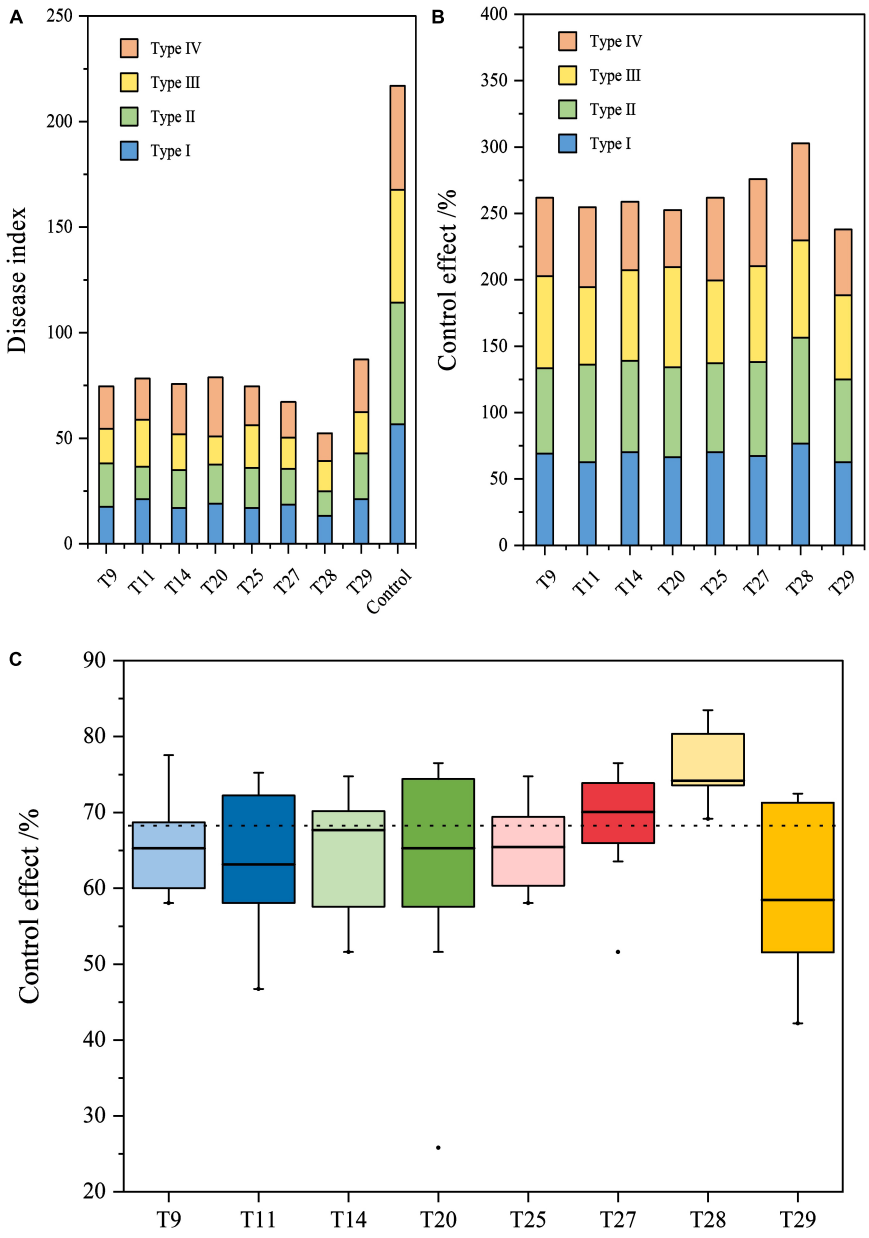

FIGURE 4
Efficiency of biocontrol strain combinations against Fusarium wilt of banana in different types of soil. **(A)** Disease index banana seedlings treated with the eight combinations. **(B)** Control effect of the eight combinations against Fusarium wilt of banana. **(C)** Stability of the control effect on the eight combinations. Boxplots depict minimum and maximum values (whiskers), the interquartile range (box), outliers (dots), and median (line).

combination, the greater the potential of the combination to improve the control ability of the disease during the construction of a compound microbial agent. It means the diversity of strains is beneficial in improving disease prevention. Similar to this is that the high *Pseudomonas* diversity reduced pathogen density in the tomato rhizosphere and decreased the disease incidence of tomato bacterial wilt (Hu et al., 2016). The similar result was also illustrated in the experiment of controlling corn stalk rot with a compound bacterial agent composed of 7 different strains (Niu et al., 2017). This may occur because the biological characteristics of the strains in the combination are quite different due to their distant genetic relationships, leading to differences in disease-resistance mechanisms. Such a combination can provide a variety of disease-resistance mechanisms, a more stable community structure, and the occupation of complementary niches in complex environments to play a greater synergy role in disease control.

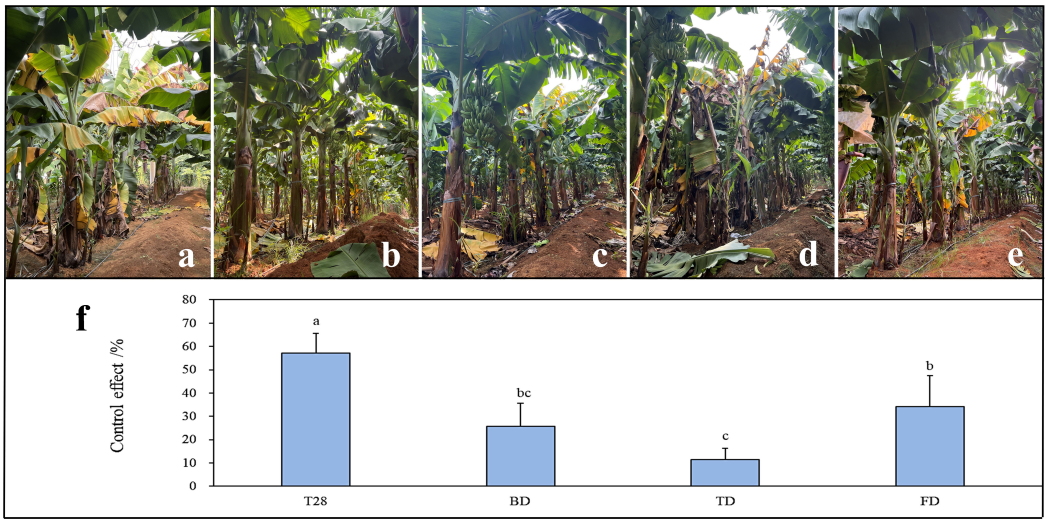

FIGURE 5
Control effect of different treatments against Fusarium wilt of banana in the field. **(a)** Control, **(b)** the biocontrol strain combination of T28 (T28), **(c)** *Bacillus subtilis* (BD), **(d)** *Trichoderma harzianum* (TD), and **(e)** Carbendazim (FD). **(f)** Data presented are the control effects of the four treatments against Fusarium wilt of banana after four consecutive applications. Values are the means (± SEM) of the three replications. Different lowercase letters above the bars denote significant differences among treatments ($P < 0.05$).

To imitate the field conditions, biocontrol strain combinations were evaluated for their ability and stability for controlling FWB in different types of banana plantation soil under pot conditions. Soil samples were collected from different regions of banana plantations, and their physicochemical properties differed (Table 1). The results showed that compared with the two- and three-strain combinations, a higher control effect, and better stability were more easily available for the four-strain combination. In particular, the four-strain combination T28 showed the most stable and highest control effect in different soils. T28 was composed of four different antagonistic microorganisms, which were *Paenibacillus terrae*, *Burkholderia cepacia*, *Bacillus amyloliquefaciens* and *Trichoderma harzianum*. The member strains in the combination are abundant in their genetic, ecological, and physiological diversity, may be resulting in their different colonization abilities in different types of soil. And the different strains may play a synergistic role in competing niches, regulating soil physicochemical properties, inducting defense enzymes of host, and influencing the function of rhizosphere microbial communities. This is conducive to formatting a healthy defense system in different soils, so that the disease can be controlled more effectively.

In this study, we determined the efficiency of biocontrol strains and their combinations in controlling FWB and evaluated the efficiency of stability in 11 combinations in different types of banana plantation soil under greenhouse conditions. Combination T28 was identified by its high control effect and strong stability against FWB, and its efficiency was evaluated under field conditions. However, in a complex soil environment, different combinations of biocontrol strains may exhibit different colonization abilities, whose interactions can be extremely complex. Through metagenomic and transcriptomic analysis, we can subsequently investigate the effects of compound microbial agents on the diversity and community structure function of the soil microorganisms around banana roots, mine their disease resistance-related genes, and explore the mechanisms of disease prevention and control. This can provide a theoretical basis for the biological control of FWB.

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 author.

Author contributions

CD performed the experiments, analyzed the data, and wrote the manuscript. DY and YY conducted the experiments and revised the manuscript. LP and JZ collected samples and conducted the experiments. SJ revised the manuscript. GF designed the experiments, supervised the project, and wrote the manuscript. All authors have 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.

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Bio-priming of banana tissue culture plantlets with endophytic *Bacillus velezensis* EB1 to improve Fusarium wilt resistance

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Tissue culture techniques have been routinely used for banana propagation and offered rapid production of planting materials with favorable genotypes and free of pathogenic microorganisms in the banana industry. Meanwhile, extensive scientific work suggests that micropropagated plantlets are more susceptible to *Fusarium oxysporum* f. sp. *cubense* (*Foc*), the deadly strain that causes Fusarium wilt of bananas than conventional planting material due to the loss of indigenous endophytes. In this study, an endophytic bacterium *Bacillus velezensis* EB1 was isolated and characterized. EB1 shows remarkable *in vitro* antagonistic activity against *Foc* with an inhibition rate of 75.43% and induces significant morphological and ultrastructural changes and alterations in the hyphae of *Foc*. Colony-forming unit (c.f.u.) counting and scanning electron microscopy (SEM) revealed that EB1 could colonize both the surface and inner tissues of banana tissue culture plantlets. Banana tissue culture plantlets of late rooting stage bioprimed with EB1 could efficiently ward off the invasive of *Foc*. The bio-priming effect could maintain in the acclimatized banana plants and significantly decrease the disease severity of Fusarium wilt and induce strong disease resistance by manipulating plant defense signaling pathways in a pot experiment. Our results provide the adaptability and potential of native endophyte EB1 in protecting plants from pathogens and infer that banana tissue culture plantlets bio-priming with endophytic microbiota could be a promising biological solution in the fight against the Fusarium wilt of banana.

KEYWORDS

endophyte, *Bacillus*, Fusarium wilt of banana, biological control, banana tissue culture plantlets

Introduction

As the most important fruit in the world and the major staple crop in more than 130 countries across the tropical belt, banana (*Musa* spp.) production contributes significantly to income and food security (Kema et al., 2021). However, the banana industry is under severe threat from Fusarium wilt, the most destructive disease of banana in history whose causal agent is *Fusarium oxysporum* f. sp. *cubense* (*Foc*). *Foc* is composed of different evolutionary lineages and at least 24 vegetative compatibility groups (VCGs). *Foc* race 1 wiped out the highly susceptible Gros Michel (*Musa* AAA) variety in Central America in the mid-twentieth century (O'Donnell et al., 2009; Staver et al., 2020). The plague caused by *Foc* race 1 was

mitigated by gradually adopting a resistant cultivar Cavendish (*Musa* AAA) as a replacement for Gros Michel (Dita et al., 2018). The recent emergence of *Foc* tropical race 4 (*Foc* TR4), the most destructive and uncontrollable pathogen of banana, to which Cavendish and many other cultivars are highly susceptible, has created havoc on banana production worldwide again (Dita et al., 2018). Ever since it was first reported to destroy the Cavendish-based banana industry in the 1960s in Taiwan, *Foc* TR4 has expanded across Southeast Asia, the Middle East, Africa, and most recently has been reported in Colombia and is present in 27 countries where thousands of hectares have been affected in the past several years (Ordonez et al., 2015; Galvis, 2019). A recent projection by the Food and Agriculture Organization of the United Nations (FAO) estimated that the inexorable spread of *Foc* TR4 would lead to a 2.0% drop in global output, 240,000 direct jobs loss, and induce a 9.2% rise in the global reference price for bananas by 2028 (Altendorf, 2019).

The management of Fusarium wilt is particularly challenging due to several conspiring factors. First, as a soil-borne fungus, *Foc* can survive in the soil in the form of chlamydospore for up to 30 years even in the absence of host plants and be dispersed through diverse ways (i.e., infecting plant material, soil, water, and others) (Cook et al., 2015; Dita et al., 2018). Second, the only effective measure to manage this disease is stated frequently as planting resistant cultivars, but resistant cultivars might not meet the current market demand and may be overcome by continually emerging pathogens (Ploetz, 2015). The latter cultivated banana is almost exclusively of the *Foc* TR4 susceptible Cavendish for its export properties, which facilitates the dispersion of the disease worldwide. Third, as a typical vascular wilt disease, *Foc* can escape from contacting with non-contact fungicides, non-endophytic biological control agents (BCAs), and other control measures once it penetrates the host plant (Bubici et al., 2019). Thus, it is almost impossible to eliminate the disease incidence once the field gets contaminated with *Foc*. Therefore, highly efficient and sustainable strategies should be implemented to alleviate the influences of Fusarium wilt on susceptible varieties and to improve the durability of available resistant varieties.

In recent years, the use of BCAs for the control of many plant diseases including the Fusarium wilt of banana has gained great interest as an alternative to chemical application. Among the BCAs, Among the BCAs, the pivotal role of endophyte in the health and fitness of their host plants has become evident only in recent years (Papik et al., 2020; Matsumoto et al., 2021). Endophytes refer to microbes that colonize internally in different plant tissues and perform mutualistic symbiotic associations with their hosts (Papik et al., 2020). Their unique ecological niches similar to that of vascular wilt pathogens make them better targets for biocontrol agents against wilt disease than their rhizospheric counterparts (Strobel and Daisy, 2003; Eljounaidi et al., 2016). As the second microbiological layer of plant defense, endophytes can defend plants from biotic stresses either by showing direct antagonistic activity such as parasitism, antibiosis, and competition or by inducing indirect antagonism effects (induced systemic resistance, ISR) in host plants to an array of phytopathogens (Dini-Andreote, 2020; Dubey et al., 2020). Several studies have shown that endophytic microbes may serve as environmentally safe measures to combat Fusarium wilt of banana (Cao et al., 2005; Bubici et al., 2019; Gómez-Lama Cabanás et al., 2021; Savani et al., 2021; Zhang et al., 2022). Applications of endophytic *Trichoderma asperellum Prr2* (Thangavelu and Gopi, 2015), *Pseudomonas aeruginosa* (Yu et al., 2010), *Pseudomonas* sp. UPMP3, and *Burkholderia* sp. UPMB3 (Mohd Fishal et al., 2010) have reduced the disease incidence of Fusarium wilt in banana significantly under greenhouse and field conditions.

Nowadays, the most common application strategies of endophytes in agricultural systems are adding them directly into the soil and preparing them as seed-coating agents, which are rather inefficient in practice. Thus, it is imperative to explore alternative strategies for endophyte application (Dubey et al., 2020). Even more significant is the fact that, unlike most other seed plants, the propagation of banana is mainly dependent on tissue culture with all microorganisms eliminated during the micropropagation process under strict aseptic conditions. The regenerated plants are, therefore, particularly vulnerable when transferred directly to natural conditions with multiple environmental stresses (Lian et al., 2009; Soumare et al., 2021). In this sense, the establishment of beneficial interactions between explants and beneficial microbes to offer protection for young host plantlets against environmental stress in field conditions might represent a valuable approach to efficiently solve those restrictions (Soumare et al., 2021). Unfortunately, only few studies have reported inoculation with endophytes in banana tissue culture plantlets during the rooting or acclimatization stages (Guez-Romero et al., 2008; Lian et al., 2009; Kavino and Manoranjitham, 2018). As the key components for achieving sustainable agriculture, the interactions between plants, fungi, and endophytes in tissue culture plantlets have not been sufficiently studied. In the present study, a bacterial endophytic strain EB1 was isolated from a healthy banana plant in a wilt-diseased banana field in Dongguan, Guangdong Province, China (23.045315° N, 113.546177° E). We critically aimed to decipher (i) the phylogenetic, genomic, and antagonistic effect of EB1 against *Foc* by *in vitro* test and (ii) how EB1 modulates the resistance of banana plants against *Foc* by using a banana plant–EB1system created by inoculating banana tissue culture plantlets with EB1 at the end of rooting stage. Our study was designed to lend new insights into the sophisticated mechanisms of host plants–endophytes interactions for coping with environmental stresses and to provide potential strategies to control the Fusarium wilt of banana.

Materials and methods

Strain, media, and cultural conditions

Wild-type *Foc* TR4 strain II5 (VCG01213) was cultivated on a potato dextrose agar (PDA) plate at 28°C and used in this study. Isolated endophytic bacteria were inoculated in Luria-Bertani (LB) agar (Sangong Co., Ltd., Shanghai, China) plates. Basal Murashige and Skoog (MS) medium was used for tissue culture experiments.

Isolation and selection of endophytic bacteria against *Foc* TR4 from healthy banana plant

The healthy banana plants used in this study were collected from a wilt-diseased banana field in Dongguan, Guangdong Province, China (23.045315° N, 113.546177° E). Banana plant samples were washed with tap water thoroughly to remove the airborne counterparts and soaked in 75% (v/v) ethanol for 1 min, 0.1% (v/v) NaClO for 15 min, followed by being rinsed 5 times with sterile water to deplete epiphytic microorganisms in aseptic conditions. Ten grams of plant tissue was weighed and ground with 20 ml sterilized distilled water premixed with sterilized quartz sand using a sterilized mortar and pestle for 5 min. Aliquots of 1 ml of the resulting suspension were diluted using a serial dilution method and spread evenly on an LB agar plate and incubated at 28°C for 5 days. All culturable bacterial colonies were purified and selected based on their morphological characteristics. The antagonistic efficacies of these endophytic bacterial isolates were evaluated against *Foc* TR4 by a dual-culture experiment (Fan et al., 2019). One actively growing agar plug (5 mm diameter) of *Foc* TR4 was placed on the center of a fresh PDA plate. Then, 10 μL-drop of each isolate from an overnight culture (OD_{600} = 1.0) was uniformly inoculated 2.0 cm away from the fungal inoculum. Plates inoculated only with *Foc* TR4 plug were served as control. Plates were incubated at 28°C for 5 days and recorded with a Canon EOS 77D camera (Canon, Tokyo, Japan) with the same parameters, and the surface area of the mycelia was measured using the Image J software (Image J, NIH, USA). The inhibitory effect was evaluated by calculating the percentage of area inhibition using the following formula: (Sc - St)/Sc × 100, where Sc and St represent the growth area of *Foc* TR4 in the control and treated plates, respectively. The experiment was repeated 3 times, with 4 replicates each time. Strain EB1 was isolated through the above screening and stored at −80°C with glycerol (50%, v/v). The antifungal efficiency of EB1 against *Foc* TR4 was further measured by observing the morphology and ultrastructure characteristics of *Foc* TR4 in the dual-culture experiment by applying a scanning electron microscope (SEM, Hitachi Model S-3400N, Hitachi, Tokyo, Japan) and a transmission electron microscope (TEM, Hitachi HT7700, Hitachi, Tokyo, Japan).

Whole-genome sequencing of EB1

Overnight bacterial cultures of EB1 in LB broth were collected, centrifuged at 3,000 rpm for 15 min, and washed two times with sterile PBS buffer (50 μM, pH = 7.4). Whole-Genome Sequencing of EB1 was performed using a combination of the Oxford Nanopore Technologies (ONT) GridION platform (Oxford Nanopore Technologies Ltd, Oxford, UK) and Illumina MiSeq platform (Illumina MiSeq PE300, Illumina, USA) by Gene Denovo Biotechnology Co. (Guangzhou, China). DNA was extracted from Qiagen's DNeasy UltraClean Microbial Kit (Qiagen GmbH, Hilden, Germany) and its quality and concentrations were determined using a Nanodrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and Qubit Fluorometer (Thermo Fisher Scientific, MA, USA). For ONT sequencing, library preparation was conducted according to the manufacturer's protocol of the SQK-LSK109 sequencing kit (Oxford Nanopore Technologies Ltd., Oxford, UK). For Illumina sequencing, genomic DNA (gDNA) was fragmented and a paired-end library with an average DNA insert size of 300–400 bp was constructed using Illumina TruSeq Nano DNA Library Prep Kit (Illumina). The assembled sequences were deposited in the NCBI (BioProject ID: PRJNA807456). The components of coding genes, noncoding RNA (ncRNA), and functional annotation were analyzed using a range of databases including the non-redundant protein database (Nr), SwissProt, Cluster of Orthologous Groups (COGs), and Kyoto Encyclopedia of Genes and Genomes (KEGG). Gene clusters for the biosynthesis of secondary metabolites were identified by using antiSMASH.

Phylogenetic analysis of EB1

The 16S rDNA sequence of strain EB1 derived from the EB1 genome was aligned with an NCBI 16S ribosomal RNA sequences database by Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and 16S rRNA gene sequences closest to the isolates (98% sequence homology) were recovered for further phylogenetic analysis. Strain EB1 was subjected to phylogenetic analysis using MEGA version 7 (University, Pennsylvania, PA, USA) based on a full-length 16S ribosomal RNA (16S rRNA) sequence, and a phylogenetic tree was constructed using the neighbor-joining method. The reliability of this resulting tree was evaluated by the bootstrap method with 1,000 replications.

Colonization capacity of EB1 on banana tissue culture plantlets

Uniformly grown banana tissue culture plantlets ["Cavendish" banana (AAA) cv. "Brazilian"] of rooting stage were surface sterilized in 75% (v/v) ethanol for 1 min and 0.1% (v/v) NaClO for 15 min, rinsed with sterile water for 5 times, air-dried, and then transferred and grown vertically in tissue culture flasks containing 100 ml of cooled-down MS. Four plantlets were transferred to each flask. For EB1 inoculation, overnight culture of EB1 in LB broth was harvested, centrifuged, and washed in liquid MS twice, and resuspended in MS to a final optical density (OD_{600}) = 0.2. Each flask was inoculated with 0.1 ml of the bacterial suspension (~10^6 colony-forming unit, c.f.u.) or 0.1 ml MS by pipetting to the rhizosphere of banana tissue culture plantlets and cultured at 22°C and 16/8 h light/dark cycle. The colonization and reproduction of EB1 on the plantlets were quantified each day over a period of 7 days. Total c.f.u. values of EB1 were quantified per the programs described previously (de Zélicourt et al., 2018; Berlanga-Clavero et al., 2022). Briefly, 1.0 g root and pseudostem tissues of the banana tissue culture plantlets were sampled and gently washed by dipping in the distilled water to remove non-attached bacteria cells at the same time each day (±2 h). Each sample was transferred to a 2-ml microcentrifuge tube with 1 ml PBS buffer, sonicated on ice for 1 min, and vortexed for 10 min, and 100 μl of the

resulting suspensions were spread on LB agar plates after a 10-time dilution. The c.f.u. was counted after overnight incubation at 28°C, and the total c.f.u. was normalized per gram of root or pseudostem. To explore the interactions between strain EB1 and *Foc in planta*, an additional experiment was conducted in banana tissue culture plantlets by pipetting 0.1 ml *Foc* spore suspension (1×10^8 spores/L) or 0.1 ml MS to the rhizosphere of banana tissue culture plantlets after prior inoculation with EB1 for 3 days and cultured at 22°C and 16:8 h light/dark cycle for another 4 days. The experiment was conducted in triplicate, with at least eight plants per treatment. After 7 days of successive culture, SEM was used to observe the distributions of EB1 and the interactions between EB1 and *Foc* on the banana tissue plantlets.

Biocontrol efficacy of EB1 on banana plantlets

The above banana tissue culture plantlets and symbionts (banana tissue culture plantlets colonized with EB1) after 7 days of successive culture were subjected to hardening for 10 days by transferring into pots with sterilized planting soil (40 × 19 × 15 cm pots, ca. 2.0 kg soil each). Then the biocontrol efficacy of EB1 on banana plantlets was investigated in greenhouse experiments with the acclimatized banana plants (Supplementary Figure S1). Three treatments including EB1 only, TR4 only, EB1+ TR4, and control were applied in the pot. The banana plants were inoculated with or without *Foc* TR4 isolates at the concentration of 1,000 conidia/g soil, with a temperature ranging from 25 to 35°C. Three plantlets were grown in each pot and at least 20 plantlets were included in each treatment. In addition, due to the lethality of plantlets in the TR4 only, EB1 + TR4 groups, 60 plantlets were employed in each group to ensure sufficient plant material. Plant survival rates were recorded every 10 days, and observations on morphological characters such as plant height (cm) and fresh weight of shoot and root (g) were conducted after 60 days of planting. The disease index of each plantlet was assessed according to the rating scale of 0–4: 0 = no symptom; 1 = some brown spots in the inner rhizome; 2 = <25% of the inner rhizome show browning; 3 = up to 75% of the inner rhizome show browning; and 4 = entire inner rhizome and pseudostem show dark brown, dead (Liu S. et al., 2020). Harvested banana plant tissues were stored at −80°C pending for further analysis of defense-related enzymes and genes.

RNA extraction and gene expression analysis by RT-qPCR

Total RNA was extracted from the frozen banana plant using SteadyPure Plant RNA Extraction Kit (Accurate Biotechnology Co., Ltd., Hunan, China) following the manufacturer's instructions. HiScript II One Step qRT-PCR SYBR Green kit (Vazyme Biotech, Nanjing, China) was employed for qRT-PCR assays according to the manufacturer's instructions. First-strand cDNA was prepared by reverse transcription from 1 μg of DNA-free total RNA in a final reaction volume of 20 μl. RT-qPCR was conducted using a QuantStudio 5 Real-Time PCR System (Applied Biosystems, CA, USA) in four replicates. The *qTUB* gene (banana) was used as a reference for data normalization, and the target genes were amplified using the primer sets listed in Supplementary Table S1. The relative transcript abundance of each gene was estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analyses

All statistical analyses were performed using the SPSS 20.0 statistical software package (SPSS, Chicago, IL, USA). Data were analyzed using Student's *t*-test and one-way ANOVA test after being verified for normality and homogeneity of variance with Kolmogorov-Smirnov and Levene's tests. Cases with *p*-values of <0.05 were considered statistically significant.

Results

EB1 shows strong inhibitory efficiency against *Foc* TR4

The morphological observation was preliminarily carried out for strain EB1. It was found that the colony of EB1 on LB medium was dry and round with irregular protrusions at the margin, showing the typical characteristics of *Bacillus* species (Supplementary Figure S2A). The cells were short rod-shaped structures and ~1.2–1.6 μm in length, 0.6–0.7 μm in width, as revealed by SEM (Supplementary Figure S2B). EB1 showed strong inhibitory activities with the inhibition rates of mycelium growth area 75.43% against *Foc* TR4 (Figure 1A) and other Fusarium pathogens (Supplementary Figure S3) during co-cultivation compared to control. To confirm the antagonistic activity of EB1, the morphological and ultrastructural changes of *Foc* TR4 after a confrontation with EB1 were scrutinized by SEM (Figure 1B) and TEM (Figure 1C). The untreated hyphae of *Foc* TR4 appeared straight, uniform, and well-developed tube-like structure in shape under SEM. Conversely, phenotypes of abnormalities were noted in fungal hyphae co-culture with EB1. Severe forms of abnormalities, including highly deformed, irregular distorted, inflated were observed in fungal hyphae. TEM micrographs of untreated fungal hyphae had a well-defined cell wall (CW), intact plasma membrane (PM), and normal cytoplasm containing an intact nucleus and all organelles. In reverse, noticeably disruptions such as loss of cellular integrity, thickened CW, evident plasmolysis, serious vacuolation, invaginated PM, abnormal architecture of the nucleus and degenerated organelles were observed in EB1 treated hyphae. The results indicated that the morphology and structural integrity of the treated fungal were dramatically affected during co-culture with EB1.

Genome sequence assembly and general features of EB1

The whole genome of EB1 was sequenced and analyzed, through which the 16S rRNA region was extracted, and which was 1,404 bp in length. EB1 was identified as *Bacillus velezensis*

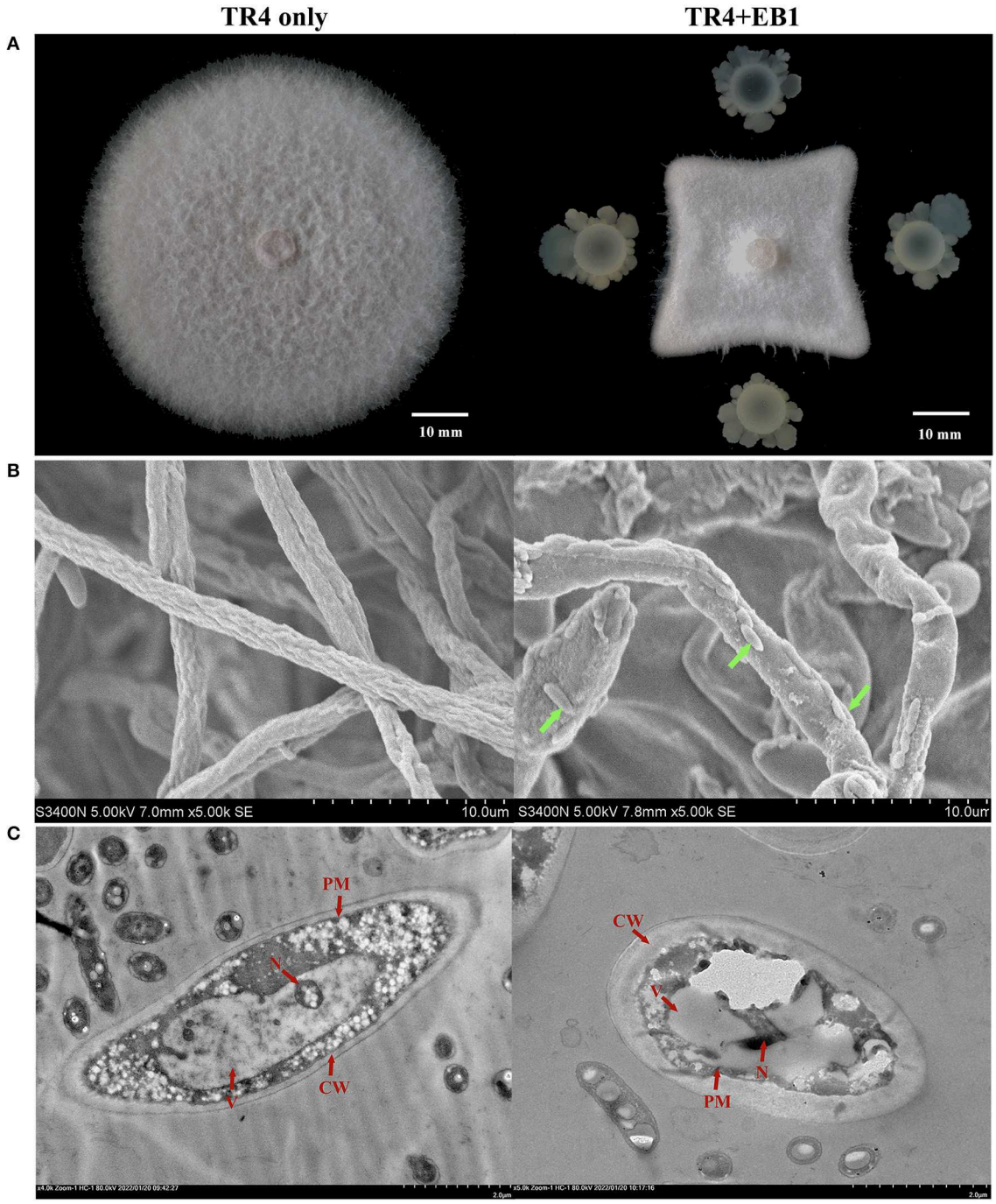

FIGURE 1
The antagonistic potential of strain EB1 against *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc* TR4). **(A)** The antagonistic potential of EB1 against TR4 *in vitro* with a dual-culture experiment. Morphological (marked by green arrow heads) and ultrastructural changes (marked by red arrow heads) of TR4 after the confrontation with EB1 were scrutinized by scanning electron microscopy (SEM) **(B)** and transmission electron microscope (TEM) **(C)**, respectively.

based on a phylogenetic tree constructed from the 16S rRNA gene (Figure 2A). The complete genome sequence of EB1 was deposited in GenBank under accession number CP093218. Accordingly, the genome of EB1 consists of a single circular chromosome of 3,929,912 bp, with an average of 46.5% GC content and a clear GC skew transition (Figure 2B). All predicted 3,622 open reading frames (ORFs) with a maximum *E*-value of 1.0 E-5 were subjected to annotation analysis by comparing with Nr, SwissProt, COG, and KEGG databases, and a total of 3,606 candidate genes had annotation information. The overall functional annotation is depicted in Supplementary Figure S4. A total of 2,756 genes were categorized into 21 functional groups using COG analysis (Figure 2C). Three main functional gene classes were revealed in the results: amino acid transport

and metabolism (329 genes), transcription (267 genes), and carbohydrate transport and metabolism (240 genes), representing 30.33% of the predicted genes in the COG analysis. Other clusters of represented genes involved in inorganic ions transport and metabolism (200 genes), energy production and conversion (188 genes), cell wall/membrane/envelope biogenesis (182 genes), signal transduction (167 genes), and translation, ribosomal structure and biogenesis (161 genes) account for 32.58% of predicted genes. In addition, a high proportion of predicted genes (26.78%) involved in general function prediction only and function unknown is poorly characterized. A total of 2,250 genes were mapped to 5 KEGG branches, including metabolism, genetic information processing, environmental information processing, cellular processes, and organismal systems, and among which, a high proportion of the annotated genes were assigned to metabolism, especially the pathways belonging to global and overview maps (682 genes), carbohydrate metabolism (240 genes), and amino acid metabolism (201 genes) (Figure 2D). Twelve biosynthetic gene clusters (BGCs) involved in the biosynthesis of secondary metabolites including non-ribosomal peptides synthase (NRPS), bacteriocin-NRPS, trans-AT polyketide synthase (transatpks), type III polyketides synthase (t3pks), terpene, transatpks-nrps, lantipeptide, and other types of polyketide synthases (OtherKS) were identified in EB1 using AntiSMASH.

EB1 showed strong colonization ability on the banana plantlets

The growth dynamics of the EB1 population during the 7 days after banana tissue plantlets treatment in tissue culture flasks were investigated in the root and shoot by c.f.u. counts and SEM (Figure 3). In the root, the colonization of EB1 numbered at 357 c.f.u./g on day 1 and increased to 1.20×10^8 c.f.u./g following an algorithm by day 5 and remained at 1.53×10^8 c.f.u./g by day 7. In contrast, in the case of shoot, no EB1 was detected on

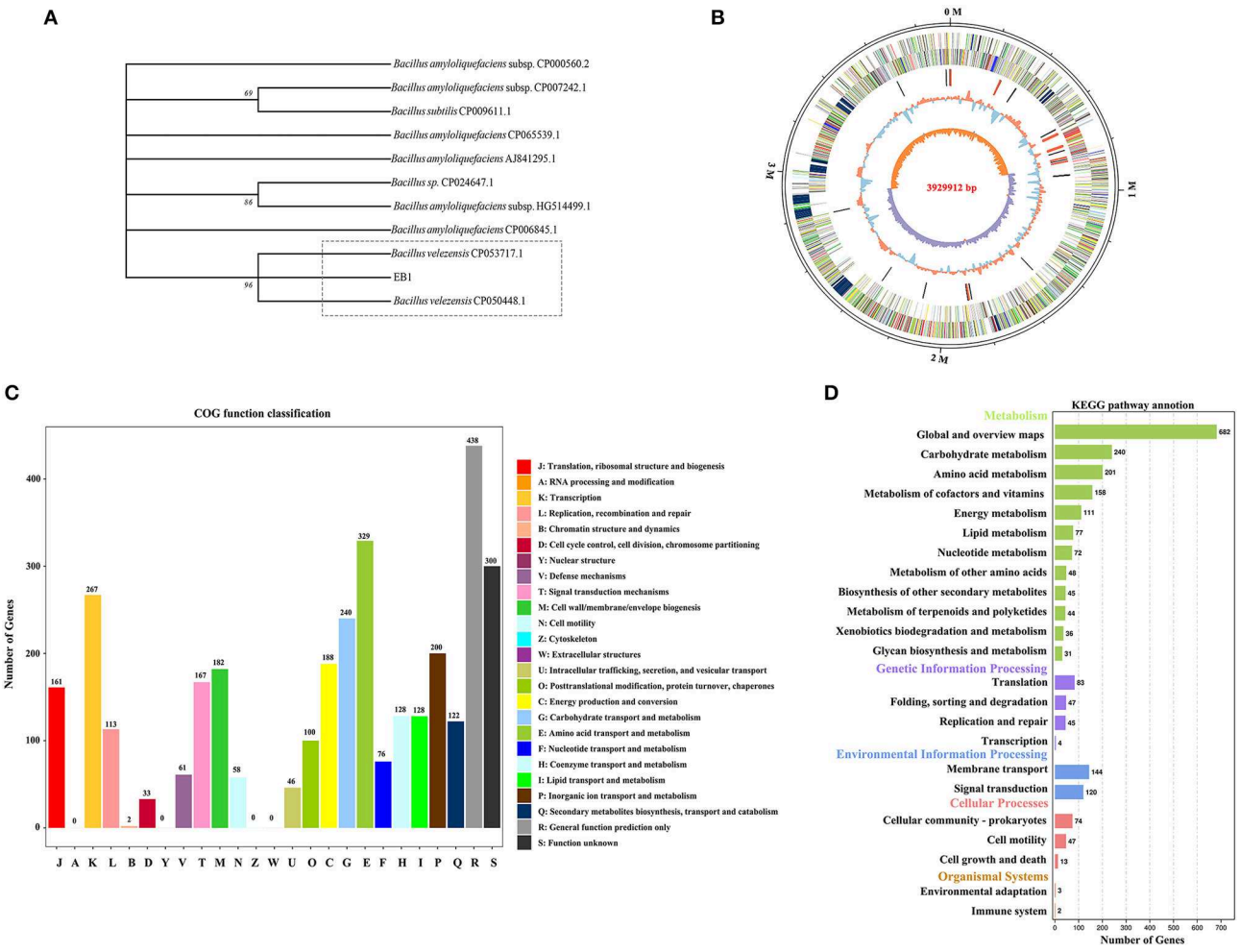

FIGURE 2
Phylogenetic and genomic analyses of strain EB1. **(A)** Phylogenetic trees of strain EB1 based on 16S rRNA gene. The tree was constructed using the MEGA software. The level of bootstrap support (1,000 repetitions) was indicated at all nodes. **(B)** Graphical circular map of EB1 genome. The distribution of the circle from the outermost to the center is (i) scale marks of the genome; (ii) protein-coding genes on the forward strand; (iii) protein-coding genes on the reverse strand; (iv) tRNA (black) and rRNA (red); (v) GC content; (vi) GC skew. **(C)** The COG annotation of strain EB1 genome. **(D)** The KEGG pathway annotation of strainEB1 genome.

day 1, after which the colonization level slowly started to increase from 9.73×10^3 c.f.u./g on day 2 to 1.96×10^7 c.f.u./g by day 7 (Figure 3A). Using SEM, no colony could be observed in the root or shoot and the pant epidermal cells were integral and smooth in the control group (Figure 3B). In the EB1-inoculated group, small rod-shaped EB1 can be seen in grooves between epidermal cells and intercellular space in root cells (Figure 3B). To explore whether EB1 inhibits *Foc in planta*, an additional experiment was conducted in banana tissue culture plantlets by inoculating with *Foc* after prior inoculation with EB1 for 7 days. The SEM observation revealed that infection of *Foc* seriously destroyed the surface structures of the root of banana tissue culture plantlets (Figure 3C). However, inoculation of EB1 prior to *Foc* treatment led to serious morphological deformities of *Foc* and the damaging effects of pathogen infection were alleviated (Figure 3C).

EB1 significantly promoted plant growth and conferred protection against *Foc* TR4

As EB1 was pre-inoculated on the culture plantlets at the rooting stage, to evaluate whether the acclimatized banana plants have been primed by EB1, the plant growth, survival, and disease severity were inspected in banana plants. Overall, pre-inoculation with EB1 at the rooting stage significantly promoted plant growth and conferred protection against *Foc* TR4 compared with the non-inoculation groups (Figure 4). No differences were detected in the height of banana plants between the control and EB1 groups. By contrast, the heights of banana plants were significantly reduced when subjected to *Foc* TR4 infections ($p < 0.001$), and this inhibitory effect was dramatically reversed upon co-inoculation with EB1 (TR4 + EB1) (Figures 4A, B). Compared with control, EB1 inoculation (EB1) significantly increased the plant biomass in both above-ground (shoot) and below-ground (roots) by 1.22- ($p = 0.002$) and 1.49-fold ($p = 0.007$), respectively. Plants in the EB1 + TR4 treatment group also had increased biomass compared with those treated by *Foc* TR4 only (shoot: 1.35-fold, $p = 0.002$ and root: 1.94-fold, $p = 0.004$). Moreover, EB1 did not cause any mortality or disease symptoms in banana plants when inoculated alone and greatly enhanced the survival rates and reduced the disease severity caused by *Foc* TR4 in banana plants who had been pre-inoculated with EB1 prior to inoculation with *Foc* TR4 (EB1 + TR4) compared with those inoculated only with *Foc* TR4 (Figures 4D, E; Supplementary Figure S5). Overall, these findings reveal that EB1 is a bacterial endophyte of banana plants that efficiently suppresses Fusarium wilt caused by *Foc* TR4.

EB1 manipulated the SA and JA pathways in banana plants

To further examine whether the EB1 could activate defense signaling in the banana plant, the expression patterns of the defense-related marker genes involved in SA and JA pathways including *NPR1*, *PR1*, *LOX2*, and *MYC2* were analyzed (Figure 5). Compared with the control, EB1 inoculation showed no induction in the expression of *NPR1* and *PR1* genes of the SA signaling pathway but showed an increase in the expression of the *MYC2* gene of the JA signaling pathway by 1.67-fold ($p = 0.016$). Whereas, the degree of expression changes in the *Foc* TR4 treatment group varied from gene to gene. *Foc* TR4 downregulated the expressions of *NPR1* and *LOX2* by 0.46- ($p = 0.0024$) and 0.63-fold ($p = 0.016$) and upregulated the expressions of *PR1* and *MYC2* by 16.62- ($p < 0.001$) and 2.23-fold ($p < 0.001$), respectively. Notably, both SA and JA signaling pathways were significantly upregulated by 1.40- ($p = 0.015$), 35.80- ($p < 0.001$), 1.50- ($p = 0.0028$), and 2.44-fold ($p < 0.001$) for *NPR1*, *PR1*, *LOX2*, and *MYC2*, respectively. The above results have indicated that EB1 primes the plants for enhanced immunity following a subsequent attack by *Foc* TR4.

Discussion

Besides resistant cultivar breeding, BCA comprising endophytic bacteria has been considered another promising control strategy against Fusarium wilt that reached the field-testing stage (Bubici et al., 2019). However, their control efficacy has been always unstable due to the varying environmental conditions, accentuating the need to develop a new efficient strategy for endophytes to harness their maximum benefits (Dubey et al., 2020; Papik et al., 2020; Savani et al., 2021; Jana et al., 2022). Plant tissue culture is the main strategy for banana propagation with the advantages of high multiplication rates and the production of disease-free planting materials with high genetic fidelity and high standards of hygiene (Pegg et al., 2019). More recently, tissue-cultured banana plantlets are questioned to be more susceptible to Fusarium wilt than vegetative planting materials because the first three stages (Stage I: establishment of explants, Stage II: elongation and multiplication, and Stage III: rooting) of the plant tissue culture process are taken place in aseptic conditions without the possibility of interaction with beneficial microbes (Orlikowska et al., 2017). In this context, co-cultivation of the plant tissue culture plantlets with beneficial microorganisms may be indispensable to improve the adaptive ability of plantlets in the acclimation stage, which has accentuated the urgent to develop new technologies based on endophytes as microbial inoculants in tissue culture banana plantlets. Therefore, in the current study, the endophytic bacterial strain *B. velezensis* EB1 was isolated and selected for in-depth analysis based on its antifungal activity against *Foc* TR4 and strong colonization ability in banana plants to support a new approach using EB1 to control Fusarium wilt of banana by introducing into banana tissue culture plantlets at the end of rooting stage.

As one of the largest bacterial genera, *Bacillus* strains coexist with plants and are among the most studied microorganisms in the biological control of various plant diseases (Shafi et al., 2017; Fira et al., 2018). For instance, endophytic bacteria *B. mojavensis* and *B. cereus* exhibited potent inhibition activities against various rice Fusarium pathogens such as *F. proliferum, F. verticillioides,* and *F. fujikuroi* (Etesami and Alikhani, 2017). Consistent with these findings, the *in vitro* dual-culture experiment demonstrated that the growth of *Foc* TR4 could be significantly inhibited by EB1. Interestingly, considerable ultra-structural alterations such as wizened, flattened, thickened CW and plasmolysis that were observed in *Foc* TR4 cells at confronting with EB1 indicated that EB1 might be capable of producing antagonistic metabolites,

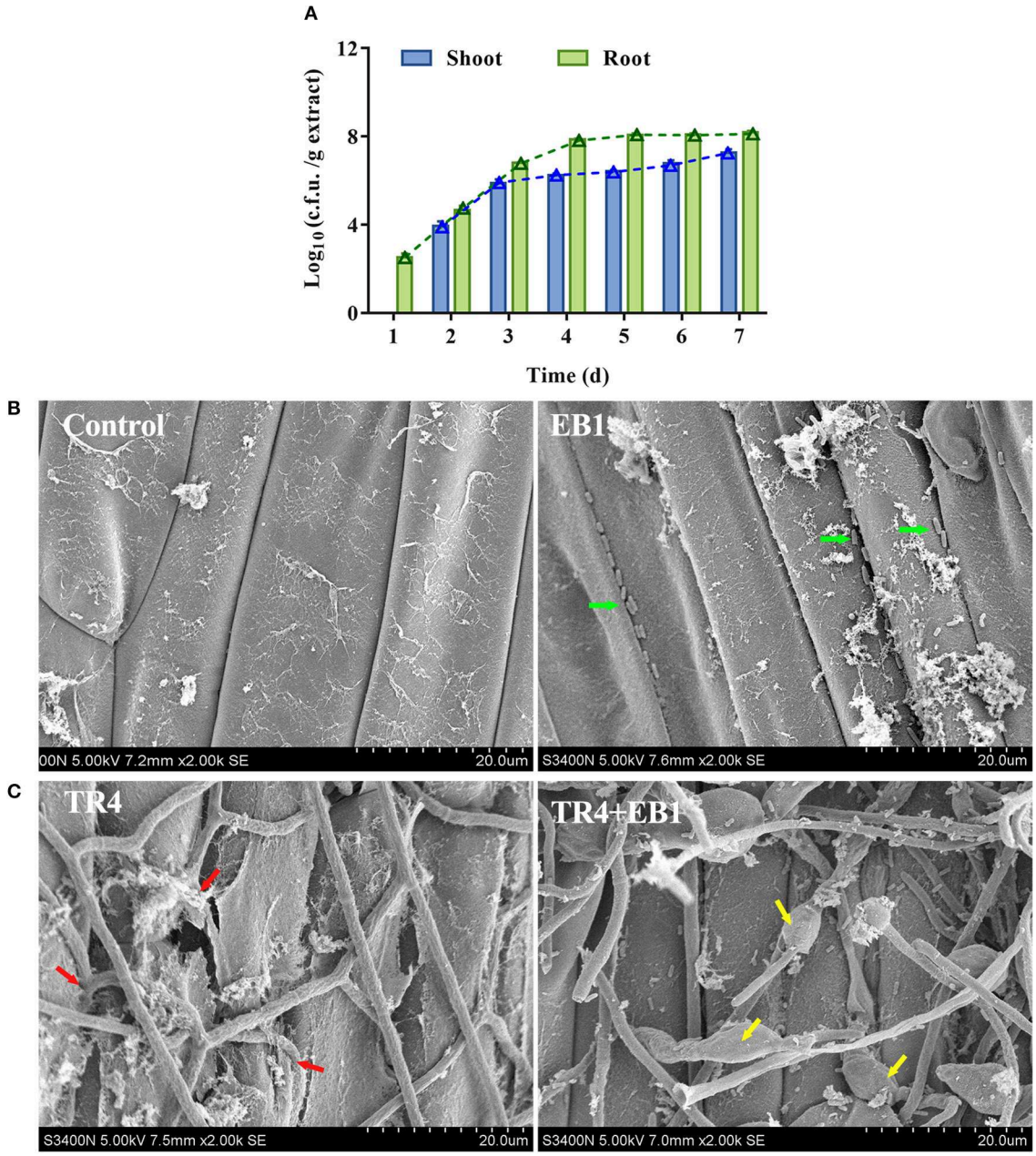

FIGURE 3
Strain EB1 showed strong colonization ability on the banana plantlets and provided protection against the infection of TR4. **(A)** EB1 dynamics (c.f.u. counts) in shoot and root extracts during the first 7 days after banana tissue culture plantlets bacterized. Bars represent average values ± SD of total c.f.u. in shoot and root extracts. Green and blue lines represent c.f.u. corresponding to the number of spores in shoot and root extracts, respectively. **(B)** Root colonization of strain EB1 visualized by scanning electron microscopy (SEM). Strain EB1 colonies occur in grooves between epidermal cells and intercellular space in root cells (marked by green arrow heads). **(C)** Strain EB1 inhibits the infection of TR4 *in planta*. TR4 seriously destroyed the surface structures of the root of banana tissue culture plantlets (marked by red arrow heads) and inoculation of EB1 prior to *Foc* treatment led to serious morphological deformities of *Foc* and the damaging effects by pathogen infection were alleviated (marked by yellow arrow heads).

penetrating into *Foc* TR4 cell, and leading to leakage of cytoplasm and disruption of internal organelles of *Foc* TR4. Accordingly, genes related to the biosynthesis of antifungal compounds such as lipopeptides and ketones, which were proven to inhibit hyphal extension and spore formation of phytopathogens, were identified in EB1 genome by the antiSMASH tool (Arrebola et al., 2010; Li et al., 2015).

Plants can act as a filter of microbial communities and select the right endophytes to maintain their normal growth and development (Dubey et al., 2020; Liu H. et al., 2020). Therefore, stable root colonization and persistence of BCAs in the plant is a key factor for their application in the biological management of microbial diseases (Shafi et al., 2017). Detections of EB1 inside both roots and shoots of banana tissue culture plantlets in our

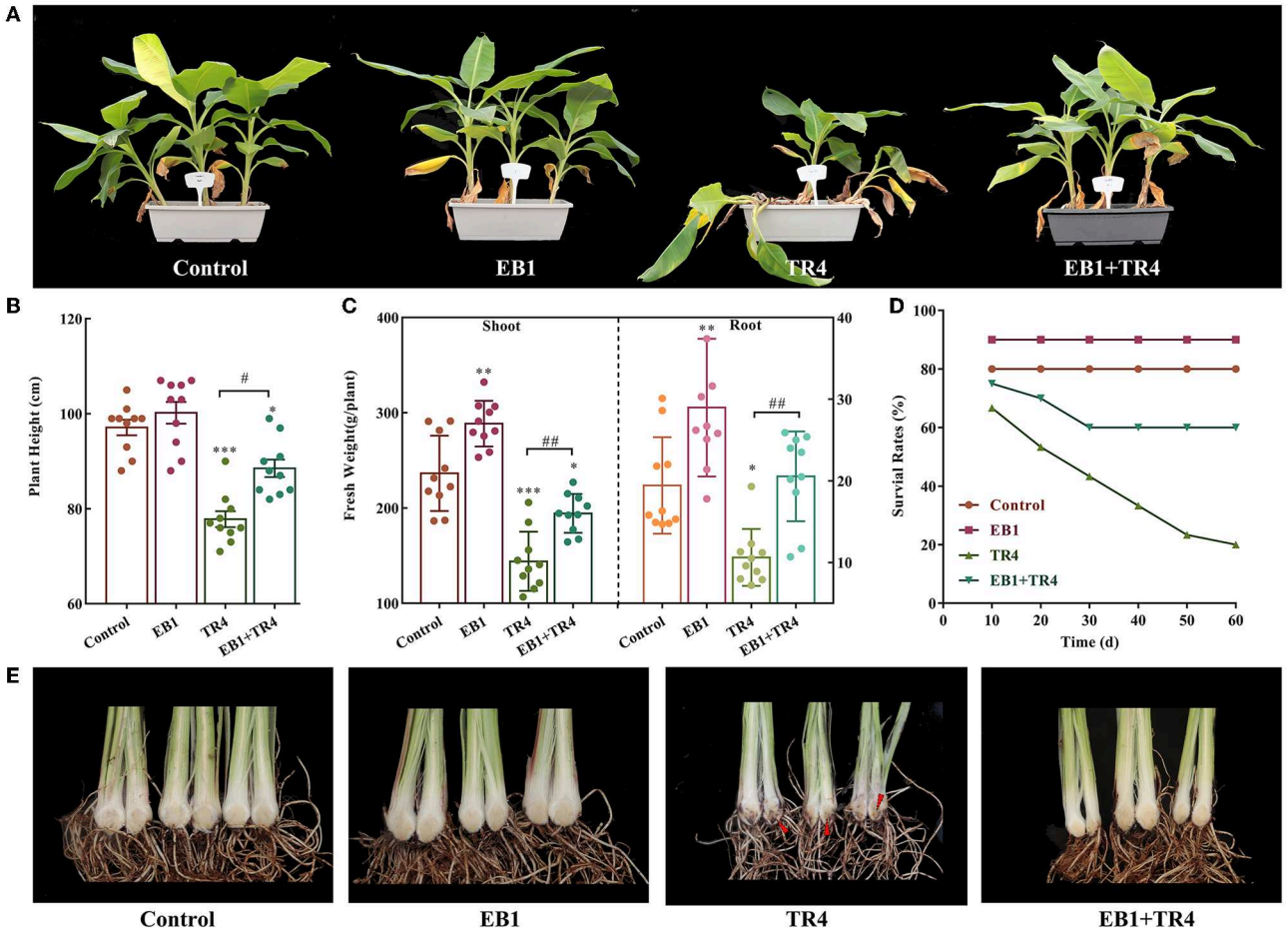

FIGURE 4
Strain EB1 enhances Fusarium wilt resistance and promoted banana growth in the pot experiment. Control, no inoculation; EB1, inoculation with EB1 only, TR4, inoculation with TR4 only; EB1 + TR4, inoculation of EB1 prior to *Foc* treatment. **(A)** Phenotypes of acclimatized banana plants after being primed with EB1 at the rooting stage of tissue culture plantlets. **(B–E)** Plant height **(B)**, biomass of shoots and roots **(C)**, survival rates **(D)**, and severity of Fusarium wilt of acclimatized banana plants under different treatments (marked by red arrow heads) **(E)**. All data are expressed as the mean ± SD of at least 10 replicate samples. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences between the treatment groups and control group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ indicate significant differences between treatment groups.

study supporting EB1 is capable of entering through the root system and migrating upwards into the pseudostem. EB1 is an endophyte isolated from banana pseudostem; thus, it is conceivable that it has evolved strategies for efficient adaptation to this niche. To determine whether EB1 inhibits *Foc* TR4 *in planta*, banana tissue culture plantlets were infected with *Foc* TR4 but only after a prior bacterization with an EB1. Using SEM, EB1 colonies were observed in grooves between root epidermal cells, indicating that the mechanism of entry of EB1 into roots occurs most probably *via* cracks, which also represent the major routes for phytopathogen to enter into plants (Compant et al., 2010; de Zélicourt et al., 2018). Correspondingly, the deformed hyphae of *Foc* TR4 and alleviated host damage were observed *in planta* due to the inoculation of EB1, suggesting that penetration of *Foc* TR4 through cellophane membranes and invasion of banana tissue were impaired upon co-inoculation with EB1. Therefore, EB1 might occupy the ecological niches and nutrition rapidly and act as an extracellular barrier for the host plant for blocking the pathogen invasion (Gao et al., 2010; Shafi et al., 2017; Dubey et al., 2020). It is noteworthy that the response of banana plants toward EB1 bacterization in the rooting stage was maintained and further amplified in the pot experiment. Plants whose roots had been pre-inoculated with EB1 at the rooting stage showed significantly higher survival rates and better growth states compared with those inoculated only with *Foc* TR4. Using antiSMASH, 12 BGCs responsible for the synthesis of 8 secondary metabolites including surfactin, bacilysin, bacillibactin, difficidin, fengycin, bacillaene, macrolactin, and butirosin have been identified in the genome of EB1. Surfactin and fengycin have been widely characterized to mediate biofilm formation and root colonization processes, which are suggested to have a role in plant development and growth promotion (Aleti et al., 2016; Berlanga-Clavero et al., 2022). In addition, putative genes involved in the production of indole-3-acetic acid (IAA), spermidine, and polyamine, which are related to plant growth-prompting activity, have also been discovered in the genome of EB1 (Xie et al., 2014; Zaid et al., 2022). Thus, our results have demonstrated the promising application of endophytic antifungal strains in agriculture to breed "microbe-optimized crops".

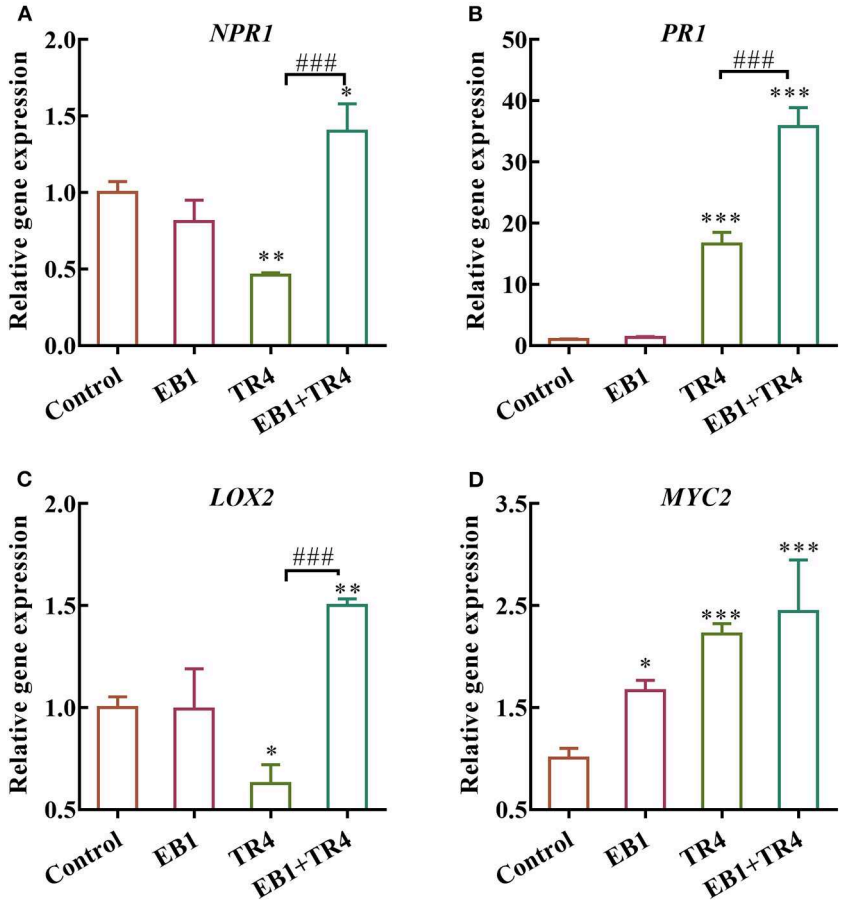

FIGURE 5
Effects of strain EB1 and TR4 inoculations on the expression of defense genes. The expression patterns of **(A)** *NPR1*, **(B)** *PR1*, **(C)** *LOX2*, and **(D)** *MYC2* were analyzed by RT-qPCR. All data are expressed as the mean ± SD ($n = 4$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences between treatment groups and control group. $^{###}p < 0.001$ indicate significant differences between treatment groups.

Different from the fighting to the death in pathogen and host relationship, recent conceptual and experimental framework has indicated that beneficial endophytes usually can evade plant defense and reach a stable harmonious commensalism with the plant (Sessitsch et al., 2012; Deng et al., 2019; Yu et al., 2019). To figure out the role EB1 plays in the three-way interactions with the host plant immunity and the fungal pathogen, expressions of genes known to be markers of plant defense signaling pathways including SA-mediated *NPR1* and *PR1* as well as JA-mediated *LOX* and *MYC2* were analyzed (Mhamdi, 2019). It is found that the expressions of these genes were stronger in plants with EB1 pretreatment and *Foc* TR4 infection than that in plants with pathogen infection only which is in accord with previous research (Chandrasekaran and Chun, 2016; Nie et al., 2017). Similarly, inoculation of wheat with endophytic bacterium *Stenotrophomonas rhizophila* SR80 increased the expressions of a range of genes in SA and JA signaling pathways, but only when the *F. pseudograminearum*, the causal agent of Crown rot disease, was present (Liu H. et al., 2020). Our findings suggested that EB1 plays a key role in the interactions with the host plant immunity and the fungal pathogen *via* a mechanism that enhances plant defense and growth (Khare et al., 2018). A few studies mentioned that beneficial microbes can quench plant immune responses by downregulating the expression of the microbial-associated molecular patterns (MAMPs) (Bardoel et al., 2011; Zamioudis and Pieterse, 2012), producing the MAMPs with a low-elicit ability (Trda et al., 2014), or minimizing the stimulation of plant defensive response (Liu et al., 2018; Deng et al., 2019). Accordingly, the whole-genome annotation data suggest that EB1 contains multiple genes that encode key components that function by these mechanisms. Combined with the localization and *in vitro* data, these observations suggest that EB1 forms a symbiotic relationship with banana plants and efficiently wards off the invasive of *Foc* TR4 *in planta* inferring the adaptability and potential of the banana tissue culture plantlets bio-primed with EB1 could be a promising biological solution for the management of Fusarium wilt of banana.

Conclusion

Our current study focused on providing a comprehensive understanding of the endophytic strain *Bacillus velezensis* EB1 isolated from a healthy banana plant in a wilt-diseased banana field and exploring its potential application in tissue culture plant of banana for the environmental sustainability management of

Fusarium wilt based on its strong antagonistic effects against the devastating fungal pathogen *Foc* and mutualistic functional roles with banana plants. To realize large-scale implementation of microbial strains in agricultural practice, new strategies for successful delivery of BCAs into plant under field conditions are needed. Therefore, in the future, we intend to (1) understand the underlying molecular mechanisms of the beneficial effect of EB1 on the growth and stress tolerance of banana plants, (2) isolate more efficient, multifunctional, stress tolerant microbes and design an artificial disease suppressive synthetic community (SynCom) which comprised by multiple microbial strains rather than mono-strain inoculums to take advantage of functional complementarity to mimic a natural disease-suppressive community in plants, and (3) develop bioformulations for sustainable application of endophytic microbes in plant tissue culture. New strategies for the successful delivery of BCAs into the plant under field conditions are needed to realize the large-scale implementation of microbial strains in agricultural practice. The introduction of endophytic microbes, as a probiotic material that enhances plant growth as well as induces defense responses of plants to cope with stress, into tissue-cultured banana plantlets, could be a novel and stable biological control method to protect bananas from *Foc* infection.

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

DX and SL: conceptualization. DX, XY, BL, and YC: experimentation. DX, XY, and CL: review and drafting. SL and CL: validation and statistical analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1146331/full#supplementary-material

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Effect of natural weed and Siratro cover crop on soil fungal diversity in a banana cropping system in southwestern China

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Introduction: Natural weed cover and a legume cover crop were examined to determine if they could impact soil fungal diversity as an indicator of soil quality in banana production.

Methods: Banana in Yunnan Province, China, was grown under three treatments: conventional tillage (bare soil), natural weed cover (primarily goosegrass (*Eleusine indica* (L.) Gaerth)), or a cover crop (Siratro (*Macroptilium atropurpureum* (DC.) Urb.)). Analysis of the soil fungal communities between 2017 and 2020 was done by Illumina Miseq high-throughput sequencing.

Results: Most significant effects were in the intercropping area for the treatments, whereas it was rarely observed in the furrow planted with banana. Based on the Shannon and Simpson diversity indices, soil fungal diversity in the intercropping area significantly decreased following planting banana in 2017 with all three treatments. However, both the Shannon and Simpson diversity indices showed that there were significant increases in fungal soil diversity in 2019 and 2020 with natural weed cover or Siratro compared to bare soil. At the end of the experiment, significant increases in fungal genera with Siratro compared to bare soil were observed with *Mortierella*, *Acremonium*, *Plectophaerella*, *Metarhizium* and *Acrocalymma*, and significant decreases were observed with *Fusicolla*, *Myrothecium*, *Exserohilum*, *Micropsalliota* and *Nigrospora*. Siratro resulted in higher stability of the soil fungal microbiome by increasing the modularity and the proportion of negative co-occurrences compared to bare soil. For fungal guilds, Siratro significantly increased saprotrophs_symbiotrophs in 2019 and 2020 and significantly decreased pathogens_saprotrophs in 2020 compared to bare soil.

Discussion: Using Siratro as a cover crop in the intercropping area of banana helped maintain soil fungal diversity, which would be beneficial for soil health with more symbiotrophs and less pathogens in the soil. However, further research is needed to determine the long-term impact of weed or Siratro cover crop on the fungal soil ecosystem and growth of banana.

KEYWORDS

cover crop, banana, soil fungal communities, soil microbial diversity, co-occurrence network

1. Introduction

Banana (*Musa* spp.) is a perennial monocotyledonous plant with the nutrient-rich fruit, which is an important cash crop in tropical and subtropical regions worldwide (Bubici et al., 2019). Conventional banana production in China can result in serious soil degradation, such as soil nutrient loss, acidification, compaction, salinization, and increased disease (Chen et al., 2018) Nevertheless, soil is usually considered an important natural resource for maintaining the function and sustainability of terrestrial ecosystems, which is a living, dynamic and non-renewable resource on a human time scale (Evans et al., 2022). Therefore, sustainability is becoming an urgent issue for agro-ecosystems (Drost et al., 2020), and maintaining beneficial soil properties to improve sustainability is a priority to ensure sustainable banana production in China and worldwide.

Soil physical and chemical properties are traditionally considered key elements of soil fertility, and are considered to be intrinsic and relatively static properties (Taboada et al., 2011). However, soil biological properties are highly dynamic playing crucial roles in soil nutrient cycling and function (Jian et al., 2020). For example, agricultural practices, such as deep tillage and high nitrogen fertilization, can negatively affect soil biology (Nivelle et al., 2016). Intensively managed mono-cropping can result in soil biodiversity losses dramatically reducing key soil functions (Tao et al., 2022). Alternative farming practices provide a way to reduce the negative effects of agricultural production on soil. These include using conservation tillage, rotation, intercrops, fallow periods and cover crops (Serebrennikov et al., 2020).

Cover crops can help retain soil moisture and nutrients, improve soil quality, and enhance the soil productivity (Blanco-Canqui and Ruis, 2020). There have been a number of examples for its use in banana. For example, intercropping banana with a mixture of *Alysicarpus ovalifolius*, *Brachiaria decumbens*, *Chamaecrista rotundifolia*, *Cynodon dactylon*, *Dichondra repens*, *Macroptilium atropurpureum*, *Neonotonia wightii*, *Paspalum notatum*, *Pueraria phaseoloides*, and *Stylosanthes* spp. as a cover crop improved crop productivity and did not result in competition with banana for nitrogen (Tixier et al., 2011). Also, planting a mixture of *Paspalum notatum*, *Neonotonia wightii*, *Pueraria phaseoloides*, and *Stylosanthes guyanensis* as a cover crop increased yield of banana and reduced nematode damage (Djigal et al., 2012). Planting *Canavalia ensiformis*, *Cajanus cajan*, *Crotalaria* spp., *Sorghum bicolor* and *Phaseolus lunatus* as cover crops resulted in significant weed control in banana and increased yield (Ávila et al., 2020).

A common cover crop is Siratro, also known as purple bush-bean [*Macroptilium atropurpureum* (DC.) Urb.] (Chatterjee, 2021). It is a perennial climbing legume with dense vines that originated in tropical and subtropical regions of the Americas (Rojas-Sandoval, 2018). It can improve soil physical–chemical properties, such as soil pH and levels of calcium and magnesium (Espindola et al., 2005), and leaves large amounts of organic residues that can increase organic carbon, nitrogen and potassium levels (Macharia et al., 2011). Such changes can also indirectly affect soil microbial diversity. Examples of its use of a cover crop include planting Siratro to improve soil fertility and maize biomass production (Abayomi et al., 2001), and using it as a green manure for passion-fruit and rice (Werasopon et al., 1998; Gama-Rodrigues et al., 2007). Examples of its use with banana include using it as a green manure to increase nutrients, particularly N, Ca and Mg (Espindola et al., 2006), and rotating it with banana to reduce plant-parasitic nematode damage (Risède et al., 2009).

Soil microbial diversity is considered to be a key parameter in evaluating soil health in agricultural ecosystems (Tahat et al., 2020). Microbial functional diversity as well depends on the diversity of the microbes present (van Capelle et al., 2012). However, the biogeochemical processes affected by soil microorganisms are also highly sensitive to variation in environmental factors (Cycoń et al., 2019). One way to increase soil microbial diversity is to increase plant diversity (Venter et al., 2016). This can be done with cover crops. For example, a cover crop of black medic resulted in higher alpha diversity in soil bacterial communities (Lupwayi et al., 2018), a cover crop mixture of triticale, rye and common vetch resulted in higher soil fungal diversity (Schmidt et al., 2019). Increasing soil fungal diversity is important as soil fungi can benefit plants, such as by increasing phosphate availability (Barroso and Nahas, 2005), decaying organic matter to release nutrients (Argiroff et al., 2022), degrading toxic compounds in soil (Chang et al., 2016), increasing antagonisms to pathogenic microbes (Miao et al., 2016) and improving soil structure by binding soil particles (Miller and Jastrow, 2000). Despite the benefits reported for cover crops in improving soil health and plant production, there is little work done on cover crops for such benefits in banana cropping systems.

In this study, a banana plantation was established in 2017 in a dry hot valley in southwestern China with the rows between the banana plants having either conventional tillage to maintain bare soil, a natural weed cover allowed to develop, or Siratro planted as a cover crop. The soil beneath the banana plants and between the plants was analyzed for fungal diversity, functional guilds and co-occurrence until 2020. The goal was to better understand how soil fungi respond to the cover crop to improve banana plant soil management.

2. Materials and methods

2.1. Site description

A site was chosen at the Science and Technology Demonstration Park of Institute of Tropical and Subtropical Cash Crops of Yunnan Academy of Agricultural Sciences, Lujiangba (N24°57′58″, E 98°53′14″) Baoshan, Yunnan, China. The climate type is dry-hot subtropical. Mean precipitation was approx. 750 mm, mean annual pan evaporation was greater than 2,100 mm, mean annual temperature was 21.3°C, maximum temperature was 40.4°C, minimum temperature was 0.2°C, annual accumulated temperature ≥ 10°C was 7,800°C, annual sunshine duration was 2,333.7 h, altitude was 700 m, and relative humidity was 70%. The soil was sandy loam soil. The soil physio-chemical parameters were pH 6.71, organic matter 11.36 g kg^{-1}, alkaline nitrogen 57.84 mg kg^{-1}, available phosphorus 28.50 mg kg^{-1} and available potassium 95.86 mg kg^{-1}.

2.2. Plot design, treatments, and soil sampling

A randomized complete block factorial design was established in 2017 with four replications with each plot was 135 m^2 with 35 banana

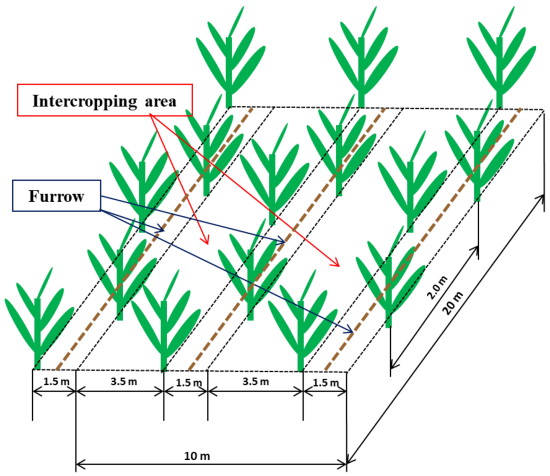

FIGURE 1
Diagram showing the 3.5m intercropping area and 1.5m furrow containing the banana plants within an individual plot.

plants (cv. Yunjiao No. 1). The treatments were conventional tillage with bare soil (CT), natural weed cover that was mainly goosegrass, *Eleusine indica* (WC) and Siratro cover (SC). Each plot contained 3.5 m and 1.5 m rows defined as the intercropping area and furrow, respectively (Figure 1). Banana seedlings were planted 26 July 2017 in the furrows, and the CT, WC and SC treatments were applied to the intercropping area. CT treatment was sprayed with herbicide monthly to remove weeds, WC treatment allowed weeds to grow into the intercropping area, except for 30 cm from the banana seedlings in year one to avoid shading of the banana plants, and SC treatment was *M. atropurpureum* planted in 2017 at a density of 22.5 kg hm^{-2} to provide more than 60% plant coverage of the intercropping area. A micro-sprinkler irrigation system was used to apply water in the furrows once a week in the dry season (October to May) as well as several times as needed in rainy transition season (August and September). No irrigation was done in the rainy season (June to July), and there was no irrigation of the intercropping area. Fertilizer (40% urea, 40% compound (N: P: K = 15: 15: 15) and 20% potash) was applied twice a month at 1.5 kg per plant per year, and there was no fertilization of the intercropping area.

To collect soil for DNA analysis, soil was sampled from five random locations in each plot using a manual soil auger to the depth of the tilled layer (30 cm) in July, which was the time of planting banana in 2017 and the period of fastest growth of the cover crop during the warm late rainy season each year until 2020. Soil from the five locations per plot were combined to form one sample per plot. The soil was sieved (≤2 mm), and approximately 20 g of soil was stored at −80°C for DNA extraction.

2.3. DNA extraction and sequencing

Total genomic DNA was extracted from each sample using the OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, United States), following the manufacturer's instructions, and stored at-20°C. The quantity and quality of the DNAs were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis. The concentration of DNA, OD 260/280 ratio, OD260/230 and the number of analyzed sequences of each sample are presented in Supplementary Table S1, and the number of total NGS reads, quality filtered reads, read length and SE/PE reads per sample are given in Supplementary Table S2.

PCR amplification of the fungal ITS1 region was performed using the forward primer ITS5F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and the reverse primer ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCRs contained 5 μL Q5 reaction buffer (5×), 5 μL Q5 HighFidelity GC buffer (5×), 0.25 μL Q5 High-Fidelity DNA Polymerase (5 U/μL), (2 μL 2.5 mM) dNTPs, 1 μL each 10 μM ITS5F and ITS1R primers, 2 μL DNA template, and 8.75 μL ddH_2O. Thermal cycling consisted of initial cycle of 98°C for 2 min, followed by 25 cycles of 98°C for 15 s, 55°C for 30 s, and 72°C for 30 s, with a final cycle of 5 min at 72°C. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, United States) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). Paired-end 300 bp sequencing was performed using the Illlumina MiSeq platform with MiSeq Reagent Kit v3 (Shanghai Personal Biotechnology Co., Shanghai, China). The sequence data for all samples were deposited at NCBI, accession number PRJNA894310.

2.4. Sequence analysis

Microbiome analysis was performed with QIIME2[1] with slight modifications. Briefly, raw sequence data were demultiplexed using the demux plugin followed by primer removal with the cutadapt plugin (Magoč and Salzberg, 2011). Sequences were then quality filtered, denoised, merged and chimeras removed using the DADA2 plugin (Callahan et al., 2016). Non-singleton amplicon sequence variants (ASVs) were aligned with mafft (Katoh et al., 2002), and trees were constructed with fasttree2 (Price et al., 2009). Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in the feature-classifier plugin (Bokulich et al., 2018) against the UNITE Release 8.0 Database.

2.5. Statistical analysis

All analysis was done with R 4.1.2.[2] Alpha diversity indices were calculated by analyzing Chao1 richness and Shannon diversity in the Vegan package in R (Oksanen et al., 2013). β-diversity and construction of non-metric multidimensional scaling (NMDS) plots were also performed in the Vegan package in R. Normalized ASVs were analyzed using Bray Curtis metrics (Bray and Curtis, 1957). Bray-Curtis distance matrices were subjected to multivariate analysis of variance (PERMANOVA) (Anderson, 2001) to compare fungal community composition and abundance using the Adonis function with a permutation number of 999 in the Vegan package in R. Linear

1 https://docs.qiime2.org/2019.4
2 https://cran.microsoft.com/snapshot/2022-02-11/bin/windows/base/

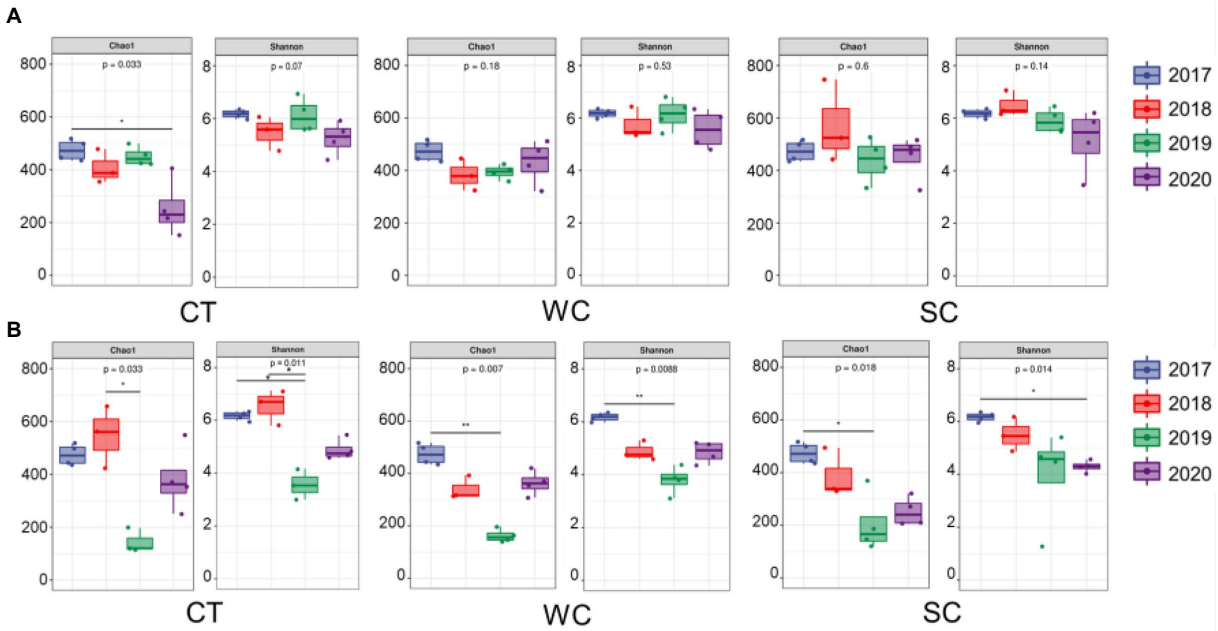

FIGURE 2
Chao1 and Shannon soil fungal diversity indices over years with conventional tillage (CT), weed cover (WC), and Siratro cover (SC) treatments in the intercropping area **(A)**, and furrow **(B)**. *, **, *** indicates $p \leq 0.05$, 0.01, and 0.001, respectively.

discriminant analysis (LDA) effect size (LEfSe) was used to compare taxonomic features between groups by the Python LEfSe package in R with an LDA threshold of 2.0 and an alpha value of 0.05. A heatmap was constructed for the statistically dominant genus (LDA $p < 0.05$) using the 'heatmaply' package in R (Galili et al., 2018). Fungal ASVs were assigned into functional guilds using the online application FUNGuild[3] (Nguyen et al., 2016). Co-occurrence patterns were reconstructed by Hmisc package in R and Gephi 0.9.2, A co-occurrence was considered to be robust if the Spearman's correlation coefficient (r) was >0.80 and $p < 0.05$. Network stability was measured by the proportion of negative or positive correlations and the modularity (Gao et al., 2021).

3. Results

3.1. Soil fungal diversity and richness

For the intercropping area with CT treatment, the Chao1 diversity index of soil fungi showed significantly less diversity after planting in 2020 compared to before planting in 2017, and the Shannon diversity index showed lower diversity in 2018 and 2020 compared to 2017 (Figure 2A). With WC treatment, there was significantly less diversity compared to 2017 in 2018 and 2019 with the Chao1 index and 2018 with the Shannon index. With SC treatment, there was only a significant difference in diversity between 2017 and 2020 with the Shannon index. Thus, soil fungal diversity in the intercropping area was generally least reduced with the SC treatment and most reduced with the CT treatment. A comparison between years showed that no treatment resulted in significant differences in soil fungal diversity in the intercropping areas (Supplementary Figure S1).

In the furrow area of the CT plots, there was a significant decrease in diversity between pre-planting in 2017 and post planting in 2019 and 2020 detected with both indices (Figure 2B). In the furrow area of the WC and SC plots, both indices showed that diversity significantly decreased detected in all years after planting compared to pre-planting Thus, soil fungal diversity was always detected to be reduced after planting, except for 2018 in the furrow with CT treatment. It appears that the treatments were less effective in maintaining fungal soil diversity in the furrow where the banana plants were located than in the intercropping area. Similar to the intercropping area, no treatment resulted in significant differences in soil fungal diversity between years in the furrow area (Supplementary Figure S1).

3.2. Soil fungal community composition

Analysis of the soil fungal community composition by NMDS (Figure 3A) and PERMANOVA (Figure 3B) showed that it was significantly affected in the intercropping area by year primarily in 2019 and 2020 for all treatment comparisons. In the furrow area, soil fungal community composition by NMDS (Figure 3C) and PERMANOVA (Figure 3D) was significantly affected only in 2020 for the CT-SC and WC-SC treatment comparisons. Figure 3A shows clustering but the separation of the clusters is much less than the intercropping area in Figure 3C. While we mostly focused on the intercropping area with the treatments, but the furrows were somewhat affected by the same management. While PERMANOVA analysis showed significant effects of the treatments on soil fungal community in both the intercropping area and furrow. These results indicate that both areas were affected by year and treatment, but more

3 http://www.stbates.org/guilds/app.php

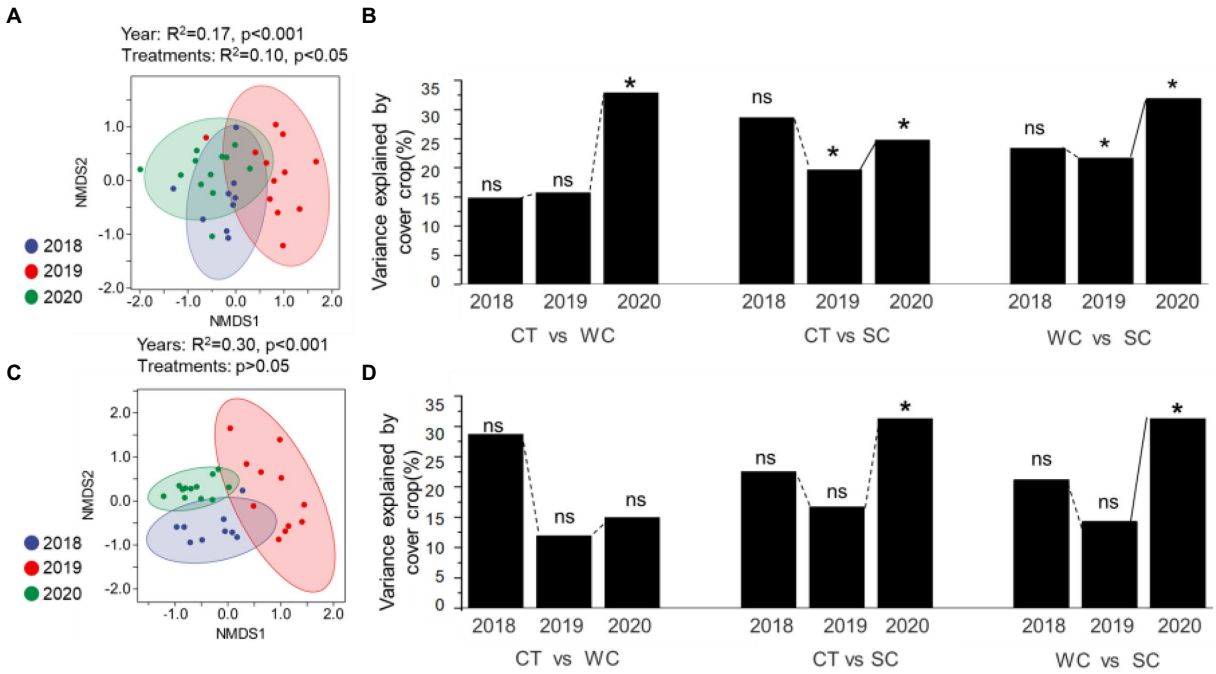

FIGURE 3
Soil fungal community composition variation over years with conventional tillage (CT), weed cover (WC), and Siratro cover (SC) treatments. NMDS and PERMANOVA analysis of the intercropping area (**A**,**B**, respectively), and furrow (**C**,**D**, respectively). *Indicates significance at $p \leq 0.05$, and ns indicates not significant.

significant differences were observed by treatment for the intercropping area, which is where the treatments were applied. Consistent with this is that the SC treatment thus appeared to have earlier effects on soil fungal community composition in the intercropping area than the furrow (Supplementary Figure S2).

3.3. Dominant soil fungal groups

At the fungal family level, there were significant differences in relative abundance between treatments using Lefse analysis in the intercropping area in 2019 and 2020 (Figures 4A,B). The number of families with the highest relative abundance remained at two in 2019 and 2020 with CT treatment, whereas it increased from three to four with the WC treatment and two to five with the SC treatment. The change over time in the number of highest abundant fungal families was mostly but not entirely due to the new families being included. There were also significant differences in relative abundance between treatments in the furrow in 2020 (Figure 4C). Unlike the intercropping area, however, most of the families with highest relative abundance in 2020 were with the CT treatment in the furrow. The only shared fungal family in this analysis between the intercropping area and furrow in 2020 was the Clavicipitaceae with the SC treatment.

At the end of the experiment in 2020, significant differences between treatments were observed among the 30 most abundant genera in the intercropping area (Supplementary Table S3). For SC compared to CT treatment, there were significantly higher levels of *Mortierella, Acremonium, Plectophaerella, Metarhizium* and *Acrocalymma*, but significantly lower levels of *Fusicolla, Myrothecium, Exserohilum, Micropsalliota* and *Nigrospora*. For SC compared to WC treatment, significantly higher levels of *Mortierella, Acremonium, Plectosphaerella* and *Acrocalymma*, but significantly lower levels of *Fusicolla, Myrothecium, Micropsalliota, Nigrospora, Pyrenochaetopsis, Corynascella, Poaceascoma*, and *Dokmaia*, were observed. For WC compared to the CT treatment, there were significantly higher levels of *Fusicolla, Metarhizium, Micropsalliota, Nigrospora* and *Dokmaia*, but significantly lower levels of *Trechispora* and *Mycosphaerella*.

At the end of the experiment in 2020, significant differences were also observed between treatments among the 30 most abundant genera in the furrow (Supplementary Table S4). There were significantly higher levels of *Trechispora* and significantly lower levels of *Acrocalymma, Micropsalliota, Fusicolla, Nigrospora* and *Metarhizium* with SC compared to CT treatment, and there were significantly higher levels of *Trechispora* and significantly lower levels of *Fusicolla* and *Metarhizium* with SC compared to WC treatment. For WC compared to CT treatment, significantly higher levels of *Micropsalliota* and *Fusicolla* were observed, but no genera significantly were observed with lower levels.

3.4. FUNguild functional prediction

Significant differences in functional prediction using FUNguild were observed due to treatments in intercropping area in 2018, 2019, and 2020 (Figures 5A–C). Compared to CT treatment, SC treatment resulted in significantly higher saprotroph_symbiotroph guild in 2019 and 2020, and significantly lower pathotroph_saprotroph guild in 2020. There was also significantly higher pathogen_saprotroph_symbiotroph guild with SC compared to WC treatment in 2020. The only significant difference between treatments in the furrow was for a

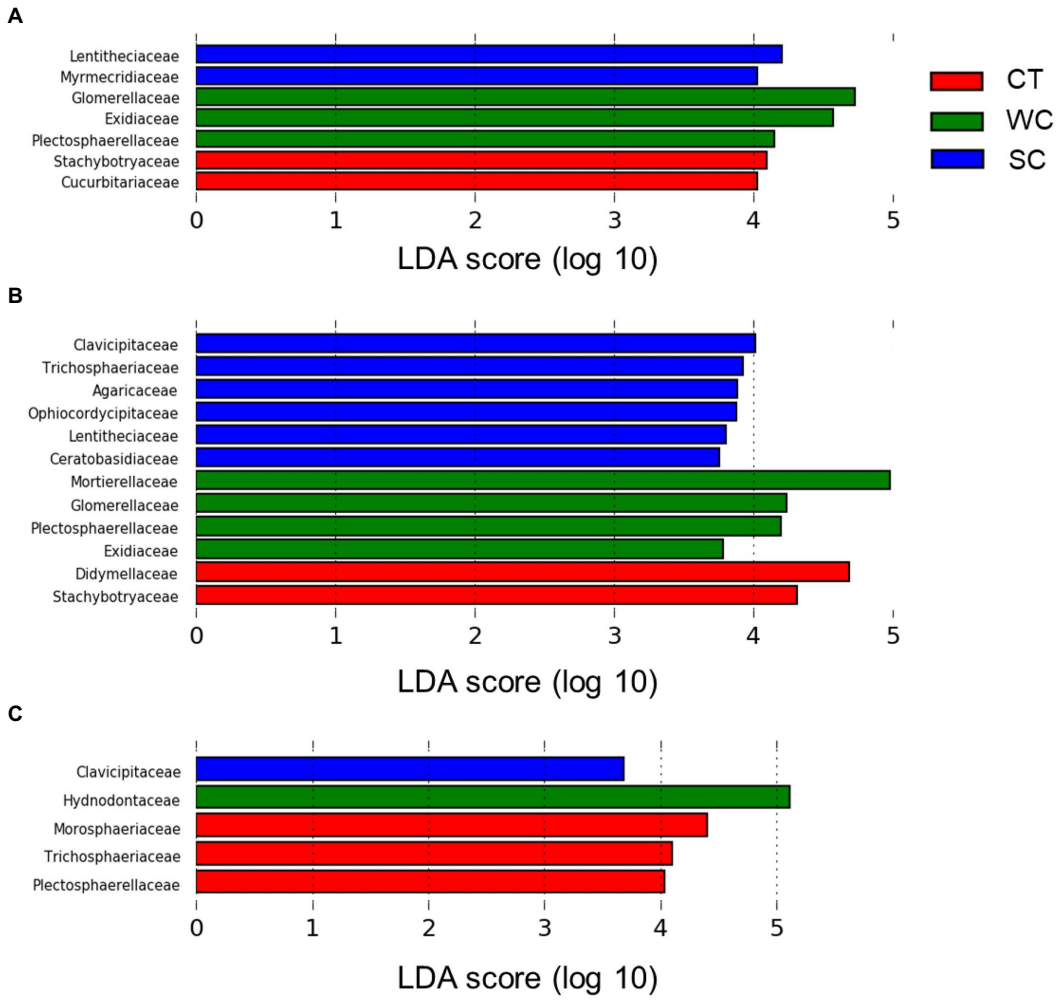

FIGURE 4
LDA score distribution at the fungal family level for conventional tillage (CT), weed cover (WC), and Siratro cover (SC) treatments in the intercropping area in 2019 **(A)**, 2020 **(B)** and the furrow in 2020 **(C)**.

significantly lower unknown guild with SC treatment in 2018, 2019, and 2020 (Supplementary Figures S3A–C).

3.5. Co-occurrence network analysis

PERMANOVA analysis of soil fungal community members with a relative abundance above 0.1% revealed significant differences between treatments in the intercropping area in 2019 and 2020, showing that the data from those years were suitable for the co-occurrence network analysis (Supplementary Table S3). SC treatment resulted in a higher stability of the soil fungal community with a modularity index of 2.00 compared to 1.47 and 1.51 for CT or WC treatments, respectively (Figure 6A). Based on edge analysis, the number of positive co-occurrences was 170, 191, and 158 for CT, WC and SC treatments, respectively, whereas the number of negative co-occurrences was 73, 93, and 94 for CT, WC, and SC treatments, respectively (Figure 6B). Thus, WC treatment had the most positive co-occurrences, and SC treatment had the most negative co-occurrences among the treatments. There were no significant differences in the degrees of the soil fungal community among the treatments indicating no significant differences in soil fungal community complexity (Figure 6C). PERMANOVA analysis of soil fungal community with a relative abundance more than 0.1% revealed no significant differences between treatments in 2018, 2019, and 2020 (Supplementary Table S5). Thus, that data in the furrow area was not suitable for the co-occurrence network analysis.

4. Discussion

Perennial cover crops are often used to improve soil properties of tropical fruit crops, even without the need to plow and incorporate the cover crop into the soil (Wei et al., 2021; Freidenreich et al., 2022). However, they are more difficult to use in banana cropping systems compared to other tropical fruits, as banana leaves create a large amount of shade limiting the growth of most cover crops. In addition, many banana producers, such as those in Yunnan province in China, do not use cover crops as they believe that cover crops would compete with banana for water and fertilizer. However, the lack of cover crops has contributed to banana soil degradation and possibly to higher levels of root diseases, such as banana Fusarium wilt disease (Shen

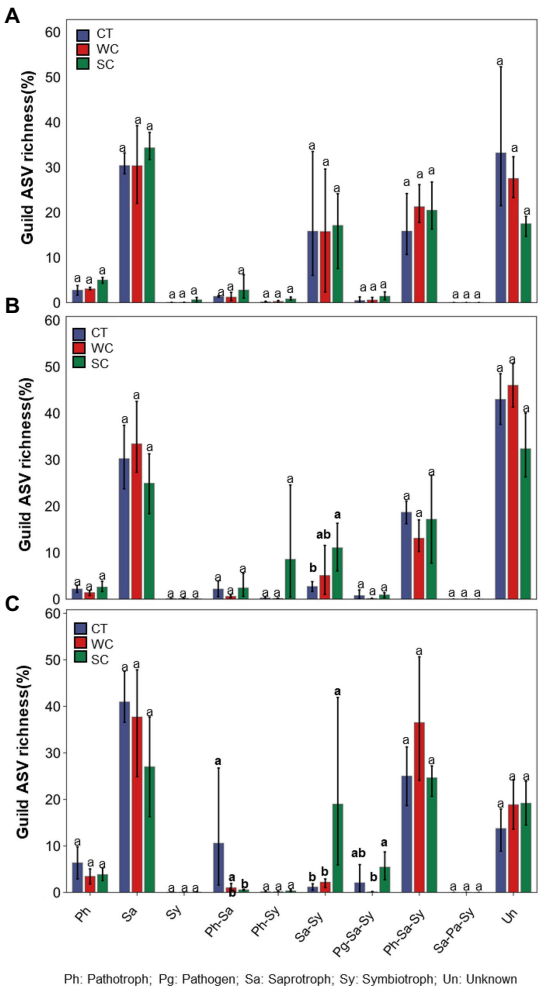

FIGURE 5
Funguild predictions of functional fungal guild ASV richness of the conventional tillage (CT), weed cover (WC) and Siratro cover (SC) treatments in the intercropping area in 2018 **(A)**, 2019 **(B)** and 2020 **(C)**. Means within the same year followed by the same letters are not significantly different at p=0.05 according to a protected LSD test.

et al., 2018; Hong et al., 2020). One benefit of cover crops is to increase soil fungal diversity and stability with crops, such as an alfalfa cover crop in apple orchards (Wang et al., 2022) and barley or vetch cover crops in sunflower or maize fields (García-González et al., 2016). Soil fungi are sensitive to the environmental factors, such as soil moisture, pH, temperature and organic matter, all of which can be affected by cover crops (Hamman et al., 2007; Wang et al., 2014).

While Siartro has been widely studied as a cover crop with a wide variety of crops worldwide including banana (Werasopon et al., 1998; Abayomi et al., 2001; Espindola et al., 2006; Gama-Rodrigues et al., 2007; Risède et al., 2009), it has been studied in China much less often, but with reports showing benefits in orange and mango orchards (Li et al., 1996; Dong et al., 2016). As this study was too short to determine effects on banana yield, the impact of the Siartro cover crop was assessed by examining the soil fungal microbiome over 3 years comparing it to conventional tillage where there was bare soil, which is the most common current practice in Yunnan province, or allowing weeds to naturally invade, primarily goosegrass, which is also often observed in banana plantations in that region.

In the intercropping area, the Siratro cover crop treatment resulted in a generally much less reduced soil fungal diversity and richness after planting banana compared to conventional tillage or a natural weed cover. In the furrow, however, soil fungal diversity and richness showed a decreasing trend with all treatments, including Siratro, compared to soil before banana was planted. Soil fungal community composition was also more rapidly affected by treatments in the intercropping area than in the furrow. The greater effect of Siratro cover crop where it was grown in the intercropping area compared to the adjacent furrow could be due to the localized nature of the rhizosphere effect where nutrients and other compounds are released in root exudates affecting microbes in the adjacent soil (Dotaniya and Meena, 2015). The rhizosphere likely was the dominant effect as the Siratro cover crop in this study was perennial and not incorporated into the soil resulting in plant residues being limited to senescent tissues. Legume root exudates contain nutrients, such as organic acids, flavonoids, and fatty acids, and signaling molecules, such as flavonoids and strigolactones, that affect soil fungi (Sugiyama and Yazaki, 2012). In contrast, the furrow had no Siratro planted and had virtually no naturally occurring weeds due to shading by the banana leaves, and thus soil fungi would be affected only by the rhizosphere effect from the banana roots.

Among two different cover plants in this study, Siratro showed a greater effect in the intercropping area than the naturally occurring goosegrass. Goosegrass is a highly invasive weed that creates significant competition to crops, negatively affecting crop yields, such as in cotton and maize (Rambakudzibga et al., 2002; Wu et al., 2015). It can also increase diseases by acting as an alternative host for pathogens, such as for the Fusarium wilt fungus of banana (Catambacan and Cumagun, 2022). However, goosegrass will still affect the soil microbiome through its rhizosphere effect, which can have beneficial impacts on soil microbes, such as increasing bacterial populations for bioremediation (Lu et al., 2010). Goosegrass belongs to the Poaceae and is an aggressively reseeding annual, while Siratro belongs to the Fabaceae and is a perennial. In addition to belonging to different plant families with different root exudate compositions and interactions with soil microbes, Siratro would thus be affecting the soil microbiome throughout the year, while goosegrass would die in the winter time, thus affecting the soil microbiome less *via* root exudates compared to Siratro and more *via* tissue decay. Goosegrass is not used as a cover crop, likely because of its aggressiveness, whereas Siratro has often been shown to be a desirable cover crop providing benefits to soil quality (Kocira et al., 2020), soil nitrogen (Virk et al., 2022) and soil microbial populations (Cattelan and Vidor, 1990).

Among the 30 most abundant fungal genera in the intercropping area, 23 would be considered saprotrophs, whereas *Acremonium* and *Trichoderma* would be considered symbiotrophs, *Metarhizium* would be considered an insect pathotroph, and *Exserohilum*, *Aspergillus*, *Plectosphaerella* and *Mycosphaerella* would be considered plant pathotrophs. The Siratro cover crop resulted in higher saprotroph_symbiotroph and lower pathotroph_saprotroph guilds compared to conventional tillage. In contrast, goosegrass weed cover resulted in only lower fungi in the unknown guild compared to conventional tillage. Among 30 most abundant fungal genera in the intercropping area, 5 genera were significantly increased with Siratro cover crop compared to conventional tillage, including *Acremonium* that could be contributing to the increase in saprotrophs_symbiotrophs. Also among those, 5 genera were significantly decreased with Siratro

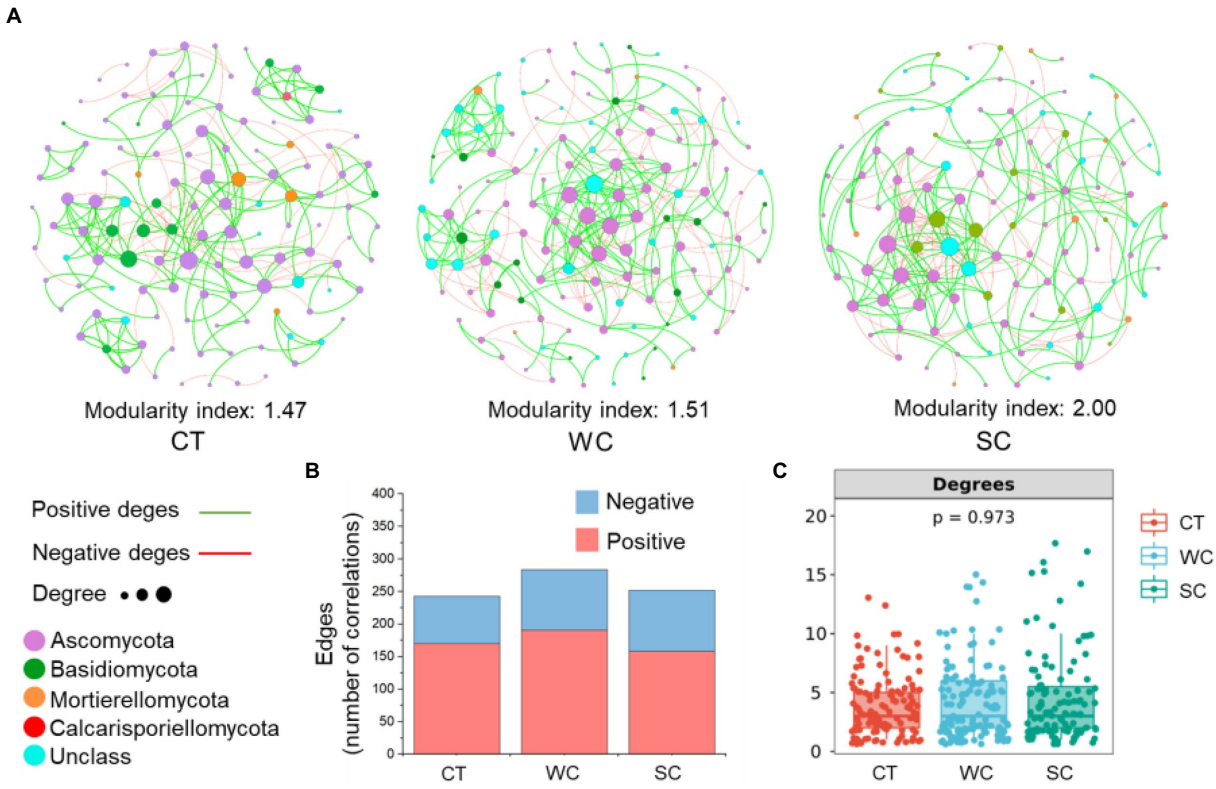

FIGURE 6
Co-occurrence networks with the conventional tillage (CT), weed cover (WC) and Siratro cover (SC) treatments in intercropping area. Modularity index and co-occurrence networks at the fungal phylum level in 2019 and 2020 **(A)**. Number of positive and negative edges of two co-occurrences **(B)**. Degrees of co-occurrences **(C)**.

relative to conventional tillage, and *Mortierella, Acrocalymma, Myrothecium* and *Exserohilum* could be contributing to the decrease in pathotrophs_saprotrophs. However, less abundant fungal genera would also have an impact. Some other examples where cover crops affected the abundance of soil fungal guilds are Wei et al. (2021) where grass cover crops increased pathotrophs and decreased saprotrophs, Benitez et al. (2016) where legume cover crops increased symbiotrophs, and Aiyer et al. (2022) where a sorghum–sudangrass cover crop decreased pathotrophs and increased symbiotrophs while an alfalfa cover crop increased pathotrophs. Thus, the effect of cover crops on soil fungal guilds appears to highly dependent upon the type of plant used. However, the use of the FUNGuild database in this and other studies has limitations as the existing literature on soil fungi is also limited with the functions of approx. 60% of soil fungi yet to be determined (Nguyen et al., 2016).

Among the 30 most abundant genera in the intercropping area, the Siratro cover crop significantly increased *Mortierella, Acremonium, Plectophaerella, Metarhizium* and *Acrocalymma* numbers relative to conventional tillage, which could impact banana production. Increased levels of *Mortierella* spp. could benefit banana, such as *Mortierella capitata* that increased available phosphorus and mycorrhizal populations with maize (Li et al., 2020) and *Mortierella elongata* that increased *Populus* biomass (Zhang et al., 2020). Increased levels of *Acremonium* spp. could benefit banana as many species can enhance plant insect resistance (Breen, 1994) and plant stress resistance, such as drought (Siegel, 1993). However, increased levels of *Plectophaerella* spp. might be harmful, such as *Plectosphaerella cucumeria* that causes root rot of banana (Kanakala and Singh, 2013). Increased levels of *Metarhizium* spp. might benefit banana as three entomopathogenic *Metarhizium* spp. increased maize yields by colonizing roots allowing seedlings to establish earlier (Liao et al., 2014). Increased levels of *Acrocalymma* spp. might also benefit banana as *Acrocalymma vagum* promoted growth of liquorice plants (He et al., 2019).

Compared to conventional tillage, the Siratro cover crop significantly decreased *Fusicolla, Myrothecium, Exserohilum, Micropsalliota* and *Nigrospora* in the 30 most abundant genera in the intercropping area. Decreased levels of *Fusicolla* spp. could be unfavorable for banana as *Fusicolla violacea* was shown to have suppressive activity against many fungal phytopathogens and a biocontrol agent of soft rot of kiwifruit (Li et al., 2021). Decreased levels of *Myrothecium* spp. could benefical as various *Myrothecium* spp. are broad host range foliar plant pathogens (Quezado Duval et al., 2010) Decreased levels of *Exserohilum* spp., could also be favorable for banana as *Exserohilum rostratum* causes banana leaf spot in China (Lin et al., 2011). However decreased levels of *Nigrospora* spp. might not be beneficial as *Nigrospora* spp. are important for leaf decay fungi acting as primary colonists of fallen banana leaves (Meredith, 1962). Decreased levels of *Micropsalliota* spp. could also be negative as is it a common a saprotrophic basidiomycete (Hussain et al., 2022), which could be involved in the decomposition of banana crop residues. However, potential positive or negative effects on banana are not conclusive until analysis can be done at the fungal species level.

Based on the co-occurrence network analysis, the Siratro cover crop resulted in a higher stability of the soil fungal community compared to the other treatments. It was also notable for the lowest number of positive co-occurrences among the treatments but had a similar number of negative co-occurrences as that with goosegrass, which was higher than with conventional tillage. Higher stability of the soil fungi could be considered a positive effect for banana production as ecosystem stability and fungal plant pathogens are negatively correlated (Liu et al., 2022). A lower number of positive co-occurrences indicates reduced cooperative relationships, and a higher number of negative co-occurrences indicates increased levels of competition and antagonism (Coyte et al., 2015). One possibility is that this is a reflection of the rhizosphere effect with a cover crop compared to no rhizosphere effect with conventional tillage. However, there were no significant differences in soil fungal community complexity between treatments, which indicates that changes in soil fungi due to Siratro, goosegrass or conventional tillage reflected shifts in the soil fungal community rather than adding new fungal genera to it.

A major problem with cover crops in banana plantations is that banana leaves can greatly inhibit the cover crop due to shading. In this study, an intercropping area was created, which would be considered wider than the standard cropping method for commercial bananas. This permitted the Siartro cover crop to have less shading from the banana, at least in the first few years of growth. While the intercropping area is wider than in typical commercial production, it still resulted in sufficient banana planting density. Another benefit of a relatively wide intercropping area was that it allowed for mechanized tillage in the intercropping area. Soil fungal diversity showed that changes differed over the years of the study depending upon whether Siratro, goosegrass or bare soil was present in the intercropping area. Further work is needed to determine which of those changes might be beneficial such as by decreasing pathogens and increasing symbionts, harmful such as by decreasing plant debris decay, or have no significant impact on banana production. However, there were no significant differences in soil fungal diversity in the intercropping area between the treatments in any particular year. Schmidt et al. (2019) reported that a mixture of cover crops including triticale, rye and common vetch significantly increased fungal community diversity in tomato-cotton rotations over 14 years. Also, soil fungal diversity was significantly increased with an alfalfa cover crop over 5 years in apple orchards (Wang et al., 2022). Thus, it may take more years to observe changes in soil fungal diversity than was used in this study. As well, it will take more years to observe if the treatments altered banana yield and quality. Despite this, this study shows that the combination of wide intercropping area with a Siartro cover crop does have an impact on soil fungi and shows potential to improve banana production.

5. Conclusion

In this study, a Siratro cover crop in the intercropping area of banana helped maintain the diversity of the soil fungal community, unlike bare soil. This correlated with higher saprotrophs-symbiotrophs and lower pathotrophs-saprotrophs compared to bare soil. This could benefit banana growth by increasing the potential for beneficial fungi forming mutualistic interactions with roots and reducing the potential for plant pathogenic fungi to damage roots. This would be beneficial for banana soil health. Based on this study, it appears that intercropping with Siratro could be a simple technique to maintain a soil supporting sustainable banana production. However, future work is needed to extend the study to understand the impacts on banana yield and quality as well as the occurrence of root diseases.

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

YW conceived, performed the experiment, analyzed the data, and wrote the manuscript. WZ conceived and analyzed the data. PG revised the manuscript. S-JZ revised the manuscript. XL conceived, designed the experiment, and revised the manuscript. SX conceived, designed, performed the experiment, wrote, and prepared the manuscript. XL and SX supervised the research and provided funding support. 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.2023.1138580/full#supplementary-material

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The genome of *Bacillus tequilensis* EA-CB0015 sheds light into its epiphytic lifestyle and potential as a biocontrol agent

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Different *Bacillus* species have successfully been used as biopesticides against a broad range of plant pathogens. Among these, *Bacillus tequilensis* EA-CB0015 has shown to efficiently control Black sigatoka disease in banana plants, presumably by mechanisms of adaptation that involve modifying the phyllosphere environment. Here, we report the complete genome of strain EA-CB0015, its precise taxonomic identity, and determined key genetic features that may contribute to its effective biocontrol of plant pathogens. We found that *B. tequilensis* EA-CB0015 harbors a singular 4Mb circular chromosome, with 3,951 protein-coding sequences. Multi-locus sequence analysis (MLSA) and average nucleotide identity (ANI) analysis classified strain EA-CB0015 as *B. tequilensis*. Encoded within its genome are biosynthetic gene clusters (BGCs) for surfactin, iturin, plipastatin, bacillibactin, bacilysin, subtilosin A, sporulation killing factor, and other natural products that may facilitate inter-microbial warfare. Genes for indole-acetic acid (IAA) synthesis, the use of diverse carbon sources, and a multicellular lifestyle involving motility, biofilm formation, quorum sensing, competence, and sporulation suggest EA-CB0015 is adept at colonizing plant surfaces. Defensive mechanisms to survive invading viral infections and preserve genome integrity include putative type I and type II restriction modification (RM) and toxin/antitoxin (TA) systems. The presence of bacteriophage sequences, genomic islands, transposable elements, virulence factors, and antibiotic resistance genes indicate prior occurrences of genetic exchange. Altogether, the genome of EA-CB0015 supports its function as a biocontrol agent against phytopathogens and suggest it has adapted to thrive within phyllosphere environments.

KEYWORDS

***Bacillus tequilensis*, comparative genomics, biocontrol, natural products, banana epiphyte**

Introduction

Endospore forming bacteria of the genus *Bacillus* are ubiquitous within terrestrial and aquatic environments (Nicholson, 2002). Industrially important species, including *B. subtilis*, *B. amyloliquefaciens*, *B. velezensis*, *B. licheniformis*, and *B. pumilus*, form a phylogenetically coherent group known as the *B. subtilis* species complex (Fritze, 2004). In addition to being an

important source of industrial enzymes, vitamins, and cofactors (Harwood et al., 2018), many of these species are used as the active ingredient in several commercial biopesticides and biofertilizers (Velivelli et al., 2014; Ngalimat et al., 2021). Well known strains including *B. amyloliquefaciens* FZB42 (reclassified as *B. velezensis*; Chen et al., 2009), *B. subtilis* QST713 (recently reclassified as *B. velezensis*; Pandin et al., 2018), *B. amyloliquefaciens* GB03 (Choi et al., 2014), and *B. subtilis* MBI600 (Samaras et al., 2021) are used to control soil and foliar bacterial and fungal diseases. These strains provide a natural alternative to synthetic agrochemicals that have detrimental effects to human health and the environment.

The utility of *Bacilli* as biocontrol and bioaugmentation agents derives from traits inherent to their natural lifestyle as microbial endo- and epiphytes. These bacteria localize to plants *via* directed movement to phytochemicals (e.g., chemotaxis to organic acids such as malic and fumaric acid; Tsai et al., 2020; Ma et al., 2021) and then use catabolic pathways to consume these compounds as carbon, nitrogen, and energy sources for growth. Colonization of plant surfaces and tissues occurs through the establishment of biofilms (Rudrappa et al., 2008). Equally significant are the natural products *Bacilli* produce and their effects on modulating plant development and the composition of resident microbiomes. *Bacilli* produce phytohormones including auxins (indole acetic acid, IAA), cytokinins, and gibberellins that regulate plant growth and differentiation. These compounds are important modulators of many plant processes ranging from abiotic stress response to flowering, fruit development, and seed germination (Poveda and González-Andrés, 2021). Other antimicrobial natural products such as cyclic lipopeptides (surfactin, iturin, and fengycin/plipastatin), siderophores (bacillibactin), bacteriocins (sublancin, subtilosin; Stein, 2005) may provide ecological advantages to the *Bacilli* by suppressing fungal and bacterial competitors. The importance of these compounds is further underscored by the abundance and conservation of natural product biosynthetic gene clusters (BGCs) within the genomes of plant-associated *Bacilli*. Indeed, *B. velezensis* QST713 harbors 15 natural product BGCs (Pandin et al., 2018), while *B. amyloliquefaciens* GB03 and *B. subtilis* MBI600 harbor 8 and 7 BGCs, respectively (Choi et al., 2014; Samaras et al., 2021).

While most species in the *B. subtilis* complex have been well characterized, significantly less is understood about the physiology and ecology of *B. tequilensis*. First described in 2006, emergent interest in *B. tequilensis* has been driven by its natural ability to suppress diverse fungal pathogens of commercial grains, vegetables, fruits, and ornamentals (Tam et al., 2020; Xu et al., 2021; Zhou et al., 2021; Kwon et al., 2022). Among *B. tequilensis*, one of the best characterized strains is EA-CB0015. Originally isolated in Uraba, Colombia, EA-CB0015 was discovered as a natural antagonist of *Pseudocercospora fijiensis* (Ceballos et al., 2012), the causative agent of black Sigatoka. This agriculturally devastating disease of banana plants causes necrotic streaks on the leaves, reduces photosynthetic capacity, and promotes premature ripening of the fruit. The resulting loses can be greater than 50% if left untreated (Noar et al., 2022). Mitigation of black Sigatoka represents a significant burden to Colombian producers, with a cost $65 million per year due to weekly application of fungicides (S. Zapata, personal communication). EA-CB0015 suppresses black Sigatoka disease through the colonization of banana leaves and the production of antifungal lipopeptides (iturins, plipastatins, and surfactins; Villegas-Escobar et al., 2013; Mosquera et al., 2014; Cuellar-Gaviria et al., 2021). In addition to bananas, the strain also reduced the severity of anthracnose (*Colletotrichum* spp.) in tamarillo fruits and gray mold (*Botrytis cinerea*) in chrysanthemum flowers (Gutierrez-Monsalve et al., 2015; Arroyave-Toro et al., 2017). Although *B. tequilensis* has yet to be commercialized, these studies collectively demonstrate the significant potential of this species for the control and prevention of agricultural diseases.

Here we sequenced the complete genome of strain EA-CB0015 to understand the genetic features integral to its success as a microbial epiphyte and biocontrol agent. Using different molecular taxonomy approaches, we show that EA-CB0015 is classified as *B. tequilensis*. Our analysis and comparisons of its genome with other plant associated *Bacilli* identified mechanisms for plant colonization and the inhibition of phytopathogens. These include genes for sporulation, biofilm formation, and the ability to metabolize diverse nutrient sources. Distinct mechanisms of protection against abiotic and biotic factors were present, including genes that direct biosynthesis of an arsenal of natural products. Lastly, features related to horizontal gene transfer including the presence of genomic islands (GEIs), insertion sequences (IS), toxin antitoxin (TA) systems, restriction modification (RM) system, and prophages are described.

Materials and methods

Genome sequencing and assembly

Bacillus tequilensis EA-CB0015 was deposited in ATCC as PTA-123533. The strain was revived from glycerol stocks onto half strength tryptic soy agar [20 g/L of TSA (Oxoid) and 9 g/l of Bacto agar (BD)] and incubated at 37°C for 24 h. A single colony was inoculated into a 100 mL Erlenmeyer containing 20 mL of tryptic soy broth (Oxoid) and grown for 8 h in a shaking incubator (250 rpm, 37°C). Genomic DNA was extracted using the UltraClean Microbial DNA Isolation kit (MoBio) for Illumina sequencing and DNeasy Blood & Tissue kit (Qiagen) for Single Molecule Real-Time (SMRT) sequencing. Samples were sequenced on an Illumina HiSeq 2000 (100 bp paired end reads) and PacBio RS II (Macrogen, Inc). Contigs less than 100 bp were discarded. Unicycler v0.4.8.0 was used for hybrid assembly using the normal bridging mode (Wick et al., 2017).

General annotation

The assembled genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v6.0 (Haft et al., 2018) and deposited under accession no. NZ_CP048852. *Skew*DB was used to predict replication origins and termini (Hubert, 2022). Genome figures were created using the CGView Server (Grant and Stothard, 2008). Predicted proteins were classified into clusters of orthologous groups (COGs) using WebMGA (Wu et al., 2011), and analyzed in the Kyoto Encyclopedia of Genes and Genomes database (KEGG; entry T07089) to assign roles in metabolic pathways (Kanehisa et al., 2016).

Multi-locus sequence analysis

All strains analyzed in this study are listed in Supplementary Table S1. Reference strains included *B. subtilis* species complex (*B. pumilus, B. subtilis*, *B. tequilensis*, *B. mojavensis*, *B. atrophaeus*, *B. velezensis*, and

B. amyloliquefaciens, B. licheniformis), the *B. cereus* species complex (*B. cereus, B. thuringiensis, B. anthracis*, *B. mycoides*), and *B. coagulans*. *Clostridium kluyveri* DSM 555^T served as the outgroup.

Genomes were downloaded from public sequence repositories and annotated using Prokka v1.14.6 (Seemann, 2014). Genes encoding *groEL* (chaperonin, large subunit), *gyrA* (DNA gyrase, subunit A), *rpoB* (RNA polymerase, beta subunit), *polC* (DNA polymerase III, alpha subunit), and *purH* (phosphoribosylaminoinidazole carboxamide formyltransferase) were retrieved from each strain, aligned using MAFFT v7.4 (Katoh, 2002), manually trimmed, and concatenated head to tail using AMAS v0.98 (Borowiec, 2016). The matrix (12,483 positions; 1,647 from *groEL*, 2,517 from *gyrA*, 3,030 from *polC*, 1,542 from *purH*, and 3,747 from *rpoB*) was analyzed using ModelTest-NG v0.1.6 (Darriba et al., 2020) to determine the best substitution model for each partition. Phylogenetic trees were constructed using maximum-likelihood (ML) and Bayesian inference (BI) methods. The ML phylogeny using the GTR + I + G4 model was best for *groEL, gyrA, purH* and *rpoB*, and GTR + G4 for *polC*, with bootstrapping (1,000) using IQ-TREE v1.6.9 (Nguyen et al., 2015). The BI phylogeny was calculated using Markov Chain Monte Carlo (MCMC) analysis and the GTR + I + G4 model using Mr. Bayes v.3.2 (Ronquist et al., 2012). Two independent runs were performed for 10 million generations (1 sampling every 1,000 generations). Effective sample size, convergency, and stationarity values were evaluated using Tracer v1.7.1 (Rambaut et al., 2018). The final phylogenetic tree was generated using DendroPy v4.4.0 (Sukumaran and Holder, 2010) and visualized using iToL v5.0 (Letunic and Bork, 2019).

Growth assays

The ability of strain EA-CB0015 to utilize different carbon sources was performed using a BioLog GEN III Microplate (Biolog Inc.). EA-CB0015 was grown on Universal Growth Agar (BUG; Biolog Inc.) and incubated at 30°C for 24 h. Isolated colonies were resuspended in the "Inoculation Fluid-A" (IF-A; Biolog Inc.) following manufacturer's instructions and this suspension was inoculated into the Gen III microplate. After 24 h incubation at 30°C, the microplate was analyzed qualitatively for color development using a MicroStation™ 2 Reader (Biolog Inc.), measuring OD at 595 and 750 nm.

Sporulation, motility, and biofilm formation assays

Colonies grown on TSA plates were stained for endospores using Schaeffer-Fulton method (Gerhardt et al., 1994). Micrographs were recorded using an Axiostar Plus microscope (Carl Zeiss) at 100X equipped with an AxioCam ICc3 (Carl Zeiss) and ZEN 2.3 lite software (Carl Zeiss). Biofilm formation was examined by inoculating a diluted culture of EA-CB0015 into 12-well plates with LBGM media. These were incubated for 48 h at 30°C (statically) and photographed (7.1 MP digital camera, Kodak EasyShare P712). Motility was assayed by inoculating 3 µl EA-CB0015 grown on tryptic soy broth TSB (Oxoid; OD 1.0, 600 nm) onto the center of 10% LB plates solidified with either 0.7% (swarming) or 0.3% agar (swimming), and incubated at 30°C for 24 h (Kearns and Losick, 2003; Ghelardi et al., 2012). Flagella were visualized using Ryu stain (Heimbrook et al., 1989).

Analysis of natural product biosynthetic gene clusters

Genomes were analyzed using antiSMASH v5.1.2 (Blin et al., 2019). BGCs with similarity scores > 70% were reported for all 58 *Bacillus* strains. Regions encoding with similarity scores less than <70% were further analyzed for strain EA-CB0015 using BLAST (Johnson et al., 2008) and HMMER v3.3.1 (Eddy, 2011). BGCs for natural products known to be produced by *Bacilli* but not detected by antiSMASH were manually identified by BLAST.

Analysis of virulence factors, antibiotic resistance, and genetic exchange

Putative phage and phage-like regions were identified using PHASTER (Zhou et al., 2011; Arndt et al., 2016). Data ordering, sub-setting and reshaping was performed using Tidyverse v1.3.0 (Wickham et al., 2019) and an in-house script uploaded on GitHub[1] (García-Botero et al., 2021). GEIs were identified using IslandViewer 4 (Bertelli et al., 2017) and ISs identified using ISfinder (e-value cutoff of 1e-07; Siguier et al., 2006). Integrative or conjugative elements (ICEBs1) were predicted with ICEBerg 2.0 (Liu M. et al., 2019). Antimicrobial resistance genes were identified using ResFinder-4.0 (70% identity threshold; minimum length of 70%; Bortolaia et al., 2020), and putative virulence factors from the Virulence Factors of Pathogenic Database (VFDB; Liu B. et al., 2019). RM systems were retrieved from REBASE (Roberts et al., 2015). Cluster BLAST analysis of identified RM systems were performed using Cblaster v1.3.0 (Gilchrist et al., 2021). Potential TA systems identified using TAfinder (Xie et al., 2018) and manually BLAST searches. Finally, CRISPRCasFinder (Couvin et al., 2018) was used to identify CRISPRs and Cas genes.

Comparative genomics

Synteny was analyzed using Easyfig v2.2.2 (Sullivan et al., 2011). ANI values (Richter and Rosselló-Móra, 2009) were calculated using GTDB-Tk v0.3.2 (Chaumeil et al., 2019). Comparisons between *B. subtilis* 168, *B. tequilensis* ATCC BAA 819^T and *B. tequilensis* EA-CB0015 were performed using proteome and protein family functions in PATRIC (Davis et al., 2020). Presence or absence of predicted genes and proteins in EA-CB0015 were compared to *B. subtilis* 168 (NBCI Reference Sequence: NC_000964.3) using SubtiWiki (Zhu and Stülke, 2018). E-values < 1e-5 and sequence identities > 70% were used as cutoffs.

Results

The genome of EA-CB0015

Hybrid assembly of Illumina and SMRT sequencing reads produced a single circularized chromosome of 4,012,371 bp with an average GC

1 https://github.com/camilogarciabotero/PHASTER-DataProcessing-scripts

content of 43.7%. In total, 4,112 CDS (3,951 genes, 161 pseudogenes), 10 copies of rRNAs (5S, 16S, and 23S), 86 tRNAs, and 5 ncRNAs were present. The forward strand encoded 2,134 genes and the reverse strand 1,978 genes (Figure 1). GC-skew suggests single symmetrical, bi-directional replication of the genome (Supplementary Figure S1). COG analysis assigned these into 5,163 protein families, most of which belonged to general or unknown function (categories R and S). Besides these, the largest groups of proteins are dedicated for amino acid transport and metabolism (E), energy production and conversion (C), translation, ribosomal structure, and biogenesis (J), DNA replication, recombination, and repair (L), and transcription (K; Supplementary Figure S2).

Molecular taxonomy classifies EA-CB0015 as *Bacillus tequilensis*

EA-CB0015 was originally identified as *B. subtilis* (Ceballos et al., 2012) and later referred to as *B. tequilensis* (Cuellar-Gaviria et al., 2021). This ambiguity derived from the use of 16S rRNA gene phylogenies, which do not provide sufficient resolution at the species level for strains within the *B. subtilis* complex (Rooney et al., 2009). To definitively determine the species identity of the strain, we performed multilocus sequence analysis (MLSA) using established marker genes shown to effectively determine speciation in *Bacillus*. EA-CB0015 was more closely related to *B. tequilensis* ATCC BAA 819^T than to other *B. subtilis* subspecies, including *subtilis*, *natto*, *stercoris*, *spizizenii* and *inaquosorum* (Figure 2A). This is supported by high ANI values between the genomes of strain EA-CB0015 and *B. tequilensis* ATCC BAA 819^T (98.6%), vs. *B. subtilis* subsp. *inaquosorum* KCTC 13429^T (92.3%) *B. subtilis* subsp. *subtilis* 168 (91.4%; Supplementary Table S2). Thus, EA-CB0015 belongs to the phylogenetically homogeneous *B. subtilis* species complex and is classified as *B. tequilensis*.

Comparative genomics of *Bacillus tequilensis* EA-CB0015, *Bacillus tequilensis* ATCC BAA 819^T, and *Bacillus subtilis* 168

The phylogenetic analysis suggests *B. tequilensis* and *B. subtilis* share a close evolutionary history. To understand the nature of this relationship, we identified genetic similarities and differences between EA-CB0015, ATCC BAA 819^T, and 168. The GC content of all three strains were similar (43.5%–44.0%) and they contained the identical number of rRNA and tRNA encoding genes. The *B. tequilensis* genomes were comparable in size (4 Mbp) and the number of protein coding sequences, but both were smaller than *B. subtilis* 168 (4.2 Mbp; Supplementary Table S3). Except for an extra 1.4 kbp plasmid in ATCC BAA 819^T, both *B. tequilensis* genomes were largely syntenic (Figure 3A). Although less conservation was observed between 168 and EA-CB0015, large segments of their genomes remained homologous with each other. A 132 kbp inversion corresponding to bacteriophage SPβ was near the replication terminus (*terC*) of both strains (Figure 3A). In strain 168, the prophage was encoded on the complementary strand 134 kb upstream *terC*, while in strain EA-CB0015 it was on the positive strand 186 kb downstream of *terC*. Interestingly, this region was highly variable between 168 and EA-CB0015, and completely absent from the genome of *B. tequilensis* ATCC BAA 819^T. Compared to 168, the SPβ prophage region of EA-CB0015 lacked the sublancin BGC and its immunity gene (Denham et al., 2019), a gene encoding for DNA cytosine-5-methyltransferase (*mtbP*), and numerous hypothetical proteins with unknown function. Most of the encoded proteins and their families were shared between all three strains (Figure 3B; Supplementary Table S4).

Motility and biofilm formation

Multicellular behavior is used by *B. subtilis* to afford survival in its natural environment. These mechanisms include motility, biofilm formation, cannibalism, quorum sensing, competence, and sporulation (González-Pastor, 2017). As *B. subtilis* 168 is well studied for its ability to assemble multicellular communities and closely related to EA-CB0015 (O'Toole et al., 2000; González-Pastor, 2017), we examined if genes for these phenotypes were also present in our strain.

Genes for flagellum-mediated motility including chemotaxis and swarming were present in the EA-CB0015 genome. All thirty-two genes required to form the flagellar basal body and hook were encoded within a 27-kb *fla/che* operon, as well as all the genes needed for filament formation (flagellin monomer protein Hag, FliD, FlgK, FlgL), assembly, and rotation (Supplementary Table S5A; Mukherjee and Kearns, 2014). Other genes required for the basal body formation (FlhO and FlhP), torque in flagellar rotation (MotA, MotB), cell separation (LytC), bistable regulation (SigD, SwrA, SwrB, DegS/DegU, YmdB, and SlrA/SinR/SlrR), chemotaxis and associated chemoreceptors (2 soluble and 7 membrane-bound) were also present (Supplementary Tables S5A,B). Microscopy showed motile, polarly flagellated cells (Supplementary Figure S3). These results suggest EA-CB0015 can sense and swim toward a wide range of attractants (pH, amino acids, oxygen, etc.) that are present in its local environment.

To form a biofilm, cells must transition from motile to sessile states, aggregate, and embed themselves within a self-produced extracellular matrix (EM; Arnaouteli et al., 2021). EA-CB0015 formed biofilms in static culture (Supplementary Figure S3), and as expected, the genome of EA-CB0015 encoded genes essential for this process including exopolysaccharide biosynthesis (*epsA-O*), protein fiber TasA (*tapA*sip*WtasA* operon), the hydrophobin-like protein BslA, and genes for poly-γ-glutamic acid (γ-PGA) biosynthesis (*pgsBCAE*; Supplementary Table S5C). Production of EM is also linked to multiple regulatory proteins that were all present within the genome (Supplementary Table S5C). The Spo0A pathway (KinC, KinD, Spo0A, SinI/SinR/SlrR, AbrB) controls the expression of more than 100 genes including biofilm matrix gene expression and sporulation, while the YwcC-SlrA stress response pathway facilitates adaptation to changing environmental conditions. The DegS–DegU two-component system regulates competence, motility and secretion of degradative enzymes. Lastly, genes that mediate expression of *slrR*, an essential regulator of biofilm formation were also present (*abh*, *ymdB*, *remA*, *remB*; Vlamakis et al., 2014).

Interestingly, EA-CB0015 does not harbor the *yitPOM* operon. The paralogous *sdpABC* operon, is also absent from the genome of the strain. The *yitPOM* operon expresses the biofilm associated toxin (YitM) and the extracellular protease (NprB), both of which suppress competitors in *B. subtilis* biofilms (Kobayashi and Ikemoto, 2019). In

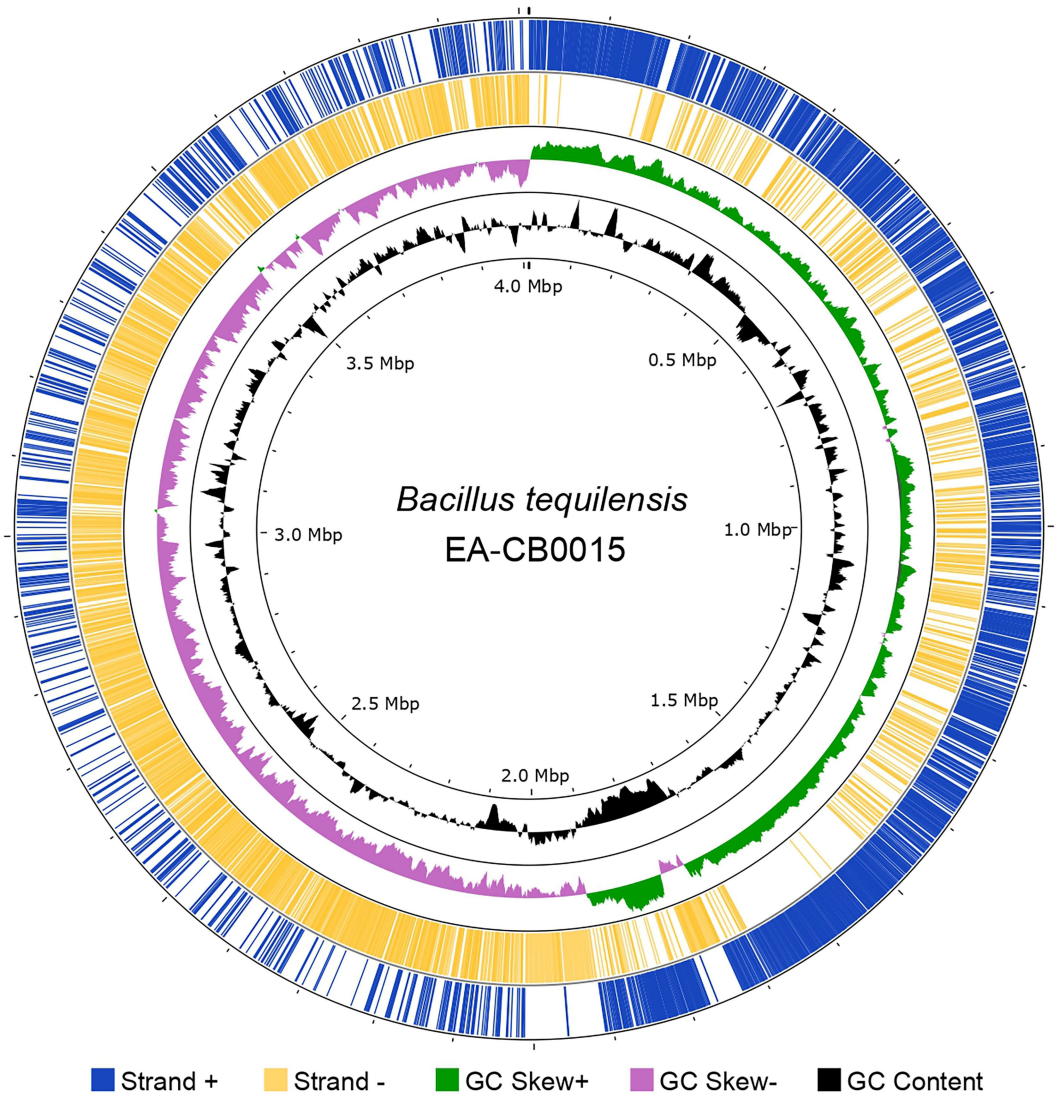

FIGURE 1
The genome of *Bacillus tequilensis* EA-CB0015. GenBank features (CDS) in the forward strand (blue) and reverse strand (yellow); GC skew (green and purple) and GC content (black). Image created with Proksee.

addition, surfactin, encoded in the *srfA* operon (Supplementary Table S5B), may induce potassium leakage that stimulates the sensor kinase KinC. This may also activate expression of biofilm formation genes (López et al., 2009).

Cannibalism

Cannibalistic behavior delays the entry into sporulation within a subpopulation of cells. This is controlled by the production of sporulation delaying protein-Sdp and sporulation killing factor-Skf, which lyse and kill sensitive siblings (González-Pastor et al., 2003). Interestingly, EA-CB0015 contained genes for Skf (*skfA-H*), but not Sdp (*sdpABC* and *sdpRI* operons were absent; Supplementary Table S5D). These results suggest EA-CB0015 cells may exhibit an accelerated sporulating phenotype. As Spo0A-inactive cells are not lysed, the pool of nutrients released into the environment from cell death is reduced. Thus, neighboring cells experience starvation to initiate spore formation (González-Pastor et al., 2003).

Quorum sensing and competence

Genes encoding mechanisms of quorum sensing were present in the EA-CB0015 genome (Supplementary Table S5E). These may allow strain EA-CB0015 to coordinate physiological processes such as the synthesis of exoproteases and other extracellular enzymes in response to cell density. The lipopeptide surfactin is also positively regulated by the phosphorylated form of ComA, which is part of the quorum sensing (QS) system (ComQXPA) in *B. subtilis* (Nakano et al., 1991; Kalamara et al., 2018). Interestingly, the putative ComQ, ComX, and ComP proteins shared low sequence identity with their corresponding homologs in 168 (Supplementary Table S5E). This suggests the Com system may handle separate social communication groups or

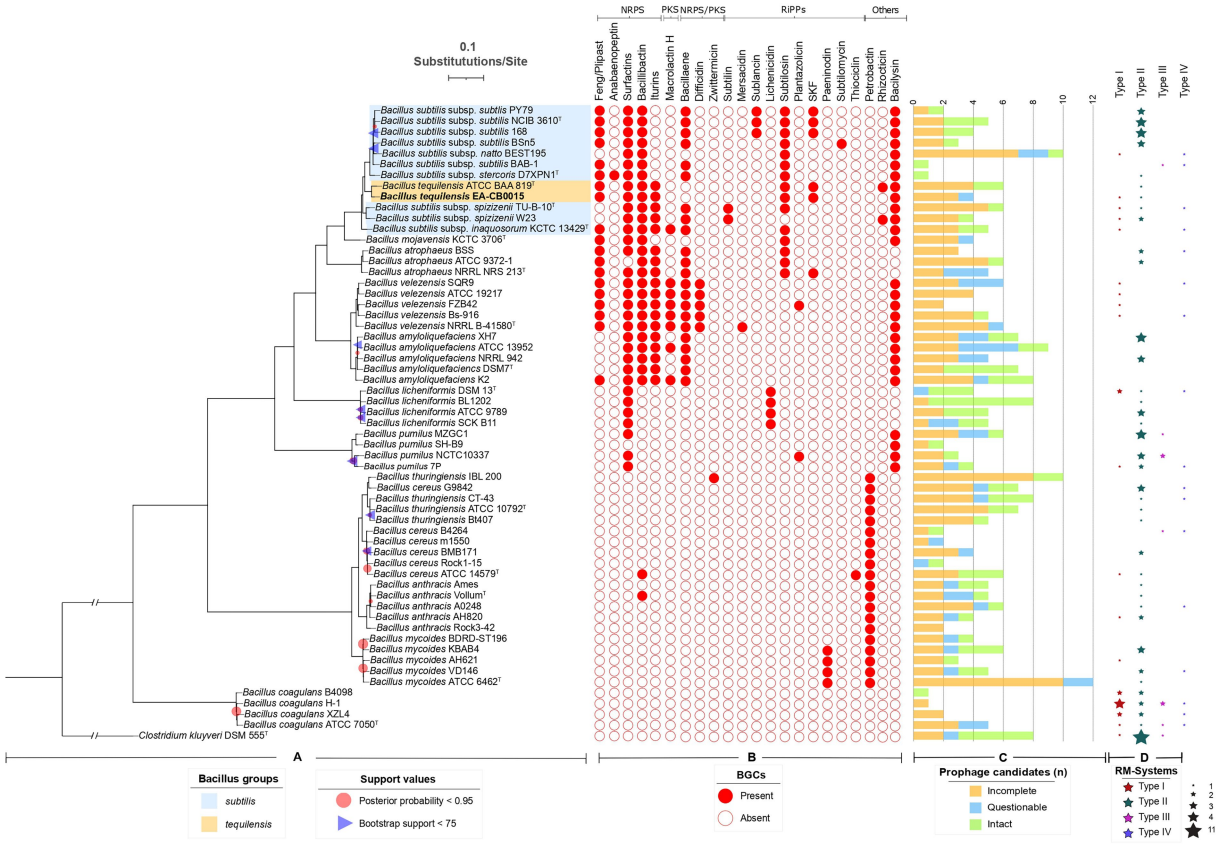

FIGURE 2
Phylogeny of EA-CB0015 and related *Bacilli* with their natural product BGCs, prophages and RM systems. **(A)** Consensus MLSA phylogeny from maximum likelihood (ML) and Bayesian inference (BI). Node supports with posterior probability values <0.95 and bootstrap support values < 75 are shown. **(B)** Natural product BGCs predicted by antiSMASH. NRPs, non-ribosomally synthetized peptides; PKs, polyketides; RiPPs, ribosomally produced post-translationally modified peptides. **(C)** Number of regions annotated as prophages according to PHASTER. Green bars indicate intact prophages (scores > 90); blue bars, questionable (70–90); orange bars, incomplete (<70). **(D)** Distribution and abundance of RM-Systems according to REBASE or manually searched.

pherotypes (Stefanic and Mandic-Mulec, 2009; Oslizlo et al., 2014). The genome also encoded six putative receptor-signal pairs of the Rap-Phr system (Rap-Phr A, C, E, F, H; Supplementary Table S5E). Similar to 168, accumulation of Phr peptides in EA-CB0015 may suppress effects of Rap proteins to allow expression of genes for swarming motility, biofilm formation, exoprotease secretion and genetic competence (Kalamara et al., 2018). Related to quorum sensing, EA-CB0015 also contained genes for the acquisition and incorporation of extracellular DNA into the host cell (genetic competence). These include the master competence regulator *comK*, genes encoding proteins essential for DNA binding and import (*comC*, operons *comE*, *comF*, *comG*, *bdbD*, *bdbC*), and cytosolic proteins that modulate recombination and transformation efficiency (RecA, SsbB, DprA, CoiA, NucA). Genes encoding proteins for transcriptional (Rok, CodY, Kre) and post-translational regulation (MecA, ComS) of ComK were also present (Supplementary Table S5F).

Sporulation

Endospores were visible when cells were subjected to Ryu stain (Supplementary Figure S3). Indeed, genes needed for sporulation were present in the genome (Supplementary Table S5G). These included genes encoding for morphogenetic proteins (SpoIVA, SpoVM, SpoVID, SafA, CotE, CotX/CotY/CotZ), their interacting partners during spore coat assembly, and spore crust proteins. Interestingly, some shared < 60% sequence identity with the homologs in 168, while others were completely absent (Supplementary Table S5G). The biosynthetic genes for legionaminic acid, which is used in crust formation, were notably missing (Supplementary Table S5G). Their absence suggests that the surface of EA-CB0015 spores may be reduced in hydrophilicity and charge (Dubois et al., 2020).

Most of the genes encoding signal transduction proteins (histidine sensor kinases KinA-E, master regulator Spo0A, phosphotransferases Spo0F and Spo0B, etc) related to sporulation were identified (Supplementary Table S5G). Homologs of *lrpAB* were missing, but other studies have indicated negligible effects on *glyA* transcription or sporulation through KinB (Dartois et al., 1997). The absence of *sivC* may result in a greater sporulation efficiency, as it functions as an inhibitor of the KinB and KinC pathway (Garti-levi et al., 2013). As expected, genes encoding proteins involved with major events in spore gemination were also present (Setlow et al., 2017). These include genes needed for germinant sensing (germinant receptors), release of dipicolinic acid (DPA) related to heat resistance (spoVA proteins,

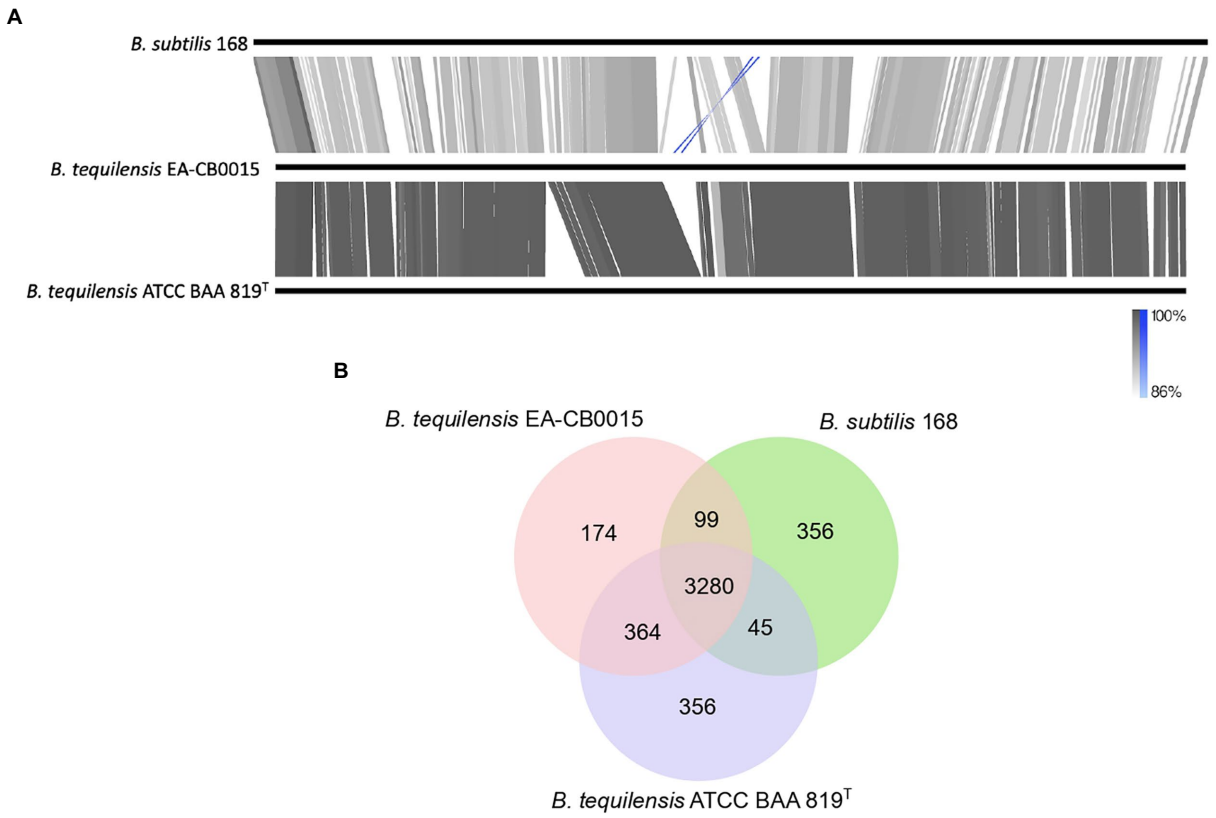

FIGURE 3
Comparison of *Bacillus subtilis* 168, *Bacillus tequilensis* EA-CB0015 and *B. tequilensis* ATCC BAA 819^T **(A)** Global genome alignments. Gray lines and blocks indicate regions and degree of shared similarity. Blue represents inversions **(B)** Shared and unique proteins between the three strains.

GerD), and hydrolysis of cortex peptidoglycan (CwlJ, SleB, SleL; Supplementary Table S5G). Altogether, these data suggest EA-CB0015 endospores can fully germinate once in favorable environmental conditions.

Pathways for carbon assimilation

Nutrient availability on leaves, especially for organic compounds, is spatially heterogeneous and limited (Leveau and Lindow, 2001; Lindow and Brandl, 2003). A major carbon source that leach from the interior of the plant are common sugars (Wildman and Parkinson, 1981; Fiala et al., 1990; Lindow and Brandl, 2003). EA-CB0015 encodes all the genes necessary for glucose assimilation into the TCA cycle (KEGG pathway map ID bteq00020) by glycolysis (bteq00010). Likewise, genes for the metabolism sucrose and starch (bteq00500), and fructose (*via* fructose-1P and frutokinase (bteq00051)) were present. Pathways for galactose, maltose, and raffinose catabolism were also predicted (bteq00052, bteq00500). However, not all the genes needed for inositol utilization were present (bteq00562), such as those that encode transporters (*iolF, iolT*). Genes needed for scyllo-inosose transformation (*iolE, iolD, iolB, iolC, iolJ*) were also absent (Supplementary Table S6).

In addition to sugars, organic acids (e.g., L-lactic acid, citric acid, and L-malic acid) are common on the foliage of plants (Morgan and Tukey, 1964). EA-CB0015 utilized these and other compounds as carbon sources in API 50 CHB/E (Villegas-Escobar et al., 2013) and Biolog GEN III Microplate growth assays (Supplementary Table S7). Although methanol is readily available on plant surfaces (Kutschera, 2007), the absence of genes for methanol dehydrogenase suggest EA-CB0015 is unable to consume it as carbon source. However, genes for formaldehyde fixation (*hxlA*, *hxlB*) shared high sequence identity (>90%) with their corresponding homologs in 168, suggesting formaldehyde may be assimilated through the ribulose monophosphate pathway (bteq_M00345, Supplementary Table S6). This may allow EA-CB0015 to scavenge formaldehyde produced by neighboring methylotrophs during growth on plant surfaces.

Hydrolytic enzymes

EA-CB0015 contained genes for β-glucanases (*bglC, bglS*), chitinase (*Csn*), extracellular proteases (*aprE*, *nprE*), bacillopeptidase F protein degradation (Bpr), endolevanase (*levC*), xylanase (*xynA, B, and C*), α-amylase (*amyE, amyX*), and pectate lyases (*pel*, *pelB*; Supplementary Table S8). These enzymes are be used to liberate sugars from complex polysaccharides that are then be assimilated by the strain. Chitinase may also function to inhibit nearby fungi by hydrolyzing their cell walls and inhibiting formation of hyphae (Kobayashi and Ikemoto, 2019; Legein et al., 2020). Lastly, a gene encoding for the quorum quenching enzyme YtnP (lactonase-homolog protein) was also found in the genome. This may degrade

quorum sensing autoinducers of competitive strains in the same plant environment (Schneider et al., 2012).

Nitrogen and phosphorous

EA-CB0015 contained genes required to reduce nitrate to ammonia *via* the dissimilatory nitrate reduction pathway (bteq_M00530, Supplementary Table S7). The strain lacks genes for a nitrogenase enzyme complex, and thus is unable to fix atmospheric dinitrogen. Genes required for metabolism of aspartate/glutamate (bteq00250), arginine/proline (bteq00330), glycine/threonine/serine (bteq00260) were present (Supplementary Table S7), all of which have been detected in the foliage of plants (Morgan and Tukey, 1964; Parangan-Smith and Lindow, 2013).

Under phosphate limiting conditions, many microorganisms can solubilize inorganic sources of phosphate or mineralize organophosphorous compounds (Hanif et al., 2015). Genes encoding for the *pho* regulon, including alkaline phosphatase, extracellular enzymes to catabolize organophosphorous compounds (PhoA, PhoB, PhoD, GlpQ), phosphate transporters, and the *tat* secretion system for protein export were indeed present within the genome (Supplementary Table S9; Allenby et al., 2005). Interestingly, phytase (*phy*) was only present as a pseudongene (Supplementary Table S9), suggesting strain is unable to hydrolyze phytate, a major source of organic phosphate.

Iron

EA-CB0015 encoded genes for the biosynthesis of the siderophore bacillibactin (*dhbACEBF*), transporters for importing it into the cell (FeuABC-YusV), and the esterase BesA to release the iron in the cytosol (Supplementary Table S10; Miethke et al., 2006). Genes encoding hydoxamate (*fhuBCDG*), petrobactin/catecholate (*fpbOPQ*), schizokinen/anthrobactin (*yfhAYZ*) transporters suggest the strain may scavenge siderophores produced by other species (Supplementary Table S10). Genes for citrate-iron transporter (*fecCDEF*), high-affinity iron transporter (*efeUOB*), and heme degradation (*hmoAB*) suggest these as additional mechanisms for iron acquisition.

Protective mechanisms against oxidative stress and UV

Residing on plant leaves and surfaces, epiphytes are continually challenged by photooxidative stress. In addition to sporulation, biofilm formation, and motility, EA-CB0015 contained genes to protect itself from desiccation, UV light, and oxidative damage (Mols and Abee, 2011). The flavin-dependent photoreceptor (*ytvA*) activates general stress response mechanisms in the presence of blue light. This includes spore-product photolyase (*splB*; Supplementary Table S11) to repair thymine dimer adducts produced from UV radiation (Herrou and Crosson, 2011; Vanhaelewyn et al., 2020). Genes for catalase, superoxide dismutase, thioredoxin reductase, hydroperoxide reductase, peroxiredoxin, and antioxidants including bacillithiol, phytoene, and sporulene (Supplementary Table S11), may help EA-CB0015 reduce damage from oxidative stress (Engelmann and Hecker, 1996; Newton et al., 2009; Zuber, 2009; Jeong et al., 2018). Production of extracellular polysaccharides and the siderophore bacillibactin (a UV-B absorbing compound) may further allow EA-CB0015 to avoid damage from UV exposure (Supplementary Table S11).

Biosynthetic pathways for natural products

Epiphytic bacteria scavenge nutrients, influence development of plants, and the composition of local microbial communities through their natural products (Legein et al., 2020). To identify the repertoire of molecules EA-CB0015 may produce, we first analyzed its genome using antiSMASH. We found seven BGCs for known natural products and two of unknown function (Supplementary Table S12). Encoded within the genome were non-ribosomal peptide synthetase (NRPS) BGCs for lipopeptides (plipastatin/fengycin C, surfactin, iturin) and the siderophore bacillibactin. The BGCs for subtilosin A (thiopeptide), sporulation killing factor (SKF; sactipeptide), and bacilysin were also detected (Figure 4). Annotation of the NRPS domains for fengycin C (Villegas-Escobar et al., 2013), suggests the strain actually produces plipastatin. Specifically, the lack of an epimerization domain in module 3 of *fenB* suggests incorporation of L-Tyr instead of D-Tyr. Moreover, the domain annotation of module 9 predicts epimerization, suggesting D-Thr instead of a L-Thr in the final peptide natural product (Hussein, 2019). The neighborhoods from EA-CB0015 were all conserved to reference BGCs for these natural products (Supplementary Figure S4).

Two genomic regions were annotated to encode for potential terpene and polyketide compounds. The first region (2,107,965 to 2,129,124 bp) contained a gene annotated as squalene-hopene cyclase, but its neighborhood lacked commonly associated genes for condensation of farnesyl diphosphate to squalene (SQase, or *hpnD*, *hpnC*, *hpnE*; Supplementary Table S13; van der Donk, 2015). The second region (2,174,022 and 2,213,530 bp) contained a putative type III polyketide synthase (chalcone synthase; BpsA) and an isoprenylcysteine carboxyl methyltraferase (BpsB), suggesting a role for biosynthesis of aliphatic polyketides, such as triketide pyrones, tetraketide pyrones and alkylresorcinols (Supplementary Table S14; Nakano et al., 2009).

Putative pathways for other natural products were identified by KEGG and manual annotation. EA-CB0015 encoded genes for the antibiotic kanosamine, which is biosynthesized in three steps from glucose-6-phosphate (enzymes NtdC, NtdA, NtdB; KEGG accession bteq00998, Supplementary Table S8). Biosynthesis of the plant hormone IAA included genes for tryptophan aminotransferase (*patB*), idole-3-pyruvate decarboxylase (*yclC*), and indole-3-acetaldehyde dehydrogenase (*dhaS*). These would produce indole 3-pyruvic acid pathway (IPyA) the predominant precursor to IAA (Shao et al., 2015). Genes related to the tryptamine-TAM pathway (*bsdC,* flavin monamine oxidase, and *dhaS*), known as an alternative pathway for the synthesis of IAA, were found (Shao et al., 2015). *YhcX,* predicted to act as a nitrilase in the last step of the indole 3-acetonitrile (IAN) pathway, was also present (Supplementary Table S15).

Genetic exchange

Leaf surfaces are proposed as hot spots for lateral gene transfer and important breeding grounds for microbial diversity (Lindow and

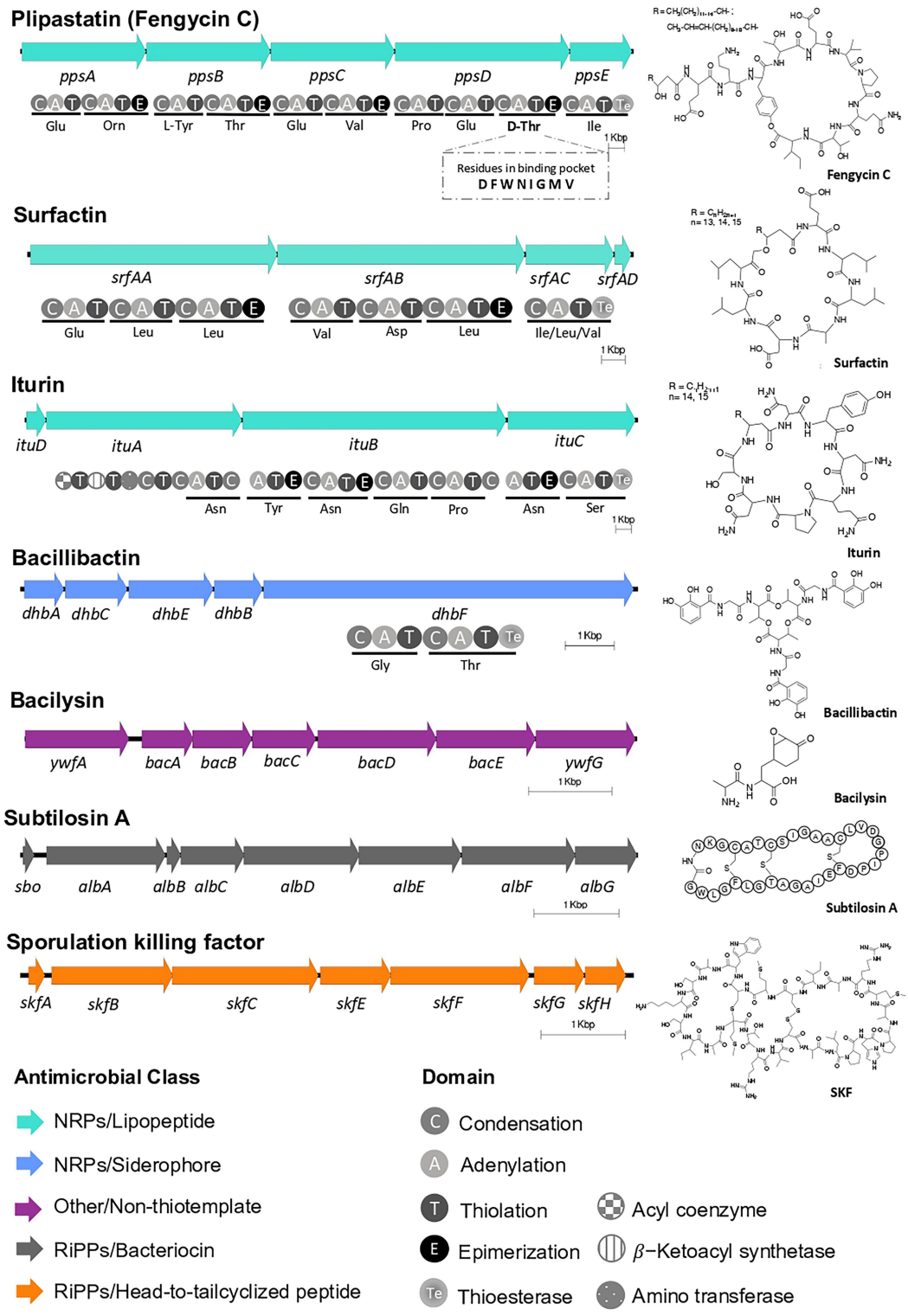

FIGURE 4
BGCs of known natural products within *Bacillus tequilensis* EA-CB0015. Predicted domains for NRPSs and amino acid specificity are shown. The third adenylation domain in the putative PpsD protein is predicted to load threonine (fengycin C) instead of tyrosine (plipastatin B). The BGCs are located on the following positions on the chromosome: SKF (209,791–215,884); surfactin (359,914–386,063); iturin* (1,919,24–1,955,499); plipastatin (fengycin C)* (1,969,256–2,007,030); bacillibactin* (3,075,812–3,087,611); subtilosin A* (3,667,130–3,674,071); bacilysin* (3,701,710–3,708,994). (*Encoded on negative strand).

Leveau, 2002). Thus, we sought to understand both the degree to which EA-CB0015 may have been affected by gene transfer events and mechanisms it may have to maintain genome integrity.

EA-CB0015 encoded genes needed to take up DNA by genetic competence (Supplementary Table S5), but did not contain plasmids nor any complete integrative or conjugative transposons (Auchtung

et al., 2016). However, some pseudogenes (*immA*, *immR*, *phrI*) or integrases (*int*) for excision of ICEBs were present in the genome (Supplementary Table S16). Four prophage regions were predicted, suggesting EA-CB0015 was susceptible to bacteriophages (Figure 5; Supplementary Figure S5). PHASTER analysis classified three as incomplete (*Bacillus* phi4J1, *Brevibacillus* Jimmer2, and *Staphylococcus* SPβ-like) and one as questionable (*Bacillus* SPβ). While insertions and recombination from prophages may disrupt important genes, they may also introduce phage resistance or prototrophy to improve the competitive fitness (Kohm and Hertel, 2021).

Other hallmarks of horizonal gene transfer include GEIs and transposable elements. GEIs encode cluster of genes for specialized functions (pathogenicity, symbiosis, metabolism, resistance, fitness) presumed to be of foreign origin (Juhas et al., 2009). Interestingly, GEIs accounted for 10% of the total genome, with many phage-related genes overlapping the same regions (Figure 5; Supplementary Figures S5, S6). Fourteen ISs (transposable elements) were present in the genome (Supplementary Table S17). However, ISs belonging to IS1182 and IS1595 families were pseudogenes and only those belonging to the IS3 family were complete. The latter was the most abundant, with 8 copies of ISBsuI and 1 copy of ISErh (Siguier et al., 2006; Figure 5). These may offer a selective advantage by accelerating genome rearrangement, introducing virulence factors, or resistance genes to antibiotics (Lindow and Leveau, 2002). Genes for putative virulence factors including hemolysin III (*hlyIII*), capsular polyglutamate and bacillibactin were encoded outside of mobile elements (Figure 5; Supplementary Table S18). Resistance genes for streptomycin (*aadK*), spiramycin and telithromycin (*mphK*), and tetracycline (*tetL*; Figure 5; Supplementary Tables S18, S19) were also detected. The streptomycin resistance gene was encoded within a genomic island.

Both type I and type II RM systems were encoded in the genome of EA-CB0015. The type I system contained genes for the HsdR endonuclease (R), specificity subunit (S), and DNA methyltransferase (M; Figure 5; Supplementary Figure S7). While genes for the endonuclease and methyltransferase of the type I system were highly conserved (>85% sequence identity), the specificity subunit (G4P54_RS16760) exhibited significantly lower sequence identity (33%–40%; Supplementary Figure S8). This suggests variability in the recognition site where the restriction endonuclease cleaves DNA. Additionally, a type II RM system with a site-specific DNA methyltransferase (locus tag G4P54_RS15065) was found.

EA-CB0015 contained 22 TA systems (Figure 5; Supplementary Figure S9; Supplementary Table S20). These included two type I TA systems (*bsrG/SR4* and *yonT-yoyJ/SR6* located in SPβ region) and three type II TA systems (*ndoA/ndoB, spoIISA/spoIISB/spoIISC* and *yeeF/yezG*) which are also in strain 168. TA systems may provide fitness advantages when the strain experiences stress from pH changes, oxygen deficiency, or iron limitation (Brantl and Müller, 2019). Lastly, no RNA-based defense systems were found using CRISPRCasFinder (Couvin et al., 2018).

Natural product BGCs, prophages and restriction modification systems among *Bacillus* spp.

To understand how EA-CB0015 compares with these closely related bacteria and the presence of any distinguishing trends, we cataloged natural product biosynthesis, prophage, and RM across type strains within the *B. subtilis* species complex, *B. cereus* species complex and *B. coagulans* (Figure 2). In general, strains that are non-pathogenic to humans (*B. subtilis*, *B. tequilensis*, *B. mojavensis*, *B. atrophaeus*, *B. velezensis*, *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus*) encoded a more natural product BGCs. Among the 22 different natural product BGCs we identified, the largest number (7–9) were associated with *B. subtilis*, *B. velezensis*, *B. amyloliquefaciens*, and *B. tequilensis* (Figure 2B). These was consistent with known biological control agents produced by these species (Chen et al., 2009; Gutierrez-Monsalve et al., 2015; Pandin et al., 2018; Samaras et al., 2021). Surfactin, bacilysin, bacillibactin, and bacillaene were the most prevalent BGCs. Except for the antifungal phosphonopeptide rhizocticin (Borisova et al., 2010), EA-CB0015 and BAA-819 share the same composition of natural product BGCs. The most common BGC in pathogenic bacilli was petrobactin. None of these 22 BGCs were present in *B. coagulans*.

Prophages account for substantial genetic variation and confer phage resistance (Casjens, 2003; Fortier and Sekulovic, 2013; Kohm and Hertel, 2021). Across all taxa, the number of candidate prophage regions and prophage CDSs was similar within the *B. subtilis* species complex (4.68 regions and 217.74 CDSs) and *B. cereus* group (5.15 regions and 196.95 CDSs; Figure 2C; Supplementary Figure S10). Intact prophages included members of the Siphoviridae, Myoviridae and Tectiviridae families (Supplementary Figure S11). Twelve phages were unique to *B. subtilis* species complex, 8 unique to the *B. cereus* species complex, and 3 were shared between both groups (Supplementary Figures S10, S12). Altogether, these results indicate phages are evolutionary conserved within species complexes.

As natural selection is one of the most important evolutionary processes, RM systems serve as an important defense against the introduction of foreign DNA (Bourniquel and Bickle, 2002). Type II RM systems were the most common among both groups, present in 68 and 81% of the strains from *B. subtilis* and *B. cereus* complexes, respectively. Other types of RM systems were also widely distributed but less abundant than type II systems (Figure 2D), but no differential patterns were observed between these two groups of strains.

Discussion

Successful biocontrol agents have been suggested to require distinct mechanisms that confer survival to its habitat and contribute to the health of its host (Legein et al., 2020). In sequencing the genome of *B. tequilensis* EA-CB0015, we uncovered the genetic basis for several physiological adaptations underlying its survival in the phyllosphere including biofilm formation, motility, competence, protection from oxidative stress, and sporulation. Spores of EA-CB0015 are predicted to lack legionaminic acid, and their reduced charge may favor adherence to the naturally hydrophobic surface of leaves. Genes for chemotaxis, and swarming suggest colonization of plant surfaces may be mediated through motility toward optimal growth conditions including microenvironments rich in nutrients. EA-CB0015 may also leverage acquisition of nutrients through its biosynthesis of IAA and surfactin. Production of IAA induces physiological changes in plants including loosening of plant cell walls and the release of nutrients (Vanderhoef and Dute, 1981). Surfactin could improve the wettability of leaves and thus facilitate adherence (Lindow and Brandl, 2003).

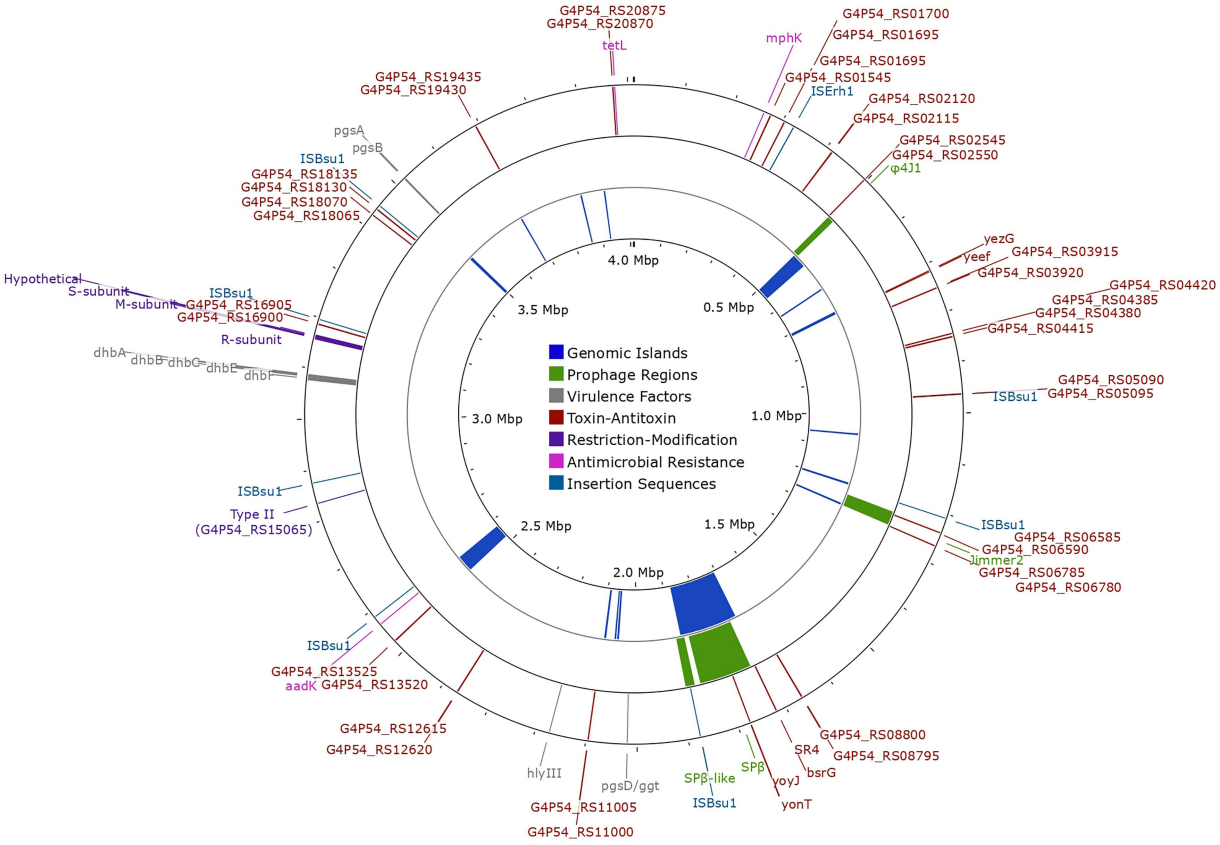

FIGURE 5
Chromosomal map of *Bacillus tequilensis* EA-CB0015 showing genetic exchange features, antimicrobial resistance genes, restriction modification systems, and virulence factors. The outer circle shows the location of insertion sequences (light-blue), antimicrobial resistance genes (pink), restriction modification systems (purple), toxin-antitoxin systems (brown) and virulence factors (gray). Intact (high confidence) prophage regions are shown in the second circle (green). The inner circle shows the location of genomic islands (dark-blue).

Both may increase access of nutrients that could then be assimilated by EA-CB0015 through its diverse pathways for carbon (sugars, organic acids, formaldehyde) nitrogen (dissimilatory nitrate reduction, amino acid catabolism), and phosphorous assimilation.

Microbial natural products contribute the health of host plants by modulating plant hormone concentrations, inducing systemic resistance, and inhibiting both growth and intercellular communication of pathogens (Legein et al., 2020). At least seven natural product BGCs were present within the genome of EA-CB0015. In addition to previously characterized lipopeptides surfactin, iturin A and fengycin C (Villegas-Escobar et al., 2013; Mosquera et al., 2014), four additional natural product BGCs for bacillibactin, bacilysin, subtilosin A, and the sporulation killing factor were identified. These later compounds could contribute to the biological control activities of this strain. In addition to iron scavenging and biofilm formation, bacillibactin has been associated with alternative functions including transport of other metals, sequestration of toxic metals, and protection from oxidative stress (Rizzi et al., 2019; Legein et al., 2020). Production of bacilysin by *B. velezensis* FZB42 was found to regulate the expression of several virulence genes in *X. oryzae* (Özcengiz and Öğülür, 2015; Wu et al., 2015), while subtilosin A was inhibitory against a variety of gram-positive and-negative bacteria (Shelburne et al., 2007). The cannibalistic peptide, SKF is known to permeabilize of cytoplasmic membranes of *E. coli* cells (Nonejuie et al., 2016) and inhibit the growth of plant pathogens such as *X. oryzae* (Lin et al., 2001). Determining the production of these compounds by EA-CB0015 on plants will provide valuable insight into their functional role in biocontrol and if beneficial synergistic effects may exist.

We identified significant variations between related *Bacilli* that arose from bacteriophage activity. Long-term associations between temperate phages may provide benefit to bacteria through resistance against infection and introduction of accessory genes for metabolism, stress tolerance, and antibiotic resistance. These may facilitate survival of EA-CB0015 when exposed to natural products produced from other epiphytic microbes and antibiotics commonly applied to commercial crops (Schnabel and Jones, 1999; Feiner et al., 2015; Howard-Varona et al., 2018; Ramisetty and Sudhakari, 2019). Moreover, mobile genetic elements in EA-CB0015 and related *Bacilli* may accelerate transfer of genes that are advantageous for their survival in the phyllosphere (Lindow and Leveau, 2002). The contribution of phages, mobile elements, and associated TA systems on the competitive fitness of epiphytic *Bacillus* spp., influencing the phyllosphere microbiome, and effecting the physiology of host plants remain important areas of future investigation.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP048852.1.

Author contributions

TC-G, K-SJ, and VV-E: conceptualization and writing the manuscript. TC-G, CG-B, K-SJ, and VV-E: methodology, investigation, and formal analysis. K-SJ and VV-E: supervision and funding 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1135487/full#supplementary-material

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The core fungal microbiome of banana (*Musa* spp.)

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Here, we report a metabarcoding (ITS2) study to define the common core fungal microbiome (mycobiome) of healthy *Musa* spp. (bananas and plantains). To identify a list of 21 core fungal taxa, we first characterised the effects of edaphic conditions and host genotype – two factors that are likely to differ between farms – on the diversity of fungal communities in bulk soil and seven plant compartments. This experiment facilitated shortlisting of core 'candidates', which were then elevated to full core status if also found to frequent a wide-range of field-grown *Musa* spp. and exhibit hub-like characteristics in network analyses. Subsequently, we conducted a meta-analysis of eleven publicly available datasets of *Musa* spp. associated fungi demonstrating that the core fungi identified in our study have close relatives in other countries. The diversity and composition of mycobiomes differed between plant compartments and soils, but not genotypes. The core mycobiome included *Fusarium oxysporum* and its relatives, which dominated all plant compartments, as well as members of the *Sordariomycetes*, *Dothideomycetes*, and *Mortierellomycota*. Our study provides a robust list of common core fungal taxa for *Musa* spp. Further studies may consider how changes in the frequencies and activities of these taxa influence host fitness and whether they can be managed to improve banana production.

KEYWORDS

fusarium, fungal diversity, plant-microbe interactions, mycobiome, network, holobiont

Introduction

Musa spp. (bananas and plantains) are one of the world's most important fruit crops but are constrained by a range of abiotic and biotic stresses, including diseases for which management options are either unavailable, or becoming less effective (Gutierrez-Monsalve et al., 2015; Bubici et al., 2019; Fu et al., 2019). Fungal diseases of *Musa* spp., such as black leaf streak, Eumusae leaf spot, freckle, and Fusarium wilt, are particularly notorious; however, many fungi benefit host fitness (Yano-Melo et al., 2003; Rodríguez-Romero et al., 2005; Drenth and Kema, 2021). If managed appropriately, banana fungal microbiomes (mycobiomes) could help growers to maintain healthy production systems. Nonetheless, plant-associated fungi are extremely diverse and may vary between locations. Hence, it is important to identify fungal taxa that are persistently associated with *Musa* spp. across a wide range of environmental conditions, *viz.* the 'common core' (Risely, 2020). This approach emphasises a relatively small subset of taxa on which to focus research efforts and helps avoid the development of microbiome management approaches that are context dependent (Toju et al., 2018). A common core mycobiome of *Musa* spp. is yet to be defined.

Different plant compartments offer unique niches for fungi and should be considered when defining a core mycobiome (Cregger et al., 2018). Previous studies of fungi associated with *Musa* spp. have focused on below-ground interactions, leaving the mycobiomes of above-ground plant compartments relatively underexplored (Wang et al., 2015; Rames et al., 2018; Shen et al., 2018). The diversity and composition of bacterial communities associated with *Musa* spp. have been shown to differ greatly between plant compartments (Birt et al., 2022). However, for fungi this association may be weaker. In cereals, legumes, *Brassicaceae*, and *Agave* spp., for example, the location in which plants are grown, rather than plant compartment has been observed to have a larger impact on the diversity and composition of fungal communities (Coleman-Derr et al., 2016; Tkacz et al., 2020).

Edaphic factors also need to be considered when defining a core mycobiome of *Musa* spp. as soil is the primary source of fungi that colonise plants (Busby et al., 2017). The diversity and composition of soil fungal communities differs depending on both abiotic factors such as nutrient availability (Vasco-palacios and Bahram, 2020) soil structure (Xia et al., 2020), and biotic factors such as plant-mediated soil feedback (Raaijmakers and Mazzola, 2016). Additionally, edaphic factors may also influence the types of relationships fungi play within the mycobiome ranging from pathogens to mutualists (Guerrero-Ariza and Posada, 2017). Hence, fungi that persistently associate with *Musa* spp. across diverse edaphic conditions are likely to be relevant to a range of production sites.

While the effects are often smaller than those associated with soil properties, the impacts of host genotype should also be considered when defining a core *Musa* spp. mycobiome (Hannula et al., 2010; Laurent et al., 2010). Changes in hormones, tissue phenotype, and life cycle timing between various genotypes can influence host-associated fungi (Vorholt et al., 2017). Finding a common core between *Musa* genotypes is important because it is estimated that there are more than 500 cultivars in use globally (Stover and Simmonds, 1987).

Here, we characterised the common core mycobiome of *Musa* spp. Our study began with a pot experiment to determine the impacts of plant compartment, edaphic conditions, and host genotype on fungal diversity using ITS2 rRNA gene amplicon sequencing. This experiment comprised more than 480 samples from eight plant compartments, three genotypes, and five distinct soils, and was used to define a list of candidate-core fungal taxa. We then characterised the fungal communities associated with more than 400 samples from field-grown *Musa* spp. comprising 52 genotypes. These results were used to refine our list of candidates and identify a final set of common core fungal taxa, which were compared with other members of the microbiome using network analysis, including bacteria from a previously published study (Birt et al., 2022). Finally, by comparing the sequences of our core fungi with those reported in 11 previous studies of banana-associated fungi, we provide evidence that they are commonly associated with *Musa* spp. in other parts of the world.

Methods

Experimental design

Pot experiment: To investigate the potential impacts of plant compartment, soil, and genotype on *Musa* spp. mycobiomes we conducted a pot experiment in a glasshouse as described previously in Birt et al. (2022). Briefly, the banana variety 'Williams' *Musa* (AAA Group, Cavendish Subgroup), Australia's most common commercial cultivar (Australian Banana Growers Council, 2021) was grown in five distinct soils collected (0–30 cm depth) from North Queensland, Australia's primary banana producing region (Supplementary Table S1). In one of these soils only, we also grew the banana variety 'Lady finger' *Musa* (AAB Group, Pome Subgroup) and the banana variety 'Goldfinger' *Musa* (AAAB Group, Prata Anã x SH-3142) to investigate the effect of genotype on *Musa* spp. mycobiomes. These genotypes represented Australia's second most common cultivar (Australian Banana Growers Council, 2021) and a Fusarium wilt resistant cultivar (De Ascensao and Dubery, 2000), respectively. Eight plant compartments were sampled using an established procedure (Birt and Dennis, 2021): bulk soil (BS), the ectorhizosphere at the apex and base of the roots (AER, BER), the endorhizosphere at the apex and base of the roots (AEnR, BEnR), the rhizome/corm (C), the pseudostem (PS), and leaves (L) (Supplementary Figure S1).

Having assessed the key drivers of fungal communities associated with *Musa* spp. and used these to define a core mycobiome, we also performed a field survey to confirm our findings under field conditions and in a wider range of genotypes. Bulk soil (BS), ectorhizosphere (ER), endorhizosphere (EnR), pseudostem (PS), and leaf (L) samples were collected from 55 plants, representing 52 genotypes in a single field from the Australian *Musa* germplasm collection, South Johnstone, Queensland (Supplementary Table S2). For each plant, we collected a set of samples (i.e., BS, ER, EnR, PS, and L) associated with a young (large sucker), medium (emerged adult stem in vegetative growth), and older (signs of active flowering) pseudostem to provide three replicates for each compartment and encompass variation that may be attributable to the age of various tissues. Strict biosecurity is in place at the site and all plants appeared healthy with no signs of pest or pathogen pressure.

DNA extraction and fungal community profiling

DNA extraction: All samples were lyophilised, homogenised by grinding, and then DNA was extracted using the high throughput Power Soil DNA Isolation kit (Qiagen) according to the manufacturer's instructions except for an extra 400 µl of Powerbead solution to moisten freeze dried samples enough for efficient extraction.

PCR: To avoid host co-amplification, we used the primers ITS-F_KYO1 (5′- CTH GGT CAT TTA GAG GAA STA A-'3) (Toju et al., 2012) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-'3) (White et al., 1990) in polymerase chain reactions (PCRs) to amplify the full ITS region (PCR 1). These regions are less conserved among fungi and plants and therefore avoid plant-associated reads. We then purified the products using magnetic beads (Rohland and Reich, 2011) and used these as template in a second PCR targeting the ITS2 region using the more universal primers gITS7 (5'- GTG AAT CAT CGA ATC TTT G-'3) (Ihrmark et al., 2012) and ITS4 to obtain an amplicon of appropriate length for DNA sequencing (PCR 2). In the second PCR, both primers were modified on the 5' end to contain the Illumina overhang adapter for compatibility with the P5 and i7 Nextera XT

indices. We tested the impact of this nested PCR (full ITS, then ITS2) on fungal community composition relative to a single step PCR (ITS2 only) and found no significant difference between protocols (see Supplementary experiment).

Thermocycling conditions were as follows: 98°C for 45 s; then 15 cycles for PCR 1 or 20 cycles for PCR 2 of 98°C for 5 s, 56°C for 5 s, 72°C for 6 s; followed by 72°C for 1 min. Amplifications were performed using a SimpliAmp® 96-well Thermocycler (Applied Biosystems). All PCRs were performed on 2 µl template in 5X Phire Green Reaction Buffer (Thermo Fisher), 100 µM of each dNTP (Invitrogen), 0.4 µl Phire Green Hot Start II DNA Polymerase (Thermo Fisher), 10 mM of each primer, made up to a total volume of 20 µl with molecular biology grade water. Amplicons were purified and then dual indexed using the Nextera XT Index Kit (Illumina) according to the manufacturer's instructions. Indexed amplicons were then purified, quantified using a PicoGreen dsDNA Quantification Kit (Invitrogen), and then pooled in equimolar concentrations prior to being sequenced on an Illumina MiSeq using 30% PhiX Control v3 (Illumina) and a MiSeq Reagent Kit v3 (600 cycles, Illumina) according to the manufacturer's instructions.

Processing of sequence data

By combining all data from the pot experiment and field survey into a single bioinformatic analysis, we were able to compare the two datasets to validate whether core taxa identified in pots were present in the field. Data was processed by using a modified UPARSE workflow (Edgar, 2013). Firstly, samples were demultiplexed using cutadapt from QIIME2 (v2017.9.0, Bolyen et al., 2019). Fungal ITS2 regions were then extracted using ITSx (v1.0.11, Bengtsson-Palme et al., 2013) and chimeric sequences were removed using uchime2_ref of USEARCH (v10.0.240, Edgar, 2010) against the UNITE 8.2 database (Nilsson et al., 2019). The resulting reads were then mapped against representative sequences using fastx_uniques and cluster_otus (sequence similarity = 0.97) from USEARCH to produce an OTU table. BLASTN (Zhang et al., 2000) from QIIME2 was used to assign taxonomy using the UNITE 8.2 database (Nilsson et al., 2019). Samples were rarefied to 1,000 reads per sample. QIIME2 was used to calculate all alpha diversity metrics. While our nested PCR approach greatly reduced host contamination, *Musa* ITS2 rRNA gene reads were still present in some samples; hence, some samples had fewer than 1,000 fungal reads and were discarded. While most treatment combinations retained all 10 replicates, some ended up with fewer, albeit not less than four (Supplementary Tables S3–S5).

Statistical analyses

Effects of compartment and soil/genotype: Differences in alpha diversity (observed OTUs, Chao1, and Shannon) were investigated using ANOVA with Tukey's HSD for *post hoc* analyses. Differences in fungal community composition between treatments (i.e., beta diversity) were investigated using PERMANOVA as implemented in the R package *vegan* (Oksanen et al., 2022). Fungal OTU relative abundances were Hellinger transformed prior to analysis. Differences in the composition of fungal communities associated with treatments shown to be significant were visualised using redundancy analysis (RDA) in *vegan*. All statistical analyses were performed using R version 3.6.0.

SourceTracker, a Bayesian statistical tool (Knights et al., 2011), was used to examine the extent to which the microbial community of a plant compartment was sourced from other compartments. Each compartment was defined as a microbial source and was compared to all others when using SourceTracker. A grand mean and standard deviation were calculated after SourceTracker had been run on the data from plants grown in each representative soil.

Definitions of core taxa: Abundance and occupancy relationships were chosen to define core OTUs as these are grounded in macroecological theory as a tool to establish the range of a species (Hanski, 1982; Gaston et al., 2000). Prevalent and abundant OTUs were defined as those present in ≥50% of samples within a plant compartment with an average relative abundance of ≥0.5% where found. These values were chosen as the minimum for defining a core OTU as they represent a level that minimises stochastic association (through prevalence thresholds) and to circumvent bias in the estimation of variability for taxa with low abundance as they would be near our detection limit. Studies examining core microbes in a range of other environments have taken similar approaches (Barnett et al., 2015; Brodie et al., 2016; Billiet et al., 2017; Adam et al., 2018). Candidate-core OTUs were identified based on those that were found within plants grown in all five soils.

Next, a final list of core taxa was produced by removing those only found in the pot dataset as these taxa were likely to occur due to the differences between the pot and field-grown plants. This was achieved by removing taxa that were not in the list of taxa found in the field dataset that were 'key constituents', defined as found in ≥50% of samples at an abundance of ≥0.5%. Through this validation process, we were able to confirm that a more stringent definition of core OTUs in the pot experiment (beyond ≥50% of samples within a plant compartment or beyond an average relative abundance of ≥0.5% where found) would miss some abundant and prevalent OTUs present in both datasets (Supplementary Figure S2).

Network analysis

Weighted co-occurrence networks were created from both the fungal and bacterial OTU tables from this and our previous study on the bacterial microbiome of *Musa* spp. (Birt et al., 2022), using samples from the independent field survey. Only OTUs found to have ≥1% relative abundance in three samples were included in the analysis to reduce computational load. Networks were calculated in R using the multi.spiec.easi function in the *SpiecEasi* package that accounts for the use of two independent but compositional datasets that are used in a single network (Kurtz et al., 2015; Tipton et al., 2018). The resulting network was then projected using Gephi (Bastian et al., 2009). Degree, betweenness centrality, Markov centrality, and closeness centrality were calculated using the *igraph* and *centiserve* R packages (Csardi and Nepusz, 2006; Jalili, 2017). A *Fusarium* sub-network and the corresponding centrality metrics were created and calculated using Gephi. All centrality metrics were assessed between candidate-core and non-core taxa using Wilcoxon rank-sum tests implemented in base R.

Meta-study of publicly available data

To determine the applicability of our core mycobiome to a worldwide context, we downloaded data from 11 previous phylogenetic marker gene studies on fungi associated with *Musa* spp. (Supplementary Table S6) using the SRA explorer (Ewels, 2020). For ITS rRNA gene high-throughput sequencing studies, the ITS1 and ITS2 regions were extracted using ITSx (Bengtsson-Palme et al., 2013). For 18S data, adapters were removed using cutadapt (Martin, 2011). Sequences were then filtered to remove low-quality reads, and an OTU table was produced using USEARCH (Edgar, 2013). The representative sequences were extracted from the top 10% of OTUs sorted by the maximum seen in any sample. BLASTN (Zhang et al., 2000) from QIIME2 was again used to assign taxonomy using against the UNITE 8.2 database for ITS rRNA gene sequences and SILVA 128 for 18S rRNA gene sequences (Quast et al., 2012; Nilsson et al., 2019). Next, the top hit based on the highest e-value was extracted for each OTU from the blast results. The taxonomy assigned for core and candidate-core OTUs was then searched for in these results using a custom R script.

Results

The effects of compartment, soil, and host genotype within the pot experiments

Compartment: The diversity and composition of fungal communities differed significantly between plant compartments, and this effect was stronger than those of soil and host genotype (Table 1; Supplementary Table S7). The least diverse fungal communities were associated with the apical endorhizosphere, followed by the basal endorhizosphere, the rhizome (Figure 1). The pseudostem and leaves were as diverse as the ectorhizosphere and bulk soil (Figure 1). In terms of composition, fungal communities were more similar in compartments that were closer to one another, with bulk soil and ectorhizosphere communities being distinct from those associated with endorhizosphere and above-ground plant compartments (Figure 2). This finding was also supported by Bayesian estimates of community provenance, which indicated that 91% of the pseudostem fungal microbiome was sourced from leaves, in contrast to 62% from bulk soil (Supplementary Table S8).

Members of the *Ascomycota* formed the majority of reads in all *Musa* spp. compartments (Figure 3). They were most abundant in the apical endorhizosphere (94.3% mean relative abundance, Figure 3) and least abundant in the leaves (70.8% mean relative abundance, Figure 3). While present in other compartments, representatives of the Basidiomycota were most common in leaves, where they comprised 10.5–45.0% mean relative abundance (Figure 3). Members of the *Mortierellomycota* and *Glomeromycota* were also detected but were relatively infrequent (Figure 3). Furthermore, c. 6.6% mean relative abundance of fungi in all communities could not be allocated a taxonomic rank below kingdom (Figure 3). Finally, while negligible in plants grown in other soils, members of the Chytridiomycota represented 11.3% mean relative abundance within the ectorhizosphere of plants grown in Tolga soil (Figure 3).

Soil and genotype: The diversity and composition of fungal communities also differed significantly between soils but not genotypes (Tables 1, 2). According to the Shannon Index, fungal diversity differed significantly between soils in the pseudostem, basal ectorhizosphere, and bulk soil (Table 2); however, these differences were not supported by all alpha diversity metrics (Supplementary Table S7). Fungal community composition was observed to significantly differ between soils in all belowground compartments, except the rhizome (Table 2).

TABLE 1 The impacts of soil, genotype, and plant compartment on the alpha diversity (Shannon's Diversity Index) and composition (Hellinger transformed OTUs) of fungal communities using ANOVA and PERMANOVA, respectively.

Predictor variable		Shannon's diversity		Community composition		
	df	*F* value	*p* value	*F* value	R^2 (%)	*p* value
Compartment	7	30.3	<0.001***	15.5	21.5	<0.001***
Soil	4	2.8	0.028*	7.4	5.9	<0.001***
Compartment: Soil	28	1.7	0.021*	2.0	11.2	<0.001***
Compartment	7	14.5	<0.001***	12.1	29.3	<0.001***
Genotype	2	0.5	0.596	1.4	1.0	0.074
Compartment: Genotype	14	0.7	0.770	1.1	5.2	0.221

These results derive from our pot experiment which included five distinct soils, three *Musa* spp. genotypes, and eight compartments.

Identifying 'candidate core' fungal taxa

Given that compartment and soil, but not genotype, were found to influence the *Musa* spp. mycobiome, we defined a list candidate-core fungal OTUs as follows. Firstly, for each soil, we identified the most abundant and prevalent OTUs on the basis that they were found in ≥50% of replicates one or more compartments, at a mean relative abundance of ≥0.5% where present (Supplementary Figures S3, S4). From the 173 OTUs that met these criteria, we then removed any that were not found in all soils (Supplementary Figures S3–S5), leaving 42 that were given 'candidate-core' status (Supplementary Figure S5).

Refining the 'core' mycobiome of *Musa* spp.

Next, we surveyed the fungal microbiomes of 52 field-grown banana plants, to assess whether the 'candidate-core' fungal OTUs identified in our pot experiment were also important under more realistic conditions. As observed in pots, field-grown plants were dominated by members of the Ascomycota and Basidiomycota (Supplementary Figure S6).

The 'importance' of 'candidate-core' fungal OTUs was considered from two key perspectives. Firstly, we inferred their importance to putative microbiome interactions using network analyses. These interactions were inferred using SPIEC-EASI for fungal ITS2 data only, and fungal ITS2 in combination with bacterial 16S data from the same samples (Birt et al., 2022; Supplementary Figures S7, S8). In both

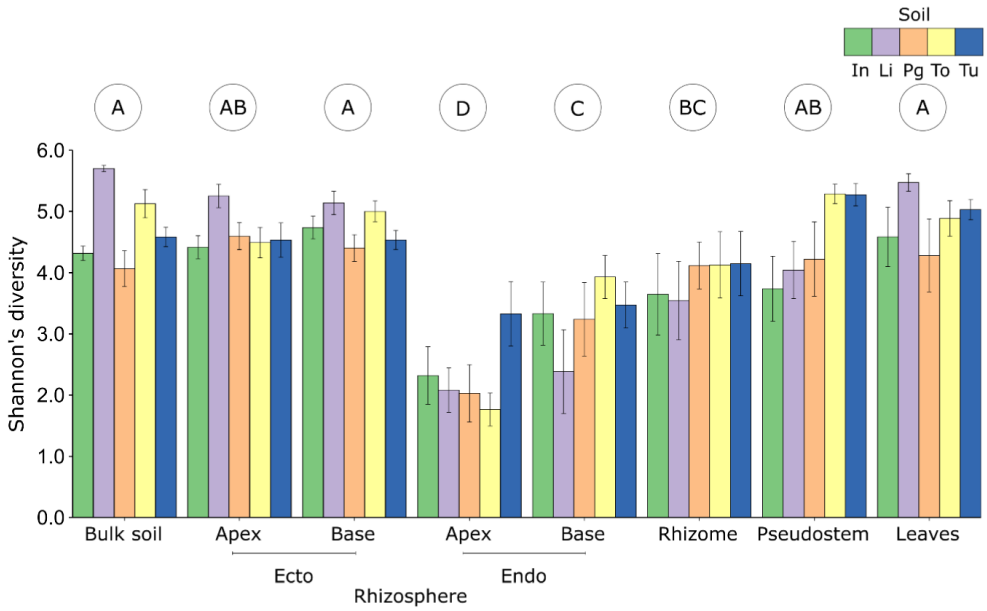

FIGURE 1
The alpha diversity (numbers of observed OTUs) of fungal communities associated with different plant compartments of *Musa* (AAA Group, Cavendish Subgroup) 'Williams' grown in pots with five distinct soils. Error bars represent standard errors of the means. Letters in circles indicate compartments that differ according to Tukey *post hoc* tests. endo, endorhizosphere; ecto, ectorhizosphere; In, Innisfail; Li, Liverpool; Pg, Pin Gin; To, Tolga; Tu, Tully.

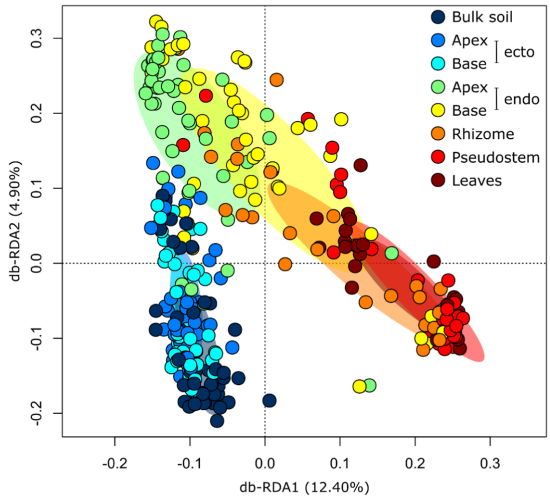

FIGURE 2
Distance-based Redundancy Analysis (RDA) ordination highlighting differences in the composition of fungal communities (Hellinger transformed OTUs) associated with *Musa* (AAA Group, Cavendish Subgroup) 'Williams' in various plant compartments. The ellipses represent standard deviations of the group centroids. endo, endorhizosphere; ecto, ectorhizosphere.

cases, 'candidate-core' fungal OTUs had significantly more connections (Degree), and larger betweenness, closeness, and Markov centrality scores than non-core OTUs (Table 3).

Secondly, we inferred the importance of candidate core OTUs based on whether they were represented among the most abundant and prevalent fungal OTUs in the field mycobiomes (i.e., those present in ≥50% of field-grown plants at ≥0.5% mean relative abundance). Of the 36 OTUs that met these criteria, 14 were classified as 'candidate core' OTUs (i.e., same OTU), and seven were considered close relatives (i.e., identical taxonomy but different OTUs). Together, these 21 OTUs were elevated to full 'core' status and represented the majority of the most abundant and prevalent OTUs in every compartment (Figure 4). In addition, while representing only 0.35% of all OTUs in the field, the 21 'core' OTUs represented c. 50–60% and c. 35–45% of sequences in roots and above-ground compartments, respectively (Figure 4). Lastly, 95% (20/21) of 'core' OTUs were represented in all 52 *Musa* spp. genotypes examined, either as the same OTU or a close relative. The only exception was *Phaeosphaeria oryzae*, which was present in 87% (45/52) *Musa* spp. genotypes.

The core mycobiome of *Musa* spp.

The 21 core fungal OTUs constitute nine distinct genera, including eight within the Ascomycota, and one within the *Mortierellomycota* (Figure 5; Supplementary Figure S9). Representative ITS2 gene sequences of the core candidate-core are provided in the supplementary information (Supplementary Table S9).

Fusarium spp. and their close relatives represent a significant proportion of the final core. Eight (38%) of the final core are members of the *Nectriacaeae* – the family containing *Fusarium* spp. OTU 1 (*Fusarium oxysporum*) was found at a high relative abundance throughout the plant and represented >50% of reads in the apical endorhizosphere (Figure 5). Core taxa that were not identified as *Fusarium* spp. were not found throughout the entire plant (Figure 5; Supplementary Figure S8). Different core taxa occupied the root-associated compartments compared to other compartments. For example, *P. oryzae* and *Cladosporium* sp. were found to be dominant only in the phyllosphere (Figure 5; Supplementary Figure S7).

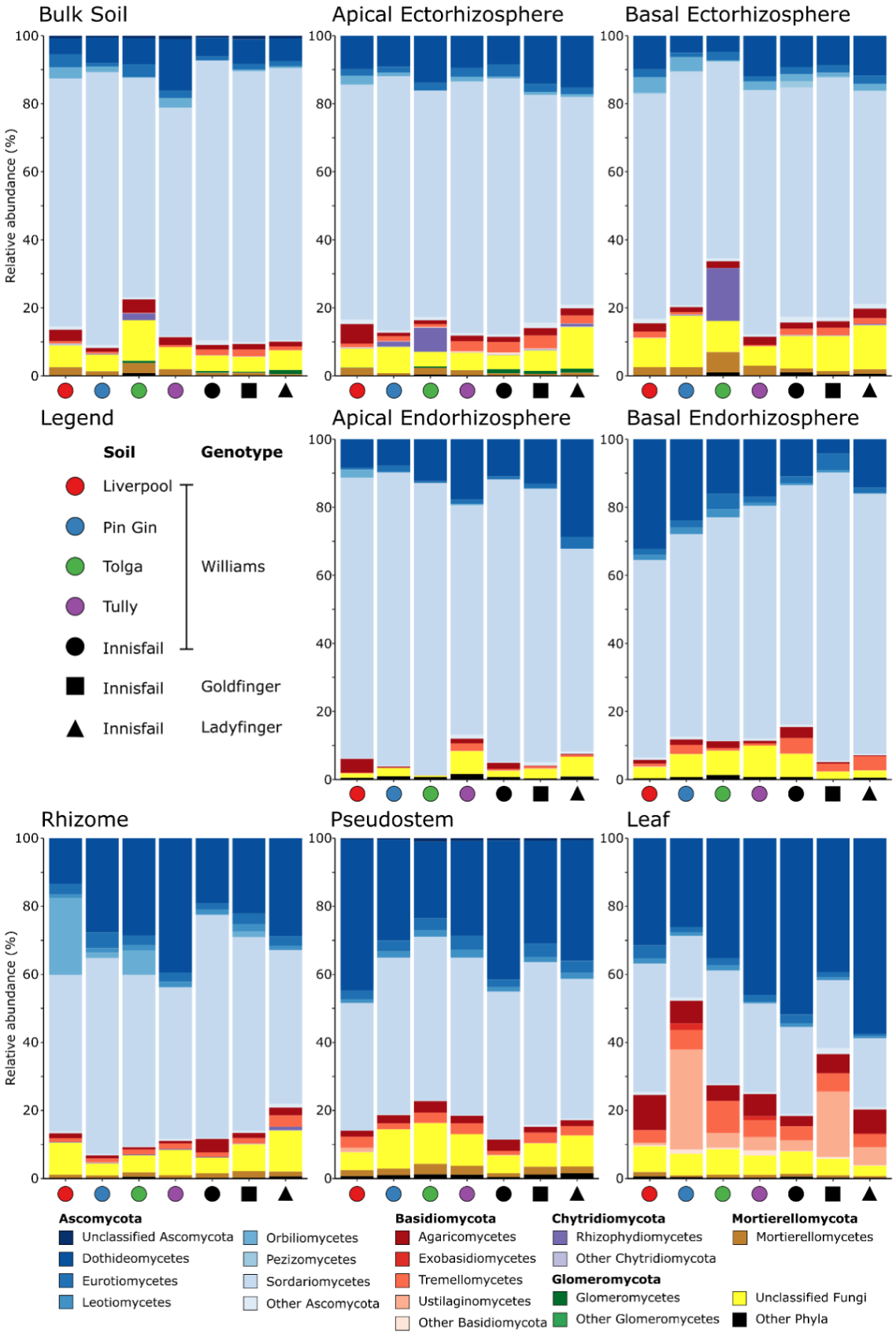

FIGURE 3
The mean relative frequencies of fungal classes in different plant compartments associated with *Musa* (AAA Group, Cavendish Subgroup) 'Williams' grown in pots with five distinct soils, and two other *Musa* spp. genotypes grown in pots containing an Innisfail series soil. Within each phylum, classes represented at <1% mean relative abundance are grouped as other.

TABLE 2 The influence of soil on the alpha diversity (Shannon's Diversity Index) and composition (Hellinger transformed OTUs) of fungal communities within each compartment, as assessed by ANOVA and PERMANOVA, respectively.

Compartment	Shannon diversity		Community composition		
	F value	*p* value	*F* value	R^2 (%)	*p* value
Bulk soil	12.7	<0.001***	6.7	38.5	<0.001***
Apical ectorhizosphere	2.2	0.083	4.5	28.4	<0.001***
Apical endorhizosphere	1.9	0.123	1.9	14.9	<0.001***
Basal ectorhizosphere	2.8	0.038*	5.6	33.6	<0.001***
Basal endorhizosphere	1.1	0.367	1.7	13.7	0.004**
Rhizome	0.2	0.912	1.3	14.3	0.169
Pseudostem	2.9	0.037*	1.5	14.3	0.086
Leaf	1.2	0.345	1.3	16.9	0.123

The results are for the *Musa* (AAA Group, Cavendish Subgroup) 'Williams' plants grown in five distinct soils in our pot experiment.

Pyrenchaetopsis tabarestanensis was dominant only in the root-associated compartments and bulk soil (Figure 5; Supplementary Figure S8).

Next, we used our microbiome networks to infer the putative interactions of core- taxa with *Fusarium* spp., which are known to influence the health of *Musa* spp. (Alabouvette, 1999; Nel et al., 2006a; Hill et al., 2021). Within this subnetwork, core taxa formed 46% of the nodes. Furthermore, core taxa were significantly more central according to the number of connections made (degree) and their tendency to connect distinct parts of the network (betweenness, Figure 6).

An assessment of the core taxa in publicly available datasets

Finally, we sought to determine whether our core fungal taxa had been detected in association with banana plants in other parts of the world. To do this, we used publicly available data from 11 other studies that considered the *Musa* spp. mycobiome (Supplementary Table S6). These studies expanded our search to China, Uganda, and other parts of Australia (Supplementary Table S6). We searched for the core taxa in the top 10% of OTUs sorted by maximum abundance: *F. oxysporum* was found in all but one study, *Fusarium* spp. were found in all studies, and *Mortierella* sp. and *Nigrospora* sp. were found in all but two and three, respectively. Every other core genus was identified in at least one study (Supplementary Figure S9). Although dropped from the final core set, *Acromonium* sp. and *Curvularia* sp. were found in seven and five studies of eleven, respectively (Supplementary Figure S10).

Discussion

To manage *Musa* spp. microbiomes, it is logical to first identify common core taxa as they are the most likely to be present across a range of conditions. Here we focussed on identifying the common core fungal taxa associated with *Musa* spp. To do this we used ITS2 amplicon sequencing to characterise fungal communities in multiple, above, and below-ground compartments of pot and field-grown plants. This was done to assess the entirety of the fungal community associated with the plant. In addition, we sought to consider variation associated with two of the key factors that may differ between farms, *viz.* soil properties and host genotype.

Musa spp. mycobiomes are diverse and differ between plant compartments and soils, but not genotypes

Compartment: Plant compartment was the strongest predictor of fungal community diversity and composition, with distinct communities associated with specific tissues. Hence, when comparing results between studies or designing new experiments, it is important to consider the compartment with which the mycobiomes are associated. For example, investigating the mycobiomes of bulk soil is unlikely to yield a result that is representative of fungi associated with roots or other tissues. Our findings also indicate that the fungal communities were diverse in both the ectorhizosphere and leaves, but declined in diversity in the root endophytic compartments. This finding differs from that of a recent investigation of the mycobiome of *Musa* explants and field-grown *Musa* spp. during Fusarium wilt disease progression (Liu et al., 2019). This study found that the endorhizosphere was more diverse than shoots (a pseudostem and rhizome pooled sample). In our study, *Fusarium* spp. were particularly dominant in the root endophytic compartments, whereas in the study of Liu et al. (2019), they were less dominant. As a result, *Fusarium* spp. in our study may have been competitively excluding other fungi in the root endophytic compartment and lowering the overall diversity; similar mechanisms have been demonstrated in other plants (Younginger et al., 2022). Although statistical comparisons of communities were not made by Liu et al., they did report the most dominant taxa being different in various compartments. We too found large differences in community composition between compartments. These distinct communities are likely to arise from compartment-specific ecological niches and environmental conditions as well as the varying ability of the plant to regulate communities in different tissues (Rossmann et al., 2017). Proximal compartments tended to be more similar and leaf communities were distinct from those found in soil-associated compartments. These findings indicate that fungi associated with *Musa* spp. may disperse into endophytic tissues from both the leaves and roots.

TABLE 3 Results from Wilcoxon sum rank tests of centrality metrics from fungal OTUs considered core and non-core.

Network	Metric	Candidate-core fungi (Median 1st quartile, 3rd quartile)	Non-core fungi (Median 1st quartile, 3rd quartile)	*W*	*p*
Fungi	Betweenness	184 (42, 793)	30 (0, 181)	2,760	0.002**
Fungi	Closeness	$1.98*10^{-4}$ ($1.95*10^{-4}$, $2.00*10^{-4}$)	$1.96*10^{-4}$ ($1.87*10^{-4}$, $1.99*10^{-4}$)	2,640	0.011*
Fungi	Degree	4 (3, 8)	3 (1, 6)	2,537	0.033*
Fungi	Markov	$7.06*10^{-4}$ ($6.0*10^{-4}$, $7.8*10^{-4}$)	$6.56*10^{-4}$ ($3.9*10^{-4}$, $7.5*10^{-4}$)	2,575	0.024*
Fungi and bacteria	Betweenness	398 (193, 1,031)	149 (34, 381)	2,978	<0.001***
Fungi and bacteria	Closeness	$2.64*10^{-4}$ ($2.58*10^{-4}$, $2.70*10^{-4}$)	$2.56*10^{-4}$ ($2.50*10^{-4}$, $2.63*10^{-4}$)	2,828	<0.001***
Fungi and bacteria	Degree	8 (6, 11)	6 (3, 8)	2,719	0.004**
Fungi and bacteria	Markov	$1.33*10^{-3}$ ($1.0*10^{-3}$, $1.7*10^{-3}$)	$2.56*10^{-4}$ ($2.5*10^{-4}$, $2.6*10^{-4}$)	2,695	0.006**

Letters indicate OTUs included in the network: F are fungi only, F and B are fungi and bacteria. Networks were built from data from an independent field survey of 52 genotypes of *Musa* spp.

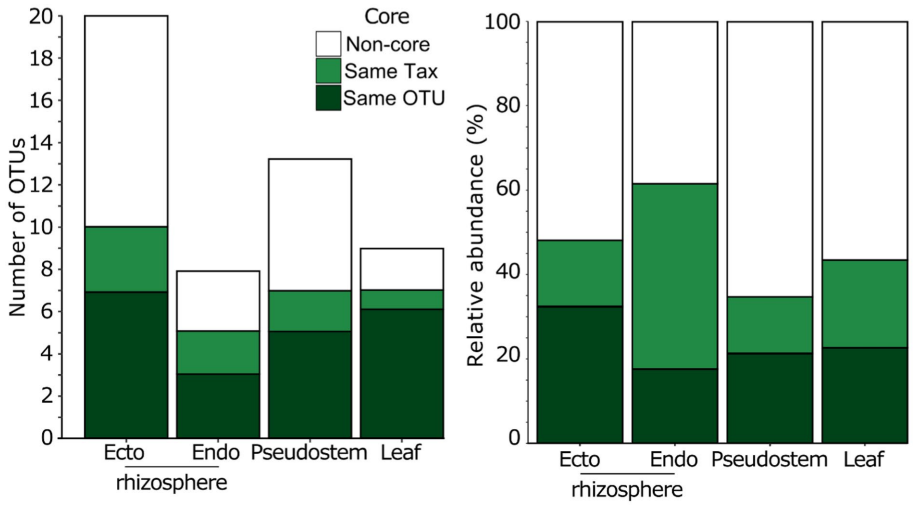

FIGURE 4
The number of abundant and prevalent OTUs from the field experiment and the relative abundance of fungi that were defined as core or non-core in each plant compartment from 52 field grown *Musa* spp. genotypes.

Edaphic factors: We also observed that the soil in which the plant is grown influences fungal diversity. This is to be expected as it is a major reservoir of fungi available to the plant (Hawksworth and Lu, 2017) and has previously been observed for bacteria associated with *Musa* spp. (Birt et al., 2022). Despite the impact of soil, we found core taxa that were persistently associated with plants grown in different soils. These would be relevant for various farms with differing edaphic conditions. Our investigation revealed differences in both alpha and beta fungal diversity among banana production soils, with larger changes in the root-associated compartments. Nevertheless, interactions between plants and fungi at the root can still have whole plant effects (Chandrasekaran et al., 2019). Differences in fungal diversity have also been observed in studies of banana plantation soils in Spain (Gómez-Lama Cabanás et al., 2021; Ciancio et al., 2022). Within one of these studies, pH was found to differ between soils, as it did in our study (Ciancio et al., 2022). Amendments to pH in banana plantation soils have been shown to influence fungal diversity and may therefore be a driver of fungal diversity in this study (Zhang et al., 2019).

Host genotype: Genotype was not found to influence the fungal microbiome of healthy *Musa* spp. in this study. Nevertheless, as some of the cultivars tested are known to be resistant to fungal pathogens (Goldfinger), clearer differences may become apparent when different genotypes experience pathogen pressure. For example, differences in the mycobiome of the leaves of various *Populus* tree genotypes were most apparent when foliar pathogens were present (Cregger et al., 2018). As genotype does not have a large impact on the *Musa* mycobiome, much of the mycobiome research that has been

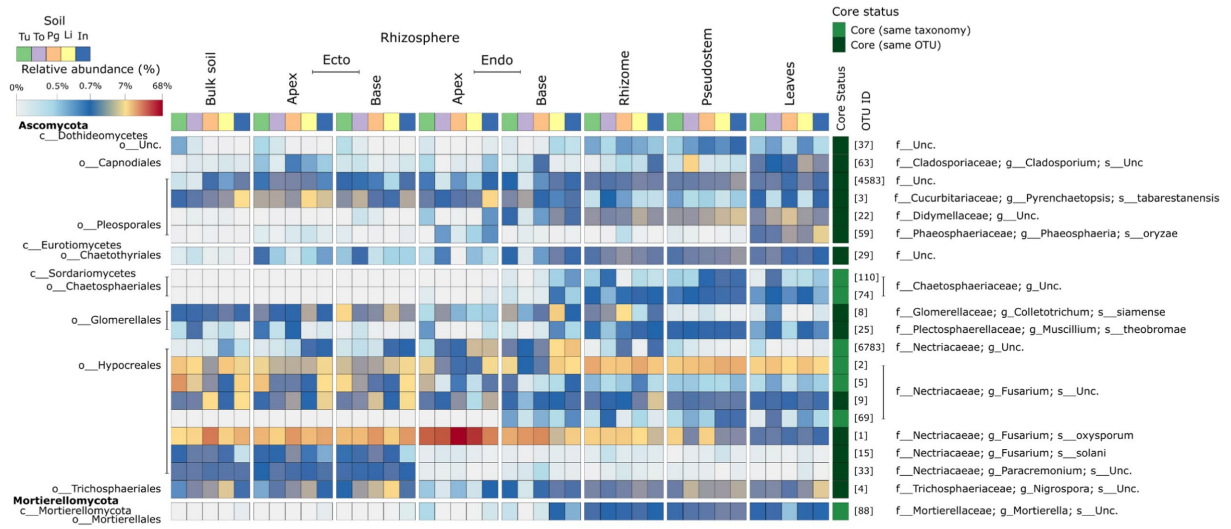

FIGURE 5
A heatmap showing the mean abundance of the core fungal microbiome taxa identified in this study in various *Musa* (AAA Group, Cavendish Subgroup) 'Williams' plant compartments and plants grown in distinct soils.

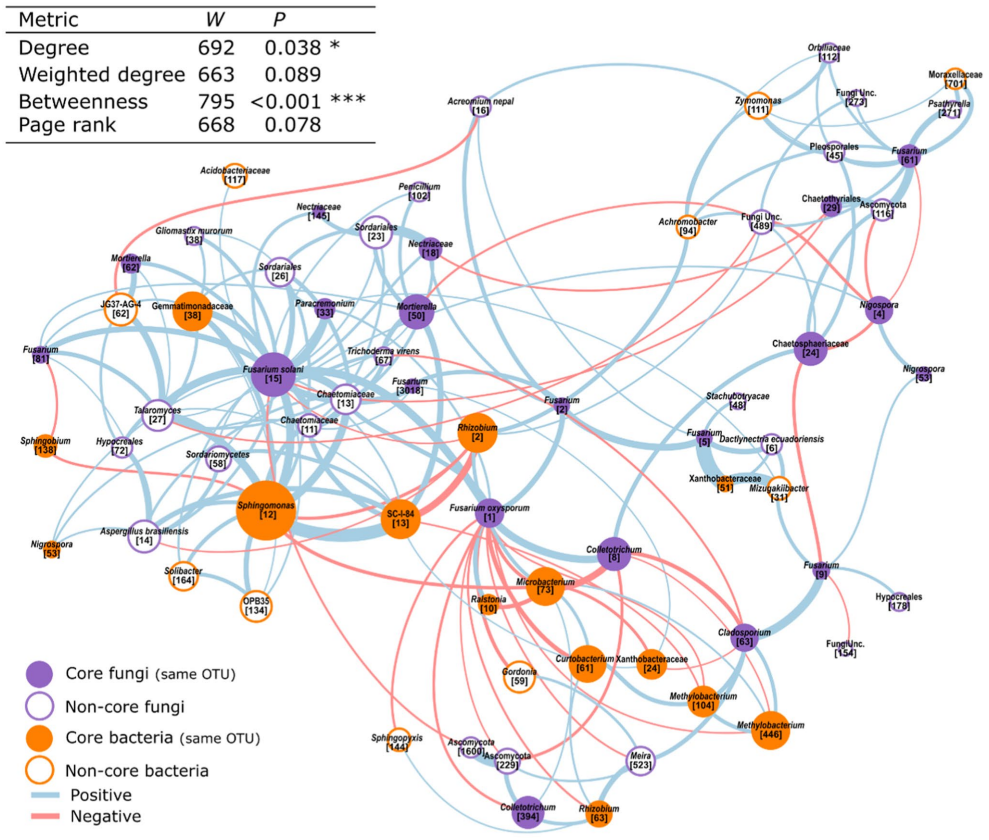

FIGURE 6
A subset of a dual amplicon co-occurrence network showing the interactions between Fusarium OTUs and their first order neighbours. Those that are core bacteria or fungi are highlighted. This network was created from the microbiome of 52 adult *Musa* spp. in a field setting. Node size is scaled by weighted degree.

conducted on specific *Musa* genotypes may be translatable to various genotypes in production (Nel et al., 2006b). We also found a similar pattern for bacterial communities in a previous study (Birt et al., 2022). However, an investigation of differences in fungi associated with the seeds of wild *Musa* spp. showed clear differences in the diversity of associated fungal species, with implications for

germination rate (Hill et al., 2021). By contrast, our study used sterile tissue culture plantlets (the industry standard) which could have prevented the vertical transmission of fungi.

The validation of core fungal taxa

The 42 candidate-core fungi microbiome were defined using a pot experiment. We validated our core taxa with a field survey of 52 genotypes because we were aware that there were other genotypes relevant to production that had not been included in the pot experiment, as well as differences between pot and field grown plants (Poorter et al., 2016). Here, we found 21 'core' OTUs, which despite only being 0.35% of OTUs, represented c. 50–60% and c. 35–45% of sequences in roots and above-ground compartments, respectively (Figure 4). All but one of these taxa were also present in all 52 genotypes. In addition, although developmental stage has been shown to influence plant microbiomes (Edwards et al., 2018), these core taxa were found in adult and juvenile plants. Work on other plant mycobiomes have found that dominant fungi often establish early in the plant lifecycle (Johnston-Monje et al., 2021). Given the sterile nature of tissue culture plantlets used in banana production, there may be ample opportunity to introduce core taxa in the plant hardening stage prior to introduction in the field.

Core OTUs have close banana-associated relatives around the world

To explore a range of climatic zones, different forms of management, and a greater diversity of genotypes and edaphic factors, we checked whether our core taxa could be found in publicly available datasets. We found evidence of the core taxa in Uganda, China, and other parts of Australia. The most dominant of these were the *Fusarium* spp., a *Mortierella* sp., and a *Nigrospora* sp. The consistent association of these fungi could imply they have strong co-evolutionary history (Gross, 2019). Another possibility is that these fungi are environmental generalists and they have been spread through plant matter transported by humans (Golan and Pringle, 2017). This is particularly likely for bananas as they are the world's most traded fruit and have a long history of being moved around the planet (Voora et al., 2020). Although our data does not allow us to deduce the ecological functions of these core taxa, we could infer their functional importance from network analysis.

Core OTUs occupy central positions in co-occurrence networks

By combining the dataset from this paper with a previous study of the bacterial microbiome of *Musa* spp. we were able to create a dual amplicon co-occurrence network. Networks such of these can provide increased network stability, higher connectivity, and similar topological re-organization patterns compared single phylogenetic marker networks (Tipton et al., 2018). We found that core fungi occupied central positions within this network (Table 3; Supplementary Figure S5). Fungi with central positions in networks have been shown to have implications for disease incidence in banana production soils (Yang et al., 2022). Interestingly, we also found that core taxa had co-occurrence relationships with *Fusarium* spp., which have important implications for plant health in *Musa* spp. (Ploetz, 2015; Hill et al., 2021).

Associations of core taxa with host fitness

Circumstantial evidence from the literature also indicates that some of the core taxa are associated with plant health in *Musa* spp. A study into the fungi associated with Fusarium Wilt in *Musa* spp. found that *Cladosporium* spp. were dominant in healthy plants found adjacent to wilting plants, indicating a possible role in disease control (Liu et al., 2019). This study also found *Cladosporium* spp. to persist in tissue culture plants, suggesting a strong association with *Musa* spp. throughout their lifecycle. Moreover, non-pathogenic strains of *Fusarium* associated with *Musa* spp. and have frequently been studied for their ability to control pathogenic *Fusarium* strains (Forsyth et al., 2006; Nel et al., 2006b; Belgrove et al., 2011). Despite being most studied for their ability to cause disease, *F. oxysporum* has been reported to be associated with the rhizosphere of healthy *Musa* spp. (Nel et al., 2006a). Of 60 *F. oxysporum* isolates found by Nel et al. (2006a) to be associated with the rhizosphere of various banana plants, only one isolate caused disease. In total, these isolates could be placed into 12 phylogenetic groups. In a subsequent study, it was demonstrated that some of these *F. oxysporum* isolates were not able to control a pathogenic *F. oxysporum* f. sp. *cubense in vitro* but were highly effective *in planta* (Nel et al., 2006b). The abundance of *Mortierella* spp., another core taxa, has been shown to be positively associated with suppression of Fusarium wilt in *Musa* spp., but decreased in relative abundance the longer a field was under monoculture conditions (Shen et al., 2018). *Mortierella* spp. have been shown to antagonise pathogens through antibiotic production and could perform similar functions for *Musa* spp. (Melo et al., 2014).

Despite being isolated from healthy plants, other close relatives of core fungi have also been associated with causing disease in *Musa* spp.: *Colletotrichum siamense, Nigrospora* sp., and *Cladosporium* spp. are known to cause foliar and post-harvest diseases (Jones, 2000; Surridge et al., 2003; Kumar et al., 2017; Uysal and Kurt, 2020). Nevertheless, non-pathogenic strains of these fungi have been reported. For example, *Colletotrichum* spp. have been isolated from healthy *Musa* spp., but their function was not elucidated (de Lapeyre de Bellaire et al., 2000). Often relatively small changes in fungal genomes can result in pathogenic lifestyle, such as the SIX genes identified in *Fusarium* spp. (Czislowski et al., 2017); without these genes, the fungi may be commensal or mutualist (Van Dam et al., 2017). Nevertheless, taxa may also switch between being mutualist and pathogenic under certain conditions (Slippers and Wingfield, 2007; Ribeiro et al., 2020).

Comparisons of the core mycobiome of *Musa* with other plant species

The size and composition of the core *Musa* mycobiome has some notable overlaps with other plants. For instance, the core mycobiome of sugarcane (*Saccharum officinarum*) also includes members of the *Cladosporium* and *Pleosporales* (De Souza et al.,

2016). Yet, this study found 45 taxa to be core; however, it did not explain core members across different soils which may account for the increased number of taxa considered core. A recent investigation of fungal taxa associated with grapevines (*Vitis vinifera*) explored core taxa across different soil types and found 15 fungal taxa to be core (Liu and Howell, 2021). This investigation also found *Cladosporium* and *Fusarium* OTUs to be core taxa. Similarly, 12 core fungal taxa were found to be associated with the healthy mycobiome of chilli pepper (*Capsicum annuum*) which again included *Cladosporium* and *Fusarium* OTUs (Gao et al., 2021). Together, these results suggest that the core fungi associated with *Musa* spp. may have a broad host range across different plant species.

Conclusion

This study has given novel insight into the drivers behind the diversity of fungi associated with *Musa* spp. By understanding these, we have defined core taxa consistently associated with *Musa* spp. in various settings. Through network analysis, these taxa have been shown to be more central in community interactions. The function of this set of organisms in conferring health to banana plants is still to be determined. However, this list of core taxa can now provide a focal point for management of these highly complex communities. Future work could investigate whether these taxa result from the style of the production system or an affinity with *Musa* spp. Investigations into their exact functions and how their abundances can be controlled will also provide a better basis for their management use. In the applied use of a core microbiome, there are also opportunities for using these candidates in microbiome manipulation, such as host-mediated microbiome engineering, large-scale bioprospecting, or the introduction of core-microbe consortiums.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA729168.

Author contributions

PD and AP secured funding and designed the study. HB, PD, and AP collected samples and performed experiments. HB and AR performed marker gene sequencing. HB, PD, and AS analyzed data. HB and PD wrote the paper with input from all authors. 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.2023.1127779/full#supplementary-material

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Metatranscriptome-based strategy reveals the existence of novel mycoviruses in the plant pathogenic fungus *Fusarium oxysporum* f. sp. *cubense*

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Fusarium oxysporum f. sp. *cubense* (Foc) is a devastating plant pathogen that caused a great financial loss in the banana's source area. Metatranscriptomic analysis was used to determine the diversity of mycoviruses in 246 isolates of *F. oxysporum* f. sp. *cubense*. Partial or nearly complete genomes of 20 mycoviruses were obtained by BLASTp analysis of RNA sequences using the NCBI database. These 20 viruses were grouped into five distinct lineages, namely *Botourmiaviridae*, *Endornaviridae*, *Mitoviridae*, *Mymonaviridae*, *Partitiviridae*, and two non-classified mycoviruses lineages. To date, there is no report of the presence of mycoviruses in this pathogen. In this study, we demonstrate the presence of mycoviruses isolated from Foc. These findings enhance our overall knowledge of viral diversity and taxonomy in Foc. Further characterization of these mycoviruses is warranted, especially in terms of exploring these novel mycoviruses for innovative biocontrol of banana Fusarium wilt disease.

KEYWORDS

***Fusarium oxysporum* f. sp. *cubense*, mycovirus, metatranscriptome, virus diversity, biocontrol**

Introduction

Bananas are cultivated in more than 120 countries and on approximately 11 million ha. In 2021, the global banana output reached 170.3 million tons, which is an increase of 3.6 million tons from 2020 (FAOSTAT, 2023). Banana Fusarium wilt, a devastating disease caused by the soil-borne, root-infecting fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is affecting global banana production. Based on the pathogenicity of banana cultivars, Foc is divided into three races, including Foc race 1 (Foc1), Foc race 2 (Foc2), and Foc race 4 (Foc4; Thangavelu, 2021). Foc 1 and Foc 4 are the most widespread physiological races in China, of which Foc 4 has been reported in almost all banana cultivation areas in China (Poon et al., 2020). According to geographical characteristics and temperature adaptation profiles, Foc race 4 is further subdivided into Tropical Race 4 (TR4) and Subtropical Race 4 (ST4; Ploetz, 2015). Banana Fusarium wilt has been known to be difficult to control because infection occurs through complex underground interactions between the fungus, the plant, and the soil microorganisms at the root-soil interface. Even after decades of research, there are few effective treatments for Fusarium wilt disease in bananas (Ploetz, 2015). Foc-resistant banana varieties are the most effective strategy for reducing Fusarium wilt

impacts (Viljoen et al., 2020; Zorrilla-Fontanesi et al., 2020). However, conventional breeding is a time-consuming process that can take at least 15 years (Tenkouano et al., 2011). Therefore, biological control of banana Fusarium wilt disease is a promising strategy.

Mycoviruses are viruses that replicate in fungi and have been found in fungal kingdoms. While most mycoviruses are latent infections having no apparent effect on the fungal host, a few have major effects on host growth, development, and reproduction. Several mycoviruses have been identified that reduce the virulence of fungal plant pathogens, the most notable being Cryphonectria hypovirus 1 (CHV1), which reduces the virulence of *Cryphonectria parasitica*, the causal agent of chestnut blight (Ghabrial et al., 2015; Zhang and Nuss, 2016; Rigling and Prospero, 2018). Rosellinia necatrix megabirnavirus 1 (RnMBV1) in *Rosellinia necatrix* has been found to have significant potential to control apple white root rot disease (Kondo et al., 2013). Recently, a fungal DNA virus, Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1), has been shown to have the potential to control *Sclerotinia* disease (Liu et al., 2016). Furthermore, spraying SsHADV-1 infected strain DT-8 in the early flowering phase was reported to reduce rapeseed stem rot disease severity by 67.6% and improve yield by 14.9% (Zhang H. et al., 2020). Given the numerous examples of mycoviruses capable of attenuating the virulence of fungal pathogens, mycoviruses are promising tools for developing biological control strategies to limit the impact of fungi on crop productivity.

Mycoviruses are generally classified by the host, genome structure, and viral particle shape. The majority of known mycoviruses possess linear double-stranded RNA (dsRNA) or positive-sense (+) single-stranded RNA (ssRNA) genomes. However, a few mycoviruses with linear negative-sense (−) ssRNA and single-stranded DNA (ssDNA) genomes have also been reported (Myers and James, 2022). In recent decades, the variety of mycoviruses has increased massively. Next-generation sequencing (NGS) is a popular method for exploring mycoviral diversity, including sequencing total RNA depleted of ribosomal RNA or small RNA. Many novel viruses have been discovered with the help of NGS. For instance, 68 partial or nearly complete genome segments have been identified in the metatranscriptomes of three major fungal pathogens of rice (He et al., 2022). The metagenomic approach was used to comprehensively characterize all mycoviruses in an international collection of the brown rot pathogen *Monilinia fructicola* (De Miccolis Angelini et al., 2022). Based on the International Committee on Taxonomy of Viruses (ICTV) Taxonomy Report and ICTV Master Species List 2021.v3 (https://ictv.global/msl, accessed on 5 January 2023), there are 26 families containing over 200 mycovirus species. With the development and widespread use of RNA deep sequencing techniques, the diversity of mycoviruses in *Fusarium* species is constantly increasing. Mycoviruses have been described in many *Fusarium* species, such as *F. asiaticum* (Li et al., 2019), *F. andiyazi* (Yao et al., 2020), *F. boothii* (Mizutani et al., 2018), *F. circinatum* (Martínez-Álvarez et al., 2014), *F. coeruleum* (Osaki et al., 2015), *F. globosum* (Sharma et al., 2018), *F. graminearum* (Chu et al., 2002, 2004; Li et al., 2016; Zhang X. et al., 2020), *F. incarnatum* (Zhang et al., 2019), *F. langsethiae* (Li et al., 2017), *F. oxysporum* f. sp. *dianthi* (Lemus-Minor et al., 2015), *F. poae* (Osaki et al., 2016), *F. pseudograminearum* (Zhang et al., 2018), *F. sacchari* (Yao et al., 2020), *F. solani* (Osaki et al., 2015), *F. virguliforme* (Marvelli et al., 2014), and so on.

The majority of *Fusarium* mycoviruses establish latent infections, but some mycoviruses, such as Fusarium graminearum virus 1 (FgV1; Paudel et al., 2022), Fusarium graminearum virus-ch9 (FgV-ch9; Sharma et al., 2018), Fusarium graminearum hypovirus 2 (FgHV2; Li et al., 2015), and Fusarium oxysporum f. sp. dianthi mycovirus 1 (FodV1; Lemus-Minor et al., 2018), cause hypovirulence. However, no mycoviruses have been reported in *Fusarium oxysporum* f. sp. *cubense* (Foc), a devastating plant fungal pathogen. To fill this gap, we screened and cultured 246 strains isolated from southern and southwestern China (Fujian, Guangdong, Guangxi, Hainan, and Yunnan provinces) for metatranscriptome sequencing to investigate mycovirus diversity in Foc. This information provides insight into the diversity and taxonomy of mycoviruses of Foc.

Materials and methods

Isolates and growth conditions

Foc was isolated and purified from diseased banana plants in Fujian, Guangdong, Guangxi, Hainan, and Yunnan provinces, China. The fungal stock cultures were maintained in a final concentration of 25% (v/v) glycerol at −80°C. All isolates were cultured on potato dextrose agar (PDA: potato 200 g/L, agar 15 g/L, and dextrose 20 g/L) at 28°C in darkness.

Total RNA extraction and purification

The total RNA of each strain was extracted from 0.5 g of fungal mycelium by using a Plant RNA Extraction Kit (Promega Code: LS1040, Beijing). The total RNA was stored at −80°C until used. 246 strains were divided into two groups, group 1 of 146 and group 2 of 100. Approximately 2 ng of RNA was taken from each sample and mixed. A pooled RNA sample was sent to Shanghai Biotechnology Corporation (China) for RNA sequencing (RNA-seq).

RNA sequencing and sequence analysis

Ribosomal RNA depletion, library preparations, and Illumina sequencing were performed by Shanghai Biotechnology Corporation (China). Zymo-Seq RiboFree Total RNA Library Prep Kit (Zymo Research, USA) was used for ribosomal RNA depletion and library preparations. Illumina sequencing (Illumina NovaSeq 6000) was carried out by Shanghai Biotechnology Corporation (China).The filtered data was spliced together from scratch, and the resulting sequences were then de-duplicated. Subsequently, the virus sequences have been annotated using Diamond software (version 0.9.21.122) and the National Center for Biotechnology Information (NCBI) Non-Redundant Protein database.[1]

Confirmation of virus-like contigs

To confirm the detection of viral-like contigs in the strains tested, the presence of each contig was confirmed by RT-PCR using the

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

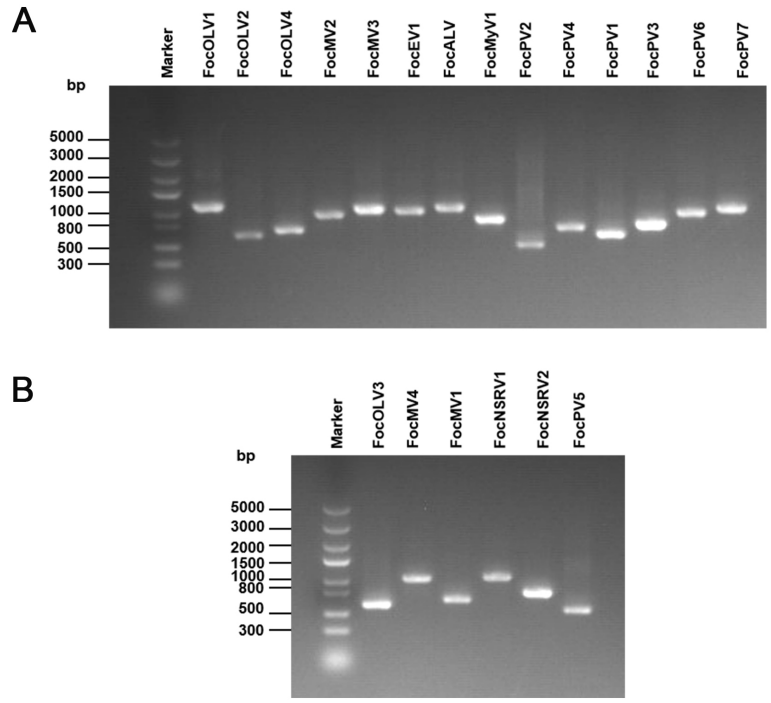

FIGURE 1
RT-PCR confirmation of 20 viral-like contigs in Foc. **(A)** RT-PCR confirmation of contigs in the RNA samples of group 1. **(B)** RT-PCR confirmation of contigs in the RNA samples of group 2. The viral primers were designed according to the contig sequences. Primers used and predicted sizes of PCR products are listed in Supplementary Table S1. Lane M, Trans5K DNA Marker (Transgen, China); Lane 1 to 20, abbreviates of viruses (see Table 1).

appropriate primer pairs. The RNA samples were used as RT-PCR templates and specific DNA bands were amplified using the Vazyme HiScript- II One Step qRT-PCR SYBR- Green Kit (no. #Q221-01, Nanjing, China) using the corresponding primers listed in the Supplementary Table S1. DsRNA extraction was also used to confirm the presence of viral-like contigs, as previously described (Li et al., 2015).

Phylogenetic analysis

Open reading frames (ORFs) were determined with the aid of the ORF Finder program from NCBI. Then, to determine the reliability of a given branching pattern, phylogenetic trees were constructed from RNA-dependent RNA polymerase (RdRp) sequences using the neighbor-joining (NJ) method and tested with 1,000 bootstrap replicates in the MEGA 11 software. Multiple sequence alignments were performed by ClustalW. Accession numbers of the species for building the evolutionary tree are listed in the Supplementary Table S2.

Result

Viral sequences in the metatranscriptome of Foc

A collection of 246 isolates (group 1 of 146 and group 2 of 100) of Foc isolated from banana samples was used. Among 246 Foc isolates, only 20 in group 1 are Foc1, while all other isolates in two groups are Foc4. A total of 178,543,328 raw reads were generated by Illumina NGS. The assembly of the high-quality reads (QS ≥ 20) yielded about 287,540 contigs (Supplementary Table S3). All contigs were subjected to BLAST analysis. In group 1, partial or nearly complete genome segments of 162 viruses were obtained from the analysis (Supplementary Table S4). Similarly, 70 viruses were obtained in group 2 (Supplementary Table S5). Viruses from different pools with >95% identity at the amino acid level were considered variants of the same virus; the sequence from each virus with the highest read coverage and sequence length was selected as representative after manual inspection of the alignment. Since low coverage and short contigs are known to be prone to error, we focus on further analyzing contigs >800 bp and high coverage contigs for RT-PCR verification. In particular, we focused on these contigs which were identified as viruses potentially related to fungi. Combined with RT-PCR amplification results, the screening identified 20 virus-related sequences from different viral families (Figure 1; Table 1). Further, we confirmed the presence of five contigs (contig9, contig5527, contig6366, contig16483, and contig20141) in each specific strain by the dsRNA extraction (Supplementary Figure S1). Based on sequence analysis, viruses were classified into different taxonomical groups, including 10 viruses that were predicted to represent +ssRNA viruses belonging to three different viral families: *Botourmiaviridae*, *Mitoviridae*, and *Endornaviridae*. The genomes of two -ssRNA viruses were associated with those of viruses in the family *Mymonaviridae* and a novel, unclassified family of the order *Bunyavirales*, respectively. The remaining dsRNA genomes were related to those of members of the family *Partitiviridae*. There is one additional virus, probably representing a member of an unassigned fungal alphavirus-like group.

TABLE 1 Assembled sequences with similarity to previously described viruses.

Number	Contig number	GenBank accession number	Contig length (bp)	Name of putative viruses	Best match[a]	aa identity (%)	Genome type	Family/genus
1	Contig97	OQ685967	2,750	*Fusarium oxysporum* f. sp. *cubense* ourmia-like virus 1 (FocOLV1)	Fusarium mangiferae botourmiavirus 2 (UBZ25887.1)	90	+ssRNA	*Botourmiaviridae*
2	Contig1565	OQ685968	3,253	*Fusarium oxysporum* f. sp. *cubense* ourmia-like virus 2 (FocOLV2)	Botoulivirus sp. (UJQ92030.1)	60	+ssRNA	*Botourmiaviridae*
3	Contig36412	OQ685969	2,766	*Fusarium oxysporum* f. sp. *cubense* ourmia-like virus 4 (FocOLV4)	Botourmiaviridae sp. (WAK77841.1)	85	+ssRNA	*Botourmiaviridae*
4	Contig120	OQ685970	2,444	*Fusarium oxysporum* f. sp. *cubense* ourmia-like virus 3 (FocOLV3)	Fusarium solani ourmia-like virus 1 (WAS28562.1)	49	+ssRNA	*Botourmiaviridae*
5	Contig9	OQ685971	2,471	*Fusarium oxysporum* f. sp. *cubense* mitovirus 2 (FocMV2)	Fusarium andiyazi mitovirus 1 (QPK91779.1)	88	+ssRNA	*Mitoviridae*
6	Contig5103	OQ685972	2,378	*Fusarium oxysporum* f. sp. *cubense* mitovirus 4 (FocMV4)	Colletotrichum fructicola mitovirus 1 (BBN51032.1)	95	+ssRNA	*Mitoviridae*
7	Contig5527	OQ685973	2,388	*Fusarium oxysporum* f. sp. *cubense* mitovirus 1 (FocMV1)	Plasmopara viticola lesion associated mitovirus 7 (WAK72427.1)	93	+ssRNA	*Mitoviridae*
8	Contig20494	OQ685974	2,356	*Fusarium oxysporum* f. sp. *cubense* mitovirus 3 (FocMV3)	Albatrellopsis flettii mitovirus 1 (QUP79368.1)	43	+ssRNA	*Mitoviridae*
9	Contig20141	OQ685975	4,012	*Fusarium oxysporum* f. sp. *cubense* endornavirus 1 (FocEV1)	Agaricus bisporus endornavirus 1 (YP_010086750.1)	25	+ssRNA	*Endornaviridae*
10	Contig2840	OQ685976	1,902	*Fusarium oxysporum* f. sp. *cubense* alphavirus-like virus (FocALV)	Fusarium graminearum alphavirus-like virus 1 (QGW08824.1)	29	+ssRNA	unclassified
11	Contig24	OQ685977	9,485	*Fusarium oxysporum* f. sp. *cubence* mymonavirus 1 (FocMyV1)	Fusarium proliferatum mymonavirus 1 (UWK02084.1)	95	-ssRNA	*Mymonaviridae*
12	Contig14157	OQ685978	10,342	*Fusarium oxysporum* f. sp. *cubense* negative-stranded RNA virus 1 (FocNSRV1)	Sanya Mymon tick virus 1 (UYL95358.1)	99	-ssRNA	*Mymonaviridae*
13	Contig14713	OQ685979	6,712	*Fusarium oxysporum* f. sp. *cubense* negative-stranded RNA virus 2 (FocNSRV2)	Grapevine-associated mycobunya-like virus 4 (QXN75455.1)	57	-ssRNA	unclassified
14	Contig11434	OQ685986	990	*Fusarium oxysporum* f. sp. *cubense* partitivirus 2 (FocPV2)	Partitiviridae sp. (UDL14404.1)	72	dsRNA	*Partitiviridae*
15	Contig6366	OQ685980	1,376	*Fusarium oxysporum* f. sp. *cubense* partitivirus 4 (FocPV4)	Flammulina betapartitivirus 1 (BDV50554.1)	61	dsRNA	*Partitiviridae*
16	Contig75904	OQ685981	1,030	*Fusarium oxysporum* f. sp. *Cubense* partitivirus 1 (FocPV1)	Trichoderma citrinoviride partitivirus 1 (AZT88592.1)	55	dsRNA	*Partitiviridae*
17	Contig16483	OQ685982	1,856	*Fusarium oxysporum* f. sp. *cubense* partitivirus 3 (FocPV3)	Gaeumannomyces tritici partitivirus 2 (AZT88604.1)	67	dsRNA	*Partitiviridae*
18	Contig99419	OQ685983	825	*Fusarium oxysporum* f. sp. *cubense* partitivirus 5 (FocPV5)	Sarcosphaera coronaria partitivirus (QLC36806.1)	54	dsRNA	*Partitiviridae*
19	First_ Contig2653	OQ685984	1,909	*Fusarium oxysporum* f. sp. *cubense* partitivirus 6 (FocPV6)	Heterobasidion partitivirus 5 (ADV15444.1)	80	dsRNA	*Partitiviridae*
20	First_ Contig2479	OQ685984	1,646	*Fusarium oxysporum* f. sp. *cubense* partitivirus 7 (FocPV7)	Podosphaera prunicola partitivirus 1 (ATS94410.1)	61	dsRNA	*Partitiviridae*

[a]The Genbank accession number for each virus is listed in the parentheses.

Botourmiaviridae-related sequences

The family *Botourmiaviridae* includes 12 genera and viruses with positive-sense RNA genomes that infect plants and fungi. Generally, members of this family, except *Ourmiavirus*, are non-encapsidated fungal viruses with a monopartite and monocistronic genome of 2,000–5,300 nucleotides containing a single ORF encoding the RNA-dependent RNA polymerase (RdRp; Ayllón et al., 2020). Contig97 was 2,750 nt in length, possessing a large ORF encoding a 642 aa peptide showing homology to the RdRp sequence of Fusarium mangiferae botourmiavirus 2, with an aa sequence identity of 90% (Table 1; Figure 2A). We have named it Fusarium oxysporum f. sp. cubense ourmia-like virus 1 (FocOLV1). Contig1565 was 3,253 nt long, encoding a putative RdRp of 705 aa, which was 60% identical to the sequence of *Botoulivirus* sp., and it was named Fusarium oxysporum f. sp. cubense ourmia-like virus 2 (FocOLV2). Contig120 was 2,444 nt in length encoding a putative RdRp of 670 aa, namely Fusarium oxysporum f. sp. cubense ourmia-like virus 3 (FocOLV3). The RdRp of FocOLV3 was most homologous to the RdRp sequence of Magnaporthe oryzae ourmia-like virus (MoOLV) with aa sequence identity of 48%. Contig36412 is 2,766 nt, with a complete ORF encoding for 747 aa. This putative protein showed 85% identity with Phomopsis longicolla RNA virus 1 (PlRV1) RdRP, and we named this

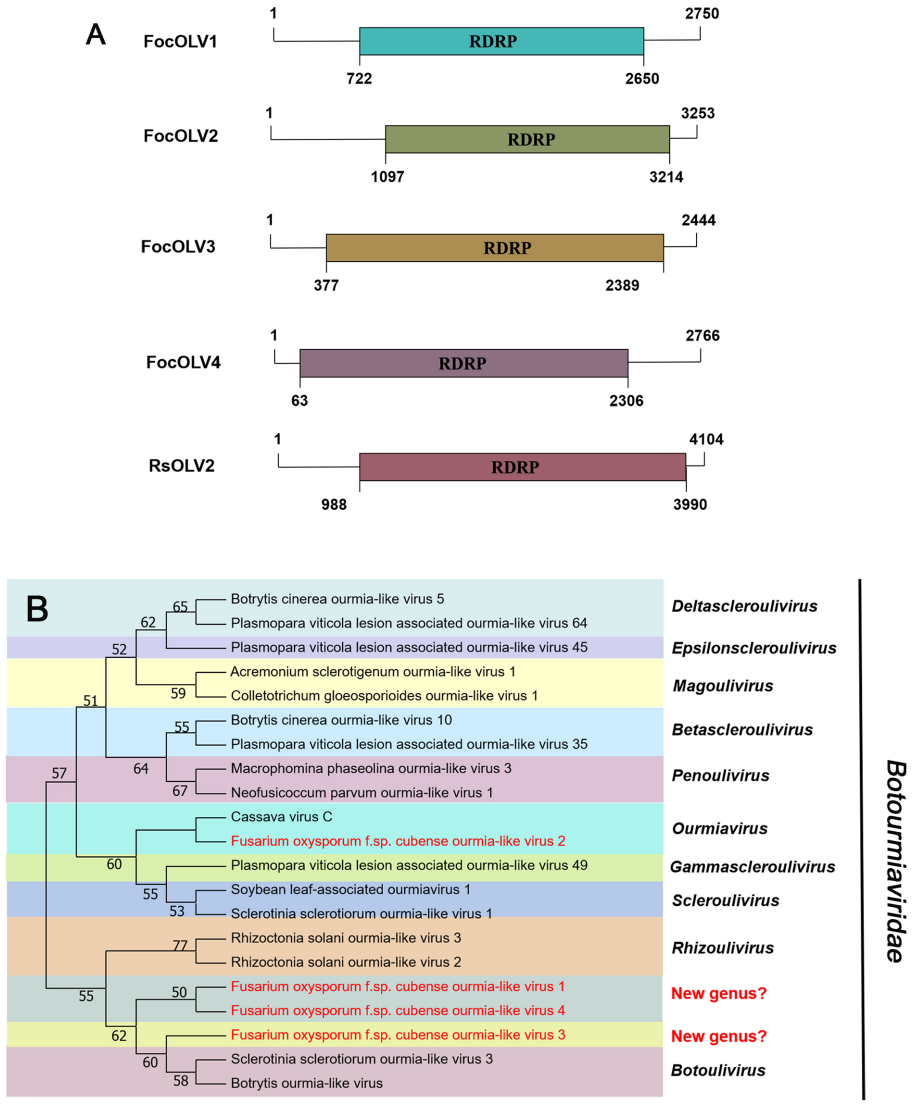

FIGURE 2
Genome organization and phylogenetic analysis of viruses in the family *Botourmiaviridae*. **(A)** Schematic diagram showing the genome organization of viruses, including Fusarium oxysporum f. sp. cubense ourmia-like virus 1 (FocOLV1), Fusarium oxysporum f. sp. cubense ourmia-like virus 2 (FocOLV2), Fusarium oxysporum f. sp. cubense ourmia-like virus 3 (FocOLV3), Fusarium oxysporum f. sp. cubense ourmia-like virus 4 (FocOLV4), and Rhizoctonia solani ourmia-like virus 2 (RsOLV2). The genomic structure of RsOLV2 was selected as a representative of the family. **(B)** Phylogenetic analysis based on the RdRP of putative viruses in the family *Botourmiaviridae* using the neighbor-joining (NJ) method. Red-marked viruses are isolated in Foc. Viruses without color annotation are representatives of known species of the viral family. Genus clades are circumscribed in coloured blocks, with genus names and the family name shown to the right of the tree. Bootstrap percentage greater than 50% are shown.

virus Fusarium oxysporum f. sp. cubense ourmia-like virus 4 (FocOLV4; Table 1; Figure 2A). To establish the phylogeny of these viruses with other viruses of family *Botourmiaviridae*, a phylogenetic tree was constructed using the neighbor-joining (NJ) method based on the viral RdRp aa sequences (Figure 2B). Results showed that FocOLV2 was mostly related to Cassava virus C, which belongs to the genus *Ourmiavirus*. Specifically, members of the genus *Ourmiavirus* were previously reported to be plant viruses with non-enveloped tri-segmented genome. This is the first report of an ourmiavirus infecting a fungus. FocOLV1 and FocOLV4 are clustered together, and form an independent branch in the evolutionary tree, suggesting that they may belong to a new genus in the family *Botourmiaviridae* (Figure 2B). FocOLV3 also form an independent branch in this phylogenetic tree, implying that it may be a novel genus in the family *Botourmiaviridae*.

Mitoviridae-related sequences

According to the current ICTV report, members of the family *Mitoviridae* are naked +ssRNA viruses that contain a single ORF encoding the RdRP, with a genome size of 2,151–4,955 nt (Ghabrial and Suzuki, 2009; Jacquat et al., 2023). Four fragments, conting9 (2,471 nt), conting5103 (2,378 nt), conting5527 (2,388 nt), and contig20494 (2,356 nt) were predicted to encode a mitoviral RdRp, namely Fusarium oxysporum f. sp. cubense mitovirus 2 (FocMV2), Fusarium oxysporum f. sp. cubense mitovirus 4 (FocMV4), Fusarium oxysporum f. sp. cubense mitovirus 1 (FocMV1), Fusarium

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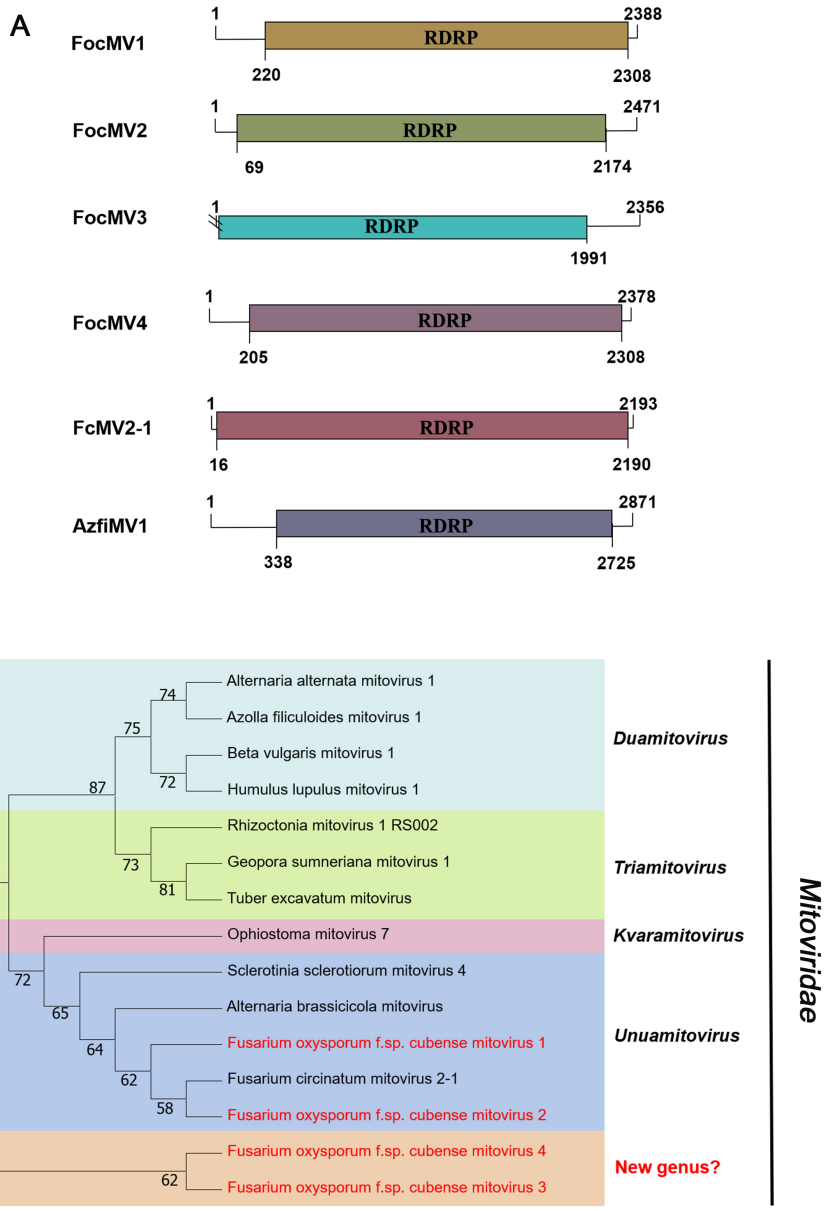

FIGURE 3
Genome organization and phylogenetic analysis of viruses in the family *Mitoviridae*. **(A)** Schematic diagram showing the genome organization of viruses, including Fusarium oxysporum f. sp. cubense mitovirus 1 (FocMV1), Fusarium oxysporum f. sp. cubense mitovirus 2 (FocMV2), Fusarium oxysporum f. sp. cubense mitovirus 3 (FocMV3), Fusarium oxysporum f. sp. cubense mitovirus 4 (FocMV4), Fusarium circinatum mitovirus 2-1 (FcMV2-1), and Azolla filiculoides mitovirus 1 (AzfiMV1). The genomic structures of FcMV2-1 and AzfiMV1 were selected as representative members of the family. **(B)** Neighbor-joining (NJ) method phylogenetic tree based on the core RNA-dependent RNA polymerase (RdRP) of putative viruses in the family *Mitoviridae*. Red-marked viruses are isolated in Foc. Viruses without color annotation are representatives of known species of the viral family. Genus clades are circumscribed in coloured blocks, with genus names and the family name shown to the right of the tree. Bootstrap percentage greater than 50% are shown.

oxysporum f. sp. cubense mitovirus 3 (FocMV3; Table 1; Figure 3A), respectively. The RdRp of FocMV2 was most similar to Fusarium andiyazi mitovirus 1 with an aa sequence identity of 88%, while FocMV4 was most similar to Colletotrichum fructicola mitovirus 1 with an aa sequence identity of 95%. FocMV1 was most similar to Plasmopara viticola lesion associated mitovirus 7, and FocMV3 was most similar to Albatrellopsis flettii mitovirus 1, with an aa sequence identity of 93 and 43%, respectively (Table 1; Figure 3A). Phylogenetic analysis using the RdRp aa sequences showed that FocMV1 and FocMV2 were clustered within the genus *Unuamitovirus* based on the NJ method. Specifically, FocMV3 and FocMV4 are clustered together, but form an independent branch, meaning that they may be members of a new genus (Figure 3B).

Endornaviridae-related sequences

Viruses in the family *Endornaviridae* have linear ssRNA genomes, ranging in length from 10–17 kb, and contain a single large ORF. The family consists of two genera: *Alphaendornavirus* and

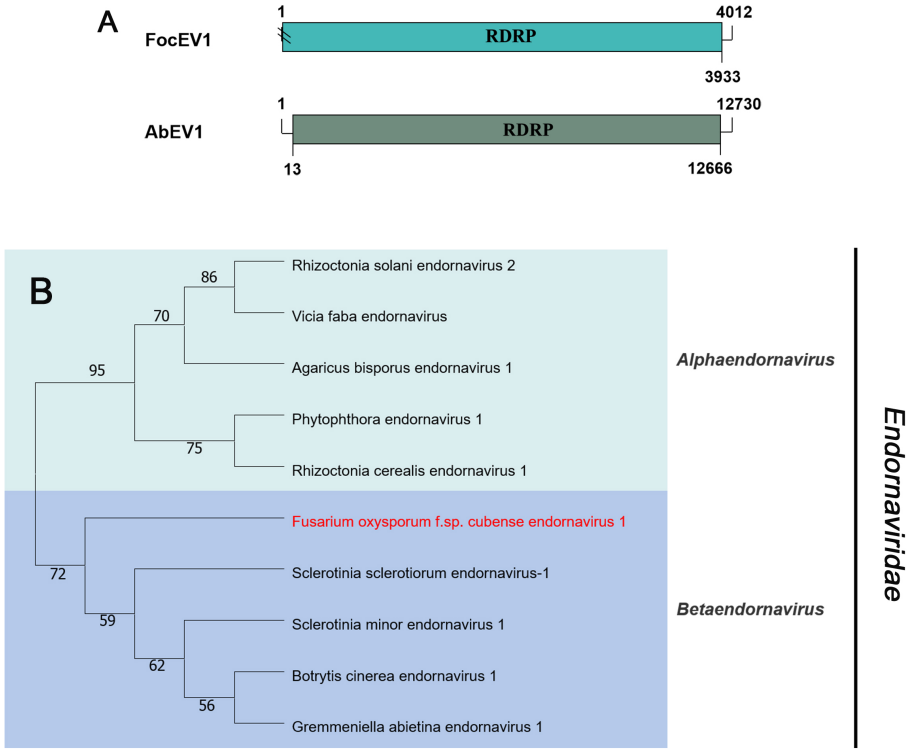

FIGURE 4
Genome organization and phylogenetic analysis of viruses in the family *Endornaviridae*. **(A)** Schematic diagram showing the genome organization of viruses, containing Fusarium oxysporum f. sp. cubense endornavirus 1 (FocEV1) and Agaricus bisporus endornavirus 1 (AbEV1). The genomic structure of AbEV1 was selected as a representative of the family. **(B)** Neighbor-joining (NJ) method phylogenetic tree based on the core RNA-dependent RNA polymerase (RdRP) of putative viruses in the family *Endornaviridae*. Red-marked viruses are isolated in Foc. Viruses without color annotation are representatives of known species of the viral family. Genus clades are circumscribed in coloured blocks, with genus names and the family name shown to the right of the tree. Bootstrap percentage greater than 50% are shown.

Betaendornavirus. Viruses are divided based on genome size, host, and presence of unique domains (Valverde et al., 2019). According to the BLASTx search, only one fragment (contig20141) of 4,012 nt, contains a large ORF encoding RdRp, showing homology to Agaricus bisporus endornavirus 1 (AbEV1) with 25% identity, namely Fusarium oxysporum f. sp. cubense endornavirus 1 (FocEV1). The phylogenetic analysis showed that FocEV1 is a new member of the genus *Betaendornavirus* (Figure 4).

Mymonaviridae-related sequences

Negative-sense RNA viruses (NSRVs) are a large and diverse group of viruses. They infect a very wide range of hosts including vertebrates (mainly mammals), invertebrates (mainly arthropods), plants, and fungi. Based on whether their RNA genomes are segmented or not, NSVs are generally classified into two large viral orders: non-segmented negative-sense RNA viruses (nsNSRVs) and segmented negative-sense RNA viruses (sNSRVs). Most nsNSRVs belong to the order *Mononegavirales*, which currently are classified into 11 families (Kuhn et al., 2020). Within the order of *Mononegavirales*, there are two families containing members that have fungi as their primary hosts: *Mymonaviridae* and *Rhabdoviridae* (Kondo et al., 2022). According to the ICTV description, the typical mymonavirus genome is approximately 10 kb, has five or six major non-overlapping ORFs, and has no poly(A) tail structure at the 3′ end (Jiāng et al., 2019). At least one virus in the family *Mymonaviridae* induces hypovirulence in its fungal host, and Sclerotinia sclerotiorum negative-stranded RNA virus 1 is one such virus (Liu et al., 2014). Contig24 was 9,485 nt in length, showing 95% identical to Fusarium proliferatum mymonavirus 1 (FpMV1), a member of an unclassified genus in the family *Mymonaviridae*, using the BLASTx search (Table 1). Thus, we named the virus Fusarium oxysporum f. sp. cubense mymonavirus 1 (FocMyV1). Sequence analysis showed that FocMyV1 has four ORFs, including a large ORF-encoding RdRP, which is similar to FpMV1 (Figure 5A). Contig14157 was 10,342 nt long, with a predicted amino-acid sequence most similar to Sanya Mymon tick virus 1 (SMtV1), with 99% identity. We named it Fusarium oxysporum f. sp. cubense negative-stranded RNA virus (FocNSRV1). Sequence analysis showed that FocNSRV1 has five ORFs, which is similar to SMtV1 (Figure 5A). Phylogenetic analysis based on the RdRP of putative viruses in the family *Mymonaviridae* suggests that FocNRSV1 likely belongs to the genus *Sclerotimonavirus*, while FocMyV1 belongs to an unclassified genus in the family *Mymonaviridae* (Figure 5B).

Partitiviridae-related sequences

The family *Partitiviridae* contains five genera, *Alphapartitivirus*, *Betapartitivirus*, *Deltapartitivirus*, *Gammapartitivirus*, and *Cryspovirus* that are a widely spread group of viruses infecting plants, fungi, and protozoa. Members of each genus have characteristic hosts: either plants or fungi for genera *Alphapartitivirus* and *Betapartitivirus*, fungi

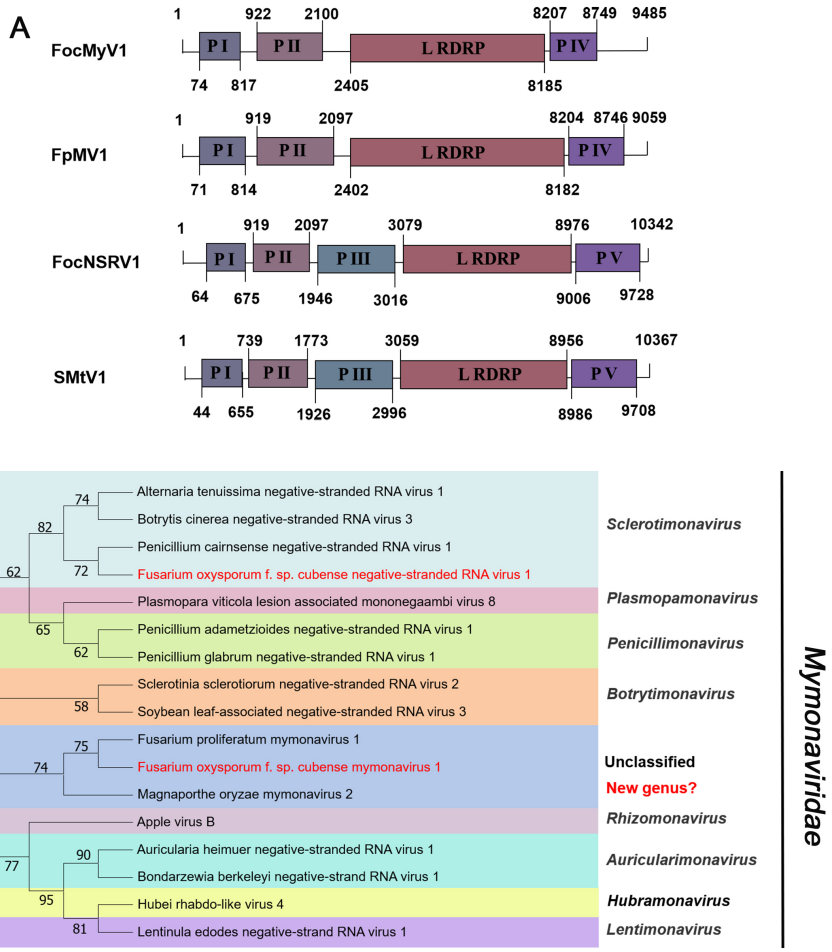

FIGURE 5
Genome organization and phylogenetic analysis of viruses in the family *Mymonaviridae*. **(A)** Schematic diagram showing the genome organization of viruses, includin Fusarium oxysporum f. sp. cubense mymonavirus 1 (FocMyV1), Fusarium oxysporum f. sp. cubense negative-stranded RNA virus 1 (FocNSRV1), Fusarium proliferatum mymonavirus 1 (FpMV1) and Sanya Mymon tick virus 1 (SMtV1). The genomic structure of FpMV1 and SMtV1 were selected as representatives of the family. **(B)** Neighbor-joining (NJ) method phylogenetic tree based on the core RNA-dependent RNA polymerase (RdRP) of putative viruses in the family *Mymonaviridae*. Red-marked viruses are isolated in Foc. Viruses without color annotation are representatives of known species of the viral family. Genus clades are circumscribed in coloured blocks, with genus names and the family name shown to the right of the tree. Bootstrap percentage greater than 50% are shown.

for genus *Gammapartitivirus*, plants for genus *Deltapartitivirus*, and protozoan for genus *Cryspovirus*. Generally, the genome of partitiviruses consists of two dsRNA segments of 1.3 to 2.4 kb each, the larger of which encodes the replicase and the smaller the structural proteins. Nevertheless, additional dsRNA segments may also be present (Vainio et al., 2018).

Seven sequences related to viruses within the family *Partitiviridae* were identified in Foc. Contig75904, contig11434, contig16483, contig6366, and first-contig2479 contained an incomplete ORF encoding RdRP (Figure 6A), which showed the greatest similarity with the Rosellinia necatrix partitivirus 1-W8 (RnPV1-W8), Fusarium solani partitivirus 2 (FsPV2), Gaeumannomyces tritici partitivirus 2 (GtPV2), Ceratocystis polonica partitivirus (CpPV) and Podosphaera prunicola partitivirus 1 (PpPV1), respectively (Table 1). They all had less than 75% similarity with known viruses. We named these novel partitiviruses as Fusarium oxysporum f. sp. cubense partitivirus 1 (FocPV1), Fusarium oxysporum f. sp. cubense partitivirus 2 (FocPV2), Fusarium oxysporum f. sp. cubense partitivirus 3 (FocPV3), Fusarium oxysporum f. sp. cubense partitivirus 4 (FocPV4), Fusarium oxysporum f. sp. cubense partitivirus 7 (FocPV7), respectively. Contig99419 with 1,815 nt contained a complete ORF that encoded a putative protein with 539 aa, and with 50% identity to the RdRP of Sclerotinia sclerotiorum partitivirus S (SsPVS), and we named this novel virus as Fusarium oxysporum f. sp. cubense partitivirus 5 (FocPV5). The first-contig2653 was 1,909 nt and had a complete ORF encoding putative RdRP with 548 aa (Figure 6A). The amino acid sequence of the predicted ORF product was identical to the RdRp of Heterobasidion partitivirus 5 (HpPV5) with 80% identity, namely Fusarium oxysporum f. sp. cubense partitivirus 6 (FocPV6). The amino-acid sequences of the RdRPs of FocPV1-7 showed that they all had less than 80% similarity with known viruses, and we tentatively speculated that they might represent novel members of the family *Partitiviridae*. FocPVs in the phylogenetic tree formed a separate branch from other known viruses, therefore, they might constitute one new separate genus within the *Partitiviridae* (Figure 6B).

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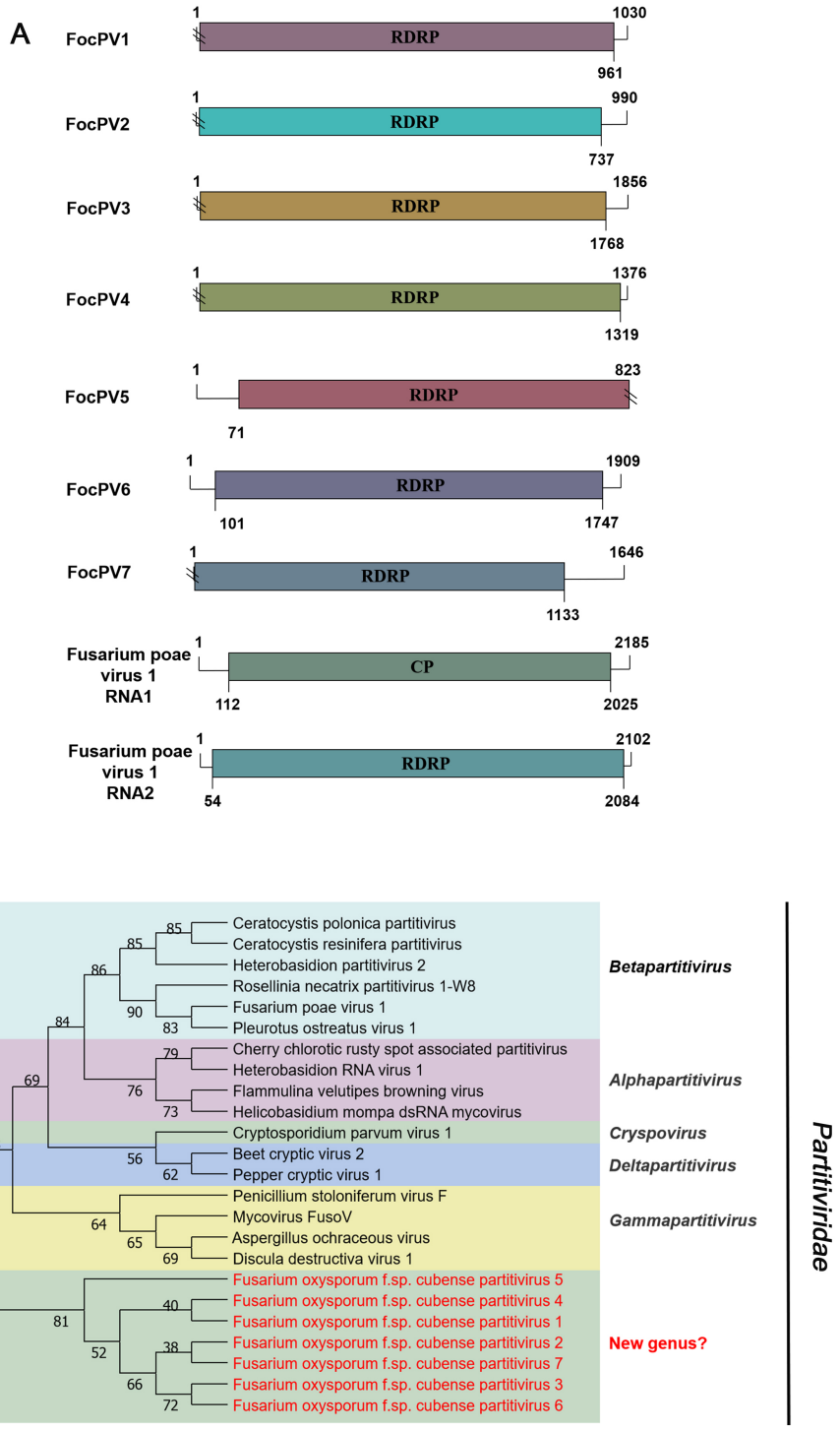

FIGURE 6
Genome organization and phylogenetic analysis of viruses in the family *Partitiviridae*. **(A)** Schematic diagram showing the genome organization of viruses, including Fusarium oxysporum f. sp. cubense partitivirus 1 (FocPV1), Fusarium oxysporum f. sp. cubense partitivirus 2 (FocPV2), Fusarium oxysporum f. sp. cubense partitivirus 3 (FocPV3), Fusarium oxysporum f. sp. cubense partitivirus 4 (FocPV4), Fusarium oxysporum f. sp. cubense partitivirus 5 (FocPV5), Fusarium oxysporum f. sp. cubense partitivirus 6 (FocPV6), Fusarium oxysporum f. sp. cubense partitivirus 7 (FocPV7) and Fusarium poae virus 1 (FpV1). The genomic structure of FpV1 was selected as representative of the family. **(B)** Neighbor-joining (NJ) method phylogenetic tree based on the core RNA-dependent RNA polymerase (RdRP) of putative viruses in the family *Partitiviridae*. Red-marked viruses are isolated in Foc. Viruses without color annotation are representatives of known species of the virus family. Genus clades are circumscribed in coloured blocks, with genus names and the family name shown to the right of the tree.

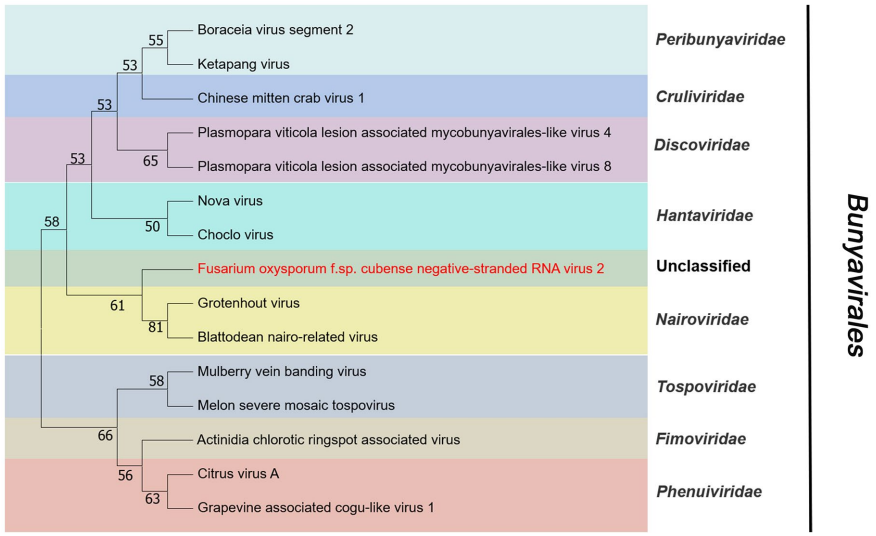

FIGURE 7
Phylogenetic analysis of viruses in the order *Bunyavirales*. Neighbor-joining (NJ) method phylogenetic tree based on the core RNA-dependent RNA polymerase (RdRP) of putative viruses in the order *Bunyavirales*. Red-marked virus is isolated in Foc. Viruses without color annotation are representatives of known species of the virus family. Family clades are circumscribed in coloured blocks, with family names and the order name shown to the right of the tree. Bootstrap percentage greater than 50% are shown.

Unclassified viral sequences

Contig2840 was 1902 nt in length, and contained an incomplete ORF encoding putative polyprotein with 588 aa. Blastx analysis showed that this putative protein was most similar to the RdRp of Fusarium graminearum alphavirus-like virus 1 (FgALV1) with 29% identity (Table 1). FgALV1, a member of a new, unclassified family in the alphavirus-like supergroup (Zhang X. et al., 2020). Thus, contig2840 represented a novel Riboviria virus. We named it Fusarium oxysporum f. sp. cubense alphavirus-like virus (FocALV). Thus, FocALV may also be a novel member of an unassigned fungal alphavirus-like group belonging to the alphavirus-like supergroup.

Contig14713 was 6,712 nt and contained a complete ORF. It encoded a RdRP with 2,203 aa. The predicted amino acid sequence of this protein was similar to Grapevine-associated mycobunya-like virus 4 (GaMLV4) with 57% identity. GaMLV4 belongs to the unclassified *Bunyavirales*. This suggests that contig14713 likely represents a novel virus of *Bunyavirales*, and we have named it Fusarium oxysporum f. sp. cubense negative-stranded RNA virus 2 (FocNSRV2). Therefore, FocNSRV2 may be a new member of the order *Bunyavirales* (Figure 7).

Discussion

In this study, a metatranscriptome -based strategy was used to investigate the virome diversity and composition associated with the plant-pathogenic fungus Foc that causes banana Fusarium wilt disease. The aim is to identify the mycoviruses which can influence the pathogenicity of the host to provide potential materials for the biological control of Foc. A total of 246 Foc isolates were collected from different diseased banana plants and the isolates were divided into 2 groups for analysis as described by Ruiz-Padilla et al. (2021) to obtain the most comprehensive overview of the viral profile. Illumina sequencing yielded a total of 287,540 contigs, and bioinformatic analysis assembled 20 mycoviruses with RNA genomes. To our knowledge, this is the first report of the mycoviral diversity in Foc. On the ground of their genomic structures, sequence sequence analyses, and phylogenetic analyses based on RdRp, these mycoviruses in Foc could be tentatively classified into five families (*Botourmiaviridae*, *Mitoviridae*, *Endornaviridae*, *Mymonaviridae*, and *Partitiviridae*) and two non-classified mycoviruses lineages.

Phylogenetic trees are widely used to study evolutionary relationships between species. To assess the phylogenetic relationship of the predicted virus with other members of the viral family, the contig sequences were compared with the RdRp sequences of members of currently known viral genera. Phylogenetic analysis revealed that four botourmiaviruses clustered into three well-supported clades, one belonging to the genus *Ourmiavirus* and others belonging to the new genus. FocOLV1 and FocOLV4 cluster together while FocOLV3 forms a monophyletic clade. Therefore, we propose the creation of two new genera within *Botourmiaviridae*. Similarly, mitoviruses are not clustered in monophyletic lineages and there are two mitoviruses in a separate branch, suggesting the creation of a new genus in the *Mitoviridae*. As shown in Figure 4, FocEV1 is tightly clustered with members of the genus *Betaendornavirus* of the family *Endornaviridae*. The genome of betaendornaviruses is less than 10.7 kb in length and absence of a site-specific nick near the 5′-end of the coding strand, according to the criteria for the classification of the two genera of Endornaviridae in ICTV (Valverde et al., 2019). As FocEV1 has an incomplete genome sequence, whether FocEV1 should be classified as a new member of the genus *Betaendornavirus* requires further work.

In 2014, the first -ssRNA mycovirus was identified in *Sclerotinia sclerotiorum*, leading to the establishment of a new family, *Mymonaviridae* (Liu et al., 2014; Jiāng et al., 2019). As

described by ICTV, Sclerotinia sclerotiorum negative stranded RNA virus 1 (SsNSRV-1), a typical member of the *Mymonaviridae*, has six major non-overlapping ORFs encoding six proteins (p I, NP, p III, p IV, L protein, and p VI; Jiāng et al., 2019). Other mymonavirus genomes contain four to seven ORFs, such as Botrytis cinerea negative-stranded RNA virus 7 (BcNSRV7), Húběi rhabdo-like virus 4 (HbRLV-4), and Lentinula edodes negative-strand RNA virus 1 (LeNSRV1; Donaire et al., 2016; Shi et al., 2016; Lin et al., 2019). Similarly, FocMyV1 contains four ORFs and ORF3 encodes the L protein, while FocNSRV1 contains five ORFs and ORF4 encodes the L protein. Moreover, the phylogenetic analysis showed that FocNSRV1 formed an independent clade of *Sclerotimonavirus* in the family *Mymonaviridae*. FocMyV1 formed a tight clustered with Fusarium proliferatum mymonavirus 1 and Magnaporthe oryzae mymonavirus 2. These three viruses formed a separated branch distant from other viruses, implying thatthey represent a new genus in the family *Mymonaviridae*. In brief, we characterized two novel mymonavirus, FocMyV1 and FocNSRV1, in the genus *Sclerotimonavirus* and a new genus, respectively. Most notably, Partitiviride-related viruses found in Foc appear to be phylogenetically on a separate branch.

Similar to other plant pathogenic fungi, mycoviruses in the Lenarviricota phylum were prevalent in Foc isolates (Chiapello et al., 2020; Ruiz-Padilla et al., 2021). Among them were 4 viruses that belong to the family *Botourmiaviridae* and 4 viruses that belong to the family *Mitoviridae*, both of which belong to the phylum Lenarviricota. Due to their mitochondrial localization, mitoviruses are conjecturably unaffected by host antiviral RNA silencing, which may contribute to their prevalence (Shahi et al., 2019). Multiple lines of evidence suggest that mycovirus have been transferred between fungi and plants due to fungus-mediated horizontal gene transfer (HGT) events and long-term co-evolution (Nerva et al., 2017; Abdoulaye et al., 2021). Indeed, there have been reports of horizontal transfer of mitoviruses from fungi to plants, as in the case of Botrytis cinerea mitovirus 10 (BcMV10), which was transmitted from *Botrytis cinerea* to cucumber plants (Wang Q. et al., 2022). Some viruses in the *Mitoviridae* family cause swelling and malformation of host mitochondria, reducing the growth and virulence of the fungus, such as Botrytis cinerea mitovirus 1 (BcMV1), Sclerotinia sclerotiorum mitovirus 1 (SsMV1/HC025) and Sclerotinia sclerotiorum mitovirus 2/KL-1 (SsMV2/KL-1; Wu et al., 2010; Khalifa and Pearson, 2013; Xu et al., 2015). The FocMV2 was most similar to Fusarium circinatum mitovirus 2–1 (FcMV2-1), with a sequence identity of 88% (Table 1). Vitro studies showed that both mycelial growth and spore germination were significantly reduced by the presence of the mitovirus FcMV2-1 (Romeralo et al., 2018). Therefore, whether or not FocMV2 plays a significant role in Foc remains to be explored.

With the application of metatranscriptomic sequencing technology in mycoviral diversity research, a great number of novel mycoviruses have been discovered and identified; and this has supported the progress of research in their related fields, such as virus pathogenesis, the control of related diseases. Further, the mining of rich mycoviral diversity could also be used to explore the interaction mechanism between mycovirus and its host fungus. Although most mycoviruses are cryptic and cause no visible abnormal symptoms in their fungal hosts, they may play an important role in their population biology. To date, at least nine mycoviruses with complete genomic sequences have been reported in *F. oxysporum*, including Fusarium oxysporum chrysovirus 1 (family *Chrysoviridae*), Fusarium oxysporum f. sp. dianthi mycovirus 1 (family *Chrysoviridae*), Fusarium oxysporum alternavirus 1 (family *Alternaviridae*), Fusarium oxysporum mitovirus 1 (family *Mitoviridae*), Fusarium oxysporum f. sp. dianthi mitovirus 1 (family *Mitoviridae*), Fusarium oxysporum f. sp. dianthi hypovirus 2 (family *Hypoviridae*), Fusarium oxysporum ourmia-like virus 1 (family *Botourmiaviridae*), Hadaka Virus 1 (family *Polymycoviridae*), and Fusarium oxysporum mymonavirus 1 (family *Mymonaviridae*; Sharzei et al., 2007; Lemus-Minor et al., 2015; Sato et al., 2020; Torres-Trenas et al., 2020; Torres-Trenas and Pérez-Artés, 2020; Zhao et al., 2020; Wang et al., 2021; Wen et al., 2021; Wang J. et al., 2022). Of these reported mycoviruses, Fusarium oxysporum f. sp. dianthi virus 1 (FodV1), Fusarium oxysporum ourmia-like virus 1 (FoOuLV1) and FoMyV1 are capable of causing hypovirulence of host and can be used as biological control agents (Lemus-Minor et al., 2019; Zhao et al., 2020; Wang J. et al., 2022). This study is the first report of seven partitiviruses from *F. oxysporum*. In fungi, partitiviruses are transmitted intracellularly during cell division, hyphal anastomosis, and sporogenesis (Nibert et al., 2014; Vainio et al., 2018). Normally, partitiviruses tend to infect host fungi asymptomatically, but some viruses significantly affect the morphology and virulence of their hosts and are therefore considered to be a potential source of biocontrol agents against pathogenic fungi (Wang et al., 2014; Xiao et al., 2014; Wang R. et al., 2022). Currently, there are less effective, environmentally friendly and sustainable control methods for Foc (Ploetz, 2015). Therefore, it is urgent that new mycoviruses that significantly reduce host virulence should be exploited as alternative biological control agents of banana Fusarium wilt caused by Foc. In conclusion, this is the first study to show the existence of various mycoviruses in Foc. This pioneering information will be a key for investigating the intricate interactions between mycovirus and Foc and will provide extensive insights into the potential use of mycoviruses as biocontrol agents.

Data availability statement

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

Author contributions

YY executed the experiments and drafted the manuscript. HL and PL designed the research and revised the manuscript. YL, XW, and YZ performed the data and bioinformatics analyses. 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.2023.1193714/full#supplementary-material

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Antimicrobial mechanisms and secondary metabolite profiles of *Streptomyces hygroscopicus* subsp. *hygroscopicus* 5–4 against banana fusarium wilt disease using metabolomics

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Fusarium wilt of bananas (FWB) is seriously affecting the sustainable development of the banana industry and is caused by the devastating soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4). Biological control is a promising strategy for controlling Fusarium wilt in bananas. We previously identified *Streptomyces hygroscopicus* subsp. *hygroscopicus* 5–4 with strong antifungal activity against the FWB. The most possible antimicrobial mechanism of strain 5–4 was explored using the metabolomics approach, light microscopy imaging, and transmission electron microscopy (TEM). The membrane integrity and ultrastructure of Foc TR4 was damaged after extract treatment, which was supported by the degradation of mycelium, soluble protein content, extracellular reducing sugar content, NADH oxidase activity, malondialdehyde content, mitochondrial membrane potential, and mitochondrial respiratory chain complex enzyme activity. The extracts of strain 5–4 cultivated at different times were characterized by a liquid chromatography–mass spectrometer (LC-MS). 647 known metabolites were detected in the extracts of strains 5–4. Hygromycin B, gluten exorphin B4, torvoside G, (z)-8-tetradecenal, piperitoside, sarmentosin, pubescenol, and other compounds were the main differential metabolites on fermentation culture for 7 days. Compared with strain 5–4 extracts, hygromycin B inhibited the mycelial growth of Foc TR4, and the EC_{50} concentration was 7.4 μg/mL. These results showed that strain 5–4 could destroy the cell membrane of Foc TR4 to inhibit the mycelial growth, and hygromycin B may be the key antimicrobial active metabolite. *Streptomyces hygroscopicus* subsp. *hygroscopicus* 5–4 might be a promising candidate strain to control the FWB and provide a scientific basis for the practical application of hygromycin B as a biological control agent.

KEYWORDS

fusarium wilt of banana, *Streptomyces hygroscopicus* subsp. *hygroscopicus*, antifungal mechanism, metabolomics, hygromycin B

Introduction

Banana is an important fruit in tropical and subtropical regions worldwide (Qi et al., 2021). Due to the fusarium wilt of bananas (FWB), the high morbidity, great depredation, and rapid spread led to a sharp reduction in the area under traditional banana cultivation (Dita et al., 2018; Yun et al., 2022). FWB, caused by the devastating soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), is seriously affecting the sustainable development of the banana industry (Prigigallo et al., 2022). Biocontrol was considered to be one of the most effective methods compared with different prevention strategies (Fu et al., 2017). This provides new opportunities for FWB control (Du et al., 2022). Biocontrol microbes such as *Pseudomonas* sp., *Bacillus* sp., and *Streptomyces* sp. have been reported to be used in controlling the FWB (Bubici et al., 2019). Although many biocontrol microbes had a good inhibitory effect on Foc *in vitro*, their control effect was often limited and unstable in the field due to the different factors of soil environmental conditions, such as pH, osmotic pressure, and salt concentration (Du et al., 2022). Therefore, it is still necessary to adapt to local conditions to develop more safe and effective biological agents.

Actinomycetes, especially *Streptomyces* with biocontrol potential, are deemed significant producers of bioactive metabolites. *Streptomyces* are believed to be unique among rhizosphere microorganisms in terms of disease management (Kaari et al., 2022). They also exist as potential endophytic microorganisms, and most colonize the tissues of plants without causing any negative effects (Kaari et al., 2022). Therefore, endophytic *Streptomyces* was considered one of the most promising strategies for the biocontrol of plant pathogens (Xu et al., 2020). In general, biocontrol microorganisms protect plants against phytopathogens through direct and indirect mechanisms. Direct mechanisms for microbial assistance against pathogens include antibiosis, lytic enzymes, hyperparasitism, predation, and competition for nutrients and space (Xu et al., 2019; Ebrahimi et al., 2022). For example, *Streptomyces corchorusii* AUH-1 could damage the cell membranes of pathogens by inhibiting ergosterol formation and increasing malondialdehyde levels (Yang et al., 2019). In Foc TR4 hyphae treated with endophytic *Streptomyces* sp. 5–10 extracts, the cell ultrastructure and membrane integrity were damaged (Yun et al., 2021). In our previous study, *Streptomyces hygroscopicus* 5–4 can secrete chitinase and β-1,3-glucanase, thus damaging the cell wall of Foc TR4 (Yun et al., 2022). In addition to the cell wall, the cell membrane is the mechanical and osmotic barrier between the cell and the environment and is a common action site of fungicides against pathogenic fungi (Kim and Lee, 2019; Xu et al., 2019; Zhang et al., 2021). To reveal the possible action mode of strain 5–4 in inhibiting Foc TR4, this study focused on the effect of strain 5–4 extracts on Foc TR4 cell membranes and the antifungal metabolic profiles of strain 5–4. The main aims of this study are as follows: (i) to reveal the possible underlying mechanism of action by studying the integrity of the cell membrane and microscopic morphology of mycelia and (ii) to determine the metabolic profiles of strain 5–4 in different fermentation culture periods by metabolomic analyses.

Materials and methods

Microorganisms and culture media

Streptomyces hygroscopicus subsp. *hygroscopicus* 5–4 (GDMCC 61679) was isolated from the roots of *Piper austrosinense* and maintained on the International *Streptomyces* Program 2 (ISP2) medium at 4°C. Banana wilt disease pathogenic fungi *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4, ATCC 76255) were kept on a Petri dish containing potato dextrose agar (PDA) at 4°C.

Antagonistic effects of strain 5–4 on mycelial development of Foc TR4 *in vitro*

Strain 5–4 was able to effectively inhibit the growth of Foc TR4 mycelium, as determined using a modified method of confrontation culture assay of detection (Getha and Vikineswary, 2002). Briefly, a sterilized cover slip (1 × 1 cm) was placed at the center of the PDA medium and inoculated with Foc TR4 and strain 5–4 at both ends of the coverslip, respectively. After being cultivated for 5 days at 28°C, the coverslips were observed using a microscope (model Axio Scope A1, Carl Zeiss AG, Germany).

Strain cultivation and metabolite extraction

Pre-cultures: strain 5–4 was inoculated in ISP2 medium and cultivated for 3 days at 28°C. Strain cultivation: 3 mL of pre-culture was inoculated in 100 mL of soybean liquid culture medium (SLM) and cultivated for 7 days at 28°C at 200 rpm (Chen et al., 2018). The strain 5–4 metabolites were extracted with 98% absolute ethanol (filtrate: ethanol = 1:1 vol/vol) from the cultural liquid. After being evaporated in a rotary vacuum evaporator, the extracts were dissolved in distilled water and dried by vacuum freezing and drying technology (Yun et al., 2022). The extracts of strain 5–4 were stored at 4°C.

Effect of extracts on Foc TR4 mycelial *in vitro*

A fungal disk of 0.5 mm diameter was inoculated on the PDA plate. The sterilized coverslips were placed near the fungal disk. When the fungal mycelia were overgrown with coverslips, the coverslips were removed and placed in sterile Petri dishes, then added 50 μL of strain 5–4 extracts to the coverslips at a concentration of 500 μg/mL (Yun et al., 2022). The mycelial morphology of Foc TR4 was investigated under a microscope after treatment at 28°C for 2 days.

Effect of extracts on the ultrastructure of Foc TR4 cells

The Foc TR4 was inoculated on the PDA plate, which contained a final concentration of 500 μg/mL extracts. After being cultured at 28°C for 5 days and collected from the edge of the inhibition zone for the Foc TR4 mycelia, the same volume of sterile water was used as a negative control. Samples were fixed, dehydrated, and embedded into Epon 812 resin according to the methods of Cao et al. (2022). The Foc TR4 mycelia were sliced by an ultra-thin slicer (Leica, UC6 CM1950, Germany). The ultrastructure of the mycelial sample was observed using a transmission electron microscope (TEM, HT7700, Hitachi, Ibaraki, Japan).

Effect of extracts on the cell membrane of Foc TR4

In addition to the cell wall, the integrity of the cell membrane is also another key factor for normal microbial life (Xu et al., 2019). Thus, the effect of extracts on the cell membrane integrity of Foc TR4 was detected.

Effects of extracts on the content of soluble protein (SP) in Foc TR4 mycelia

Briefly, an aliquot (1 mL) of Foc TR4 spore suspension (1×10^5 per/mL) was added to a 30 mL PDB and cultured at 28°C for 5 days. The fungal mycelia were centrifuged at 4000 rpm for 15 min and washed three times with sterilized water (Wang et al., 2022). A total of 0.5 g of the samples was weighed and resuspended in 10 mL of sterilized water. The extracts were added to the resuspended solution to achieve final concentrations of EC_{50} (62.79 μg/mL) and EC_{95} (869.94 μg/mL), which were obtained in previous studies (Yun et al., 2022). Subsequently, the samples were incubated for 0, 2, 4, 8, 12, and 24 h at 28°C on a rotary shaker at 200 rpm and centrifuged at 4000 rpm for 10 min to obtain supernatants. Sterile water treatment was used as a negative control. The content of SP was determined using the Bradford (1976) method based on bovine serum albumin (BSA) as the standard. Each treatment was performed in triplicate.

Effects of extracts on the content of extracellular reducing sugar (ERS) in Foc TR4 mycelia

The mycelia of Foc TR4 were cultured as described above, incubated for 3, 6, 12, and 24 h at 28°C on a rotary shaker at 200 rpm, and centrifuged at 4000 rpm for 10 min to obtain supernatants. Then, we added 0.18 mL of DNS reagent for each 0.24 mL of the supernatant. The content of ERS was determined using the 3,5-dinitrosalicylic (DNS) colorimetric method according to Yuan et al. (2014). All experiments were performed in triplicate.

Effects of extracts on the content of NADH oxidase (NOX) activity in Foc TR4 mycelia

The samples were cultured using the abovementioned method. The mycelia were washed two times with PBS; 0.1 g of the sample was ground, and the powdered tissue was dissolved in 1 mL of extraction solution, and the mixture was centrifuged at 8000 rpm for 10 min to collect supernatants.

The NADH oxidase activity content was determined using the NADH oxidase activity detection kit (Beijing Solebao Technology Inc., Beijing, China) (Chen et al., 2018).

Effects of extracts on the content of malondialdehyde (MDA) in Foc TR4 mycelia

The membrane lipid peroxidation can be analyzed by determining the content of MDA. The mycelia of Foc TR4 were cultured as described above. According to the kit instructions (the MDA kit from Beijing Solebao Technology Inc., Beijing, China), the activity of MDA was determined (Wang et al., 2019).

Effect of extracts on the mitochondrial membrane potential of Foc TR4

When Rhodamine-123 is applied to intact cells, it is predominantly localized in mitochondria and represents a reliable fluorescent probe for assessing the mitochondrial membrane potential (Anamika et al., 2021). The spore suspension of Foc TR4 (10^6 per/mL) was equally mixed with strain 5–4 extracts and incubated at 28°C for 2 h (Jiang et al., 2021; Yun et al., 2022). The samples were washed two times with PBS and incubated with cationic lipophilic rhodamine-123 at 37°C for 30 min (Zhu et al., 2012). Sterile water was used as a negative control. Effect on the mitochondrial membrane potential of Foc TR4 with extracts observed using fluorescence microscopy (MMI Cell Cut Plus, Leica DM6000B, Wetzlar, Germany).

Effect of extracts on mitochondrial respiratory chain complex enzyme activity of Foc TR4

Spore suspensions of Foc TR4 (10^6 per/mL) were incubated in a PDB liquid medium at 28°C for 5 days. The mycelia of Foc TR4 were then collected by centrifugation at 4000 r/min for 15 min and washed three times with sterile water. An ice bath was used to grind an appropriate amount of mycelium, a mitochondrial buffer, and quartz sand. The resulting homogenate was further centrifuged at 2000 rpm for 10 min at 4°C to collect the supernatants (Li et al., 2022). The supernatants were centrifuged at 12,000 rpm for 15 min, and the resulting sediments were retained. A proper amount of buffer was added to the sediments to make them fully suspended, followed by another centrifugation at 12,000 rpm for 15 min to collect the sediments, which were identified as mitochondria.

The protein content of mitochondria was adjusted to 50 μg/mL (Li et al., 2022). We then added the extracts of strain 5–4 to the mitochondria, and the final concentrations were EC_{50} (62.79 μg/mL) and EC_{95} (869.94 μg/mL), respectively. The samples were incubated at 37°C for 30 min. A negative control consisting of sterile water was included for comparison purposes. Enzyme activity assays of NADH CoQ reductase (mitochondrial complexes I), succinate dehydrogenase (mitochondrial complexes

II), cytochrome oxidase (mitochondrial complexes III), and F_1F_0-ATP synthase (mitochondrial complexes V) were performed using the Assay Kit (Solarbio Beijing, China) (Zhao et al., 2020). Each treatment group was repeated three times.

Analysis of metabolites from strain 5–4 during different culture time points by HPLC-MS

To find the metabolites with anti-Foc RT4 activity during the fermentation of strain 5–4, we analyzed the changes in metabolites under different culture times by metabolomics.

Preparation of samples

Strain 5–4 was initially cultivated in 100 mL of SLM medium. After being cultivated for the 2nd, 4th, 6th, 7th, and 8th days on a rotary shaker for 180 rpm at 28°C, the metabolites were extracted with methyl alcohol (filtrate: methyl alcohol = 1:4 vol/vol) from the cultural liquid. The methyl alcohol extracts were concentrated in vacuo to obtain the dried material. The antimicrobial activity was evaluated against Foc TR4 using the filter paper diffusion method (Yun et al., 2022) to determine the fermentation culture time points for HPLC-MS detection.

HPLC-MS

The LC-MS analysis was performed using the UHPLC-Q exertive system by Thermo Fisher Scientific. The separation was carried out using an HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm), and the eluted samples were subsequently directed into mass spectrometry detection for analysis (Yang et al., 2022; Zhang et al., 2023). The mobile phase was (A) water with 0.1% of formic acid and (B) acetonitrile/isopropanol (1:1 vol/vol) with 0.1% of formic acid (Yang et al., 2022). The samples were determined using HPLC-MS spectrometry and injected with 0.4 mL/min of mobile phase; the injection volume was 2 μL. The column temperature was 40°C. The solvent gradient started at 5% A for 3 min, was converted to 95% B for 9 min, and was maintained at 95% B for 13 min. Sample-quality spectrum signals were collected by positive and negative ion scanning modes and an electrospray ionization source. The capillary voltages of positive mode, negative mode, sheath gas flow rate, aus gas flow rate, aus gas heater temp, and normalized collision energy were set to be 3.5, 2.8 kV, 40 psi, 10 psi, 400°C, and 20-40-60 V, respectively. Data were collected in a profile mode from 70 to 1050 m/z.

Data processing

The response intensity of the sample mass spectrum peaks was normalized using the method of sum normalization and obtained as the normalized data matrix (Kaleem et al., 2022). The variables with a relative standard deviation (RSD) > 30% of QC samples were removed, and log10 logarithmization was performed to obtain the final data matrix (Kaleem et al., 2022). After data treatment, the collected data were analyzed using principal components analysis (PCA), variable influence in projection (VIP), and the Student's *t*-test ($P < 0.05$) in sequence.

Comparative analysis of antimicrobial activity between metabolites and strain 5–4 extracts

Antimicrobial activity was determined using an agar-well diffusion method (Wonglom et al., 2019). The extracts were added to the sterilized PDA medium with a final concentration of 2.5 to 200 μg/mL. After solidification, a fungal disk of 5 mm diameter was inoculated on the PDA plate. All experiments were performed in triplicate. The antimicrobial activity was measured by the inhibition rate (Cao et al., 2022). The half-maximal effective concentration (EC_{50}) was calculated from the toxicity regression equation. The mycelium characteristics of Foc TR4 treated with strain 5–4 extracts were observed under a microscope.

Statistical analysis

Statistical analysis was conducted using the SPSS software. The data were expressed as the means ± SE and analyzed using one-way ANOVA. Significance was tested using Tukey's HSD test. Statistically significant differences were determined at a *p*-value of < 0.05.

Results

Potent and cidal action against Foc TR4 by strain 5–4

The inhibitory ability of strain 5–4 on the mycelial growth of Foc TR4 was visualized using light microscopy and TEM. A confrontation culture assay showed that strain 5–4 exhibited intertwining growth with the Foc TR4 mycelium. Additionally, clear signs of lysis were evident at the contact site between their respective mycelia (Figure 1A). We speculated that the lysis of Foc TR4 mycelium was caused by the metabolites of strain 5–4. Therefore, we studied the effect of strain 5–4 extract at a concentration of 500 μg/mL on the mycelia of Foc TR4. Upon examination of the effect of extracts on the mycelia morphology of Foc TR4 under the light microscope, it was observed that the treated Foc TR4 mycelium experienced significant rupture and disintegration. In contrast, the control mycelia maintained a normal morphology (Figure 1B). Therefore, the inhibitory effect of strain 5–4 against Foc TR4 was mainly caused by its secondary metabolites. The effects of extracts on Foc TR4 cells were further studied using TEM (Figure 1C).

In untreated mycelia, normal intact fungal cell components such as cell walls (CW), cell membranes (CM), mitochondria (M), and vesicles (V) were observed to be normal and uniformly distributed. However, after treatment with extracts, notable changes were observed in the Foc TR4 mycelia. The cell wall and membrane of Foc TR4 mycelia exhibited irregular thickening,

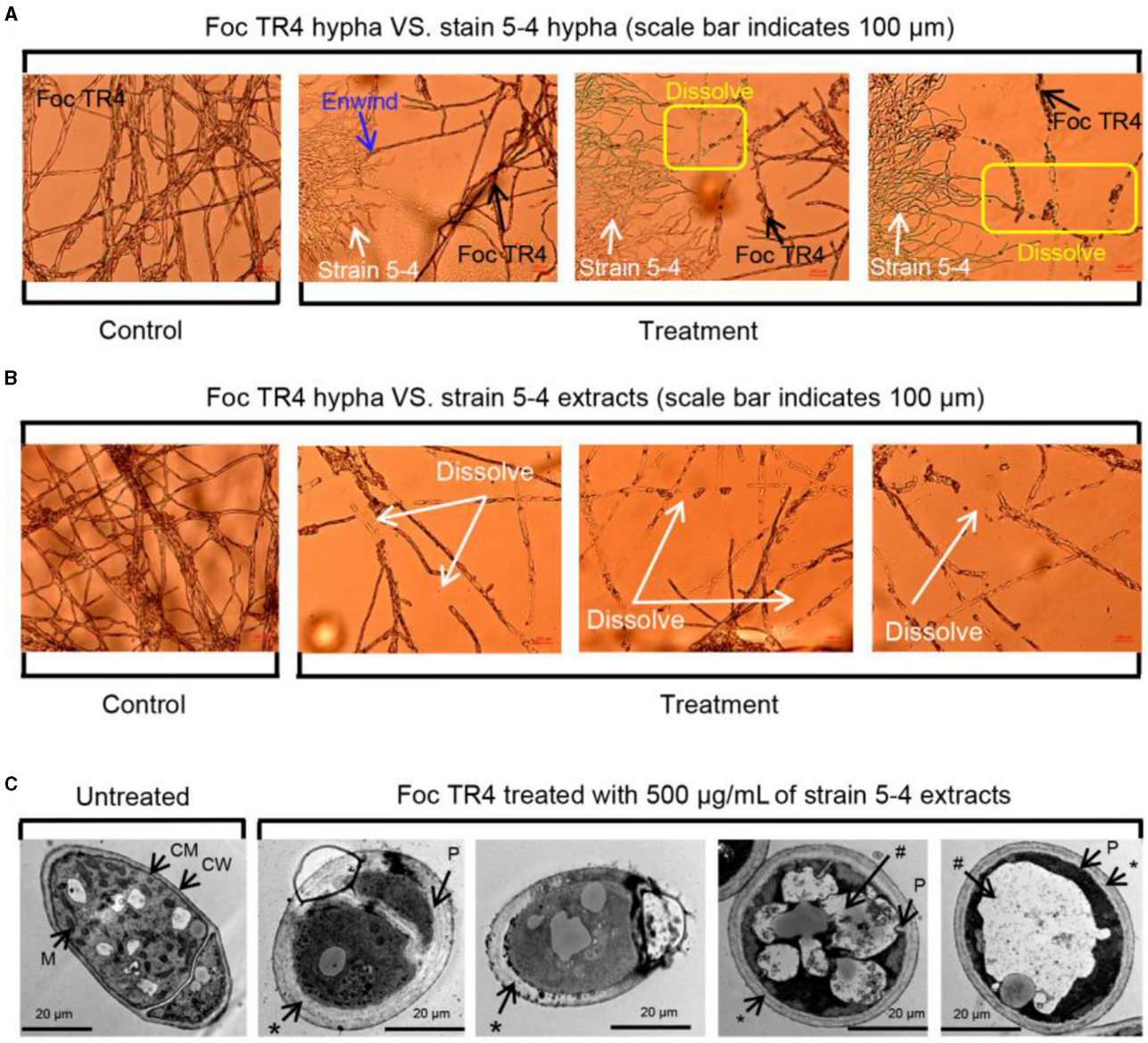

FIGURE 1
Potent and cidal action of strain 5–4 against Foc TR4 *in vitro*. **(A)** Antagonistic effects of strain 5–4 on the mycelial development of Foc TR4. **(B)** Extracts of strain 5–4 cause the disintegration of Foc TR4 mycelium. **(C)** Ultrastructure of Foc TR4 mycelial treated with extracts; mitochondria (M), cell membrane (CM), cell walls (CW), thickened cell walls (*), plasmolysis (P), and vacuolization (#).

forming a multilayered cell wall, with the outer wall showing fibrous digestion. The cytoplasm was reduced, concentrated, and clearly vacuolated. The organelles within the cells were difficult to discern, with most of them damaged and undergoing hydrolysis. These results suggest that the antagonistic metabolites penetrated the cytoplasm of Foc TR4 cells and destroyed their organelles.

Effect of strain 5–4 extracts on the cell membrane of Foc TR4

To investigate the interfering effect of strain 5–4 extracts on the Foc TR4 cell membrane function, we determined the effects of strain 5–4 extracts on the content of SP, ERS, NOX, and MDA of Foc TR4.

The SP content of Foc TR4 was reduced significantly after exposure to different concentrations of extracts compared with the control (Figure 2A). The SP content in both the controls showed a rapidly rising trend with the increase in cultured times; the maximum value was 1.68 μg/g at 24 h. The SP content of EC_{50} and EC_{95} treatments slowly increased with culture time. After 24 h of exposure to the extracts, the SP contents measured 0.81 and 0.56 μg/g at the EC_{50} and EC_{95} concentrations, respectively. These values were considerably lower compared to the control group.

The ERS content of Foc TR4 increased considerably after exposure to different concentrations of extracts, while the ERS content remained stable in the untreated cells (Figure 2B). The

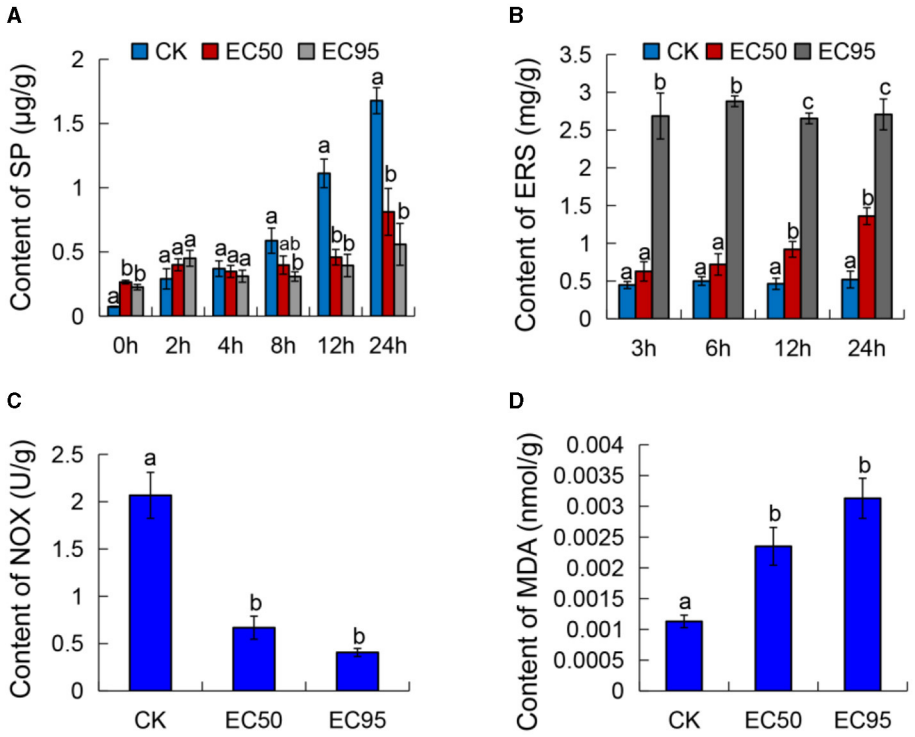

FIGURE 2
Effect of extracts on the cell membrane of Foc TR4. **(A)** The content of soluble protein (SP), **(B)** extracellular reducing sugar (ERS), **(C)** NADH oxidase activity (NOX), and **(D)** malondialdehyde (MDA) of Foc TR4. Different letters were used to indicate significant differences ($p < 0.05$).

content of ERS at the EC_{50} and EC_{95} concentrations were all higher than the control, and this change became more apparent with an increase in exposure times. At 24 h of exposure to the extracts, the ERS content of Foc TR4 treated with EC_{50} and EC_{95} concentrations were 1.36 and 2.71 mg/g, which were significantly higher than the control (0.52 mg/g). These results suggested that extracts may lead to cell membrane injury in Foc TR4 and cause ERS leakage.

The activities of NADH oxidase decreased with the increased extract concentration and showed an apparent gradient (Figure 2C). NADH oxidase activities of Foc TR4 were 0.668 and 0.41 U/g when treated with the EC_{50} and EC_{95} concentration extracts, respectively. The activities were significantly lower compared to the control group (2.067 U/g). The results showed that the NADH oxidase activity of Foc TR4 decreased in a concentration-dependent manner with an increase in the concentration of extracts.

To further investigate the damage of extracts to the Foc TR4 cell membrane, we detected the MDA content, which is often used as an index of the oxidative injury of the cell membrane (Liu et al., 2021). Figure 2D showed that the MDA content of the control was 0.00113 nmol/g. When exposed to the extracts of EC_{50} and EC_{95} concentrations, the MDA contents were significantly higher compared to the control, measuring 0.00235 and 0.00313 nmol/g, respectively. These findings demonstrated a dose-dependent increase in the MDA content of Foc TR4 as the concentration increased. These results confirmed that the cell membrane of Foc TR4 caused irreversible damage after treatment with the extracts, suggesting a direct interaction between the metabolites of strain 5–4 and the components of the membrane.

Effect of extracts on the mitochondrial membrane potential of Foc TR4

An early indication of cell apoptosis is the reduction of mitochondrial membrane potential, which can be detected by observing the fluorescence intensity produced by cell dyes (Wu et al., 2022). The fluorescence intensity of rhodamine showed a significant reduction after the EC_{95} treatment. Compared with the control, the mitochondrial membrane potential decreased (Figure S1). The mitochondrial membrane potential showed a positive correlation with the concentrations of the extract.

Effect of extracts on mitochondrial respiratory chain complex enzyme activity of Foc TR4

The mitochondrial respiratory chain can provide 95% of the energy for cellular life activities and is mainly carried out by mitochondrial respiratory chain enzymes. The respiratory chain consists of five complexes located on the inner mitochondrial membrane (Anamika et al., 2021; Tang et al., 2021). The mitochondrial respiratory chain complex I-V activity of Foc TR4

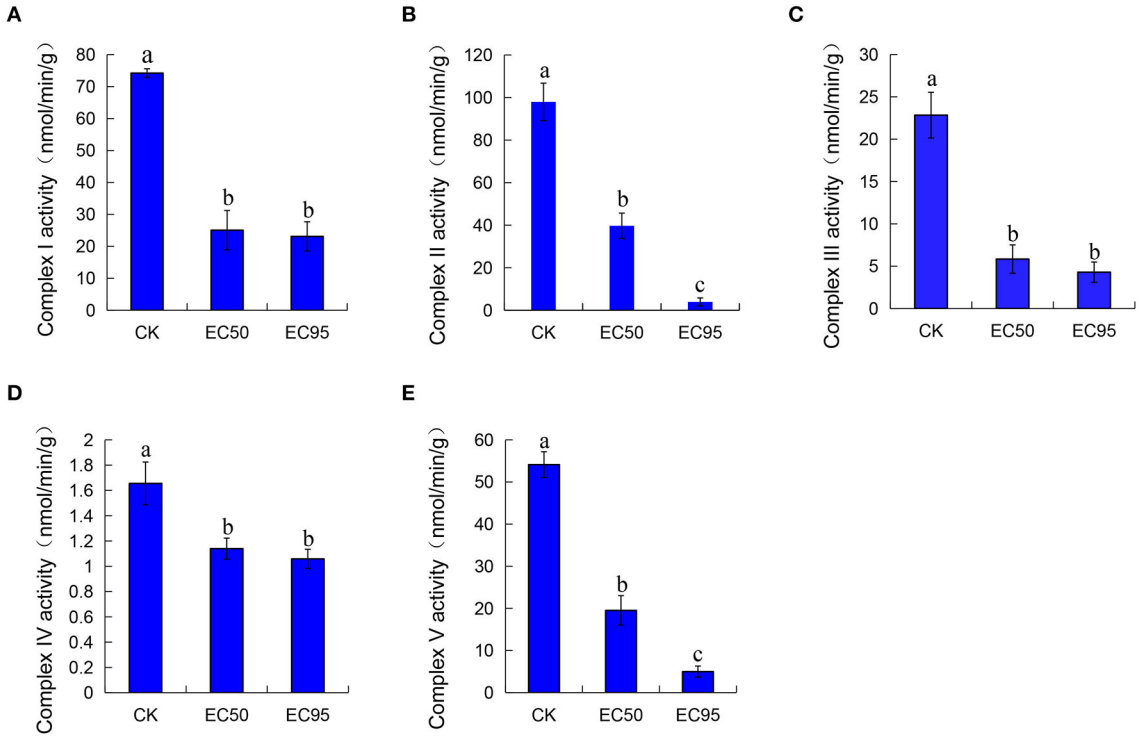

FIGURE 3
Effect of extracts on the mitochondrial respiratory chain activity complex I **(A)**, complex II **(B)**, complex III **(C)**, complex IV **(D)**, and complex V **(E)** of Foc TR4. Compared with the control groups, the groups of EC_{50} and EC_{95} showed dramatic reductions in the activity of complex I-V by approximately 30–90%. Different letters were used to indicate significant differences ($p < 0.05$).

after treatment with extracts for 30 min was determined. As shown in Figure 3, the activities of complex I-V decreased after extracts were treated compared with the control groups. At the concentration of EC_{50} treatment, the activity of complex I-V was 25.06, 39.70, 5.85, 1.14, and 19.52 nmol/min/g, which were significantly decreased compared with the control groups (74.22, 97.96, 22.84, 1.66, and 54.12 nmol/min/g). Treated with a high concentration of EC_{95} extracts, the activities of complex I-V were 23.12, 3.94, 4.30, 1.06, and 4.99 nmol/min/g, respectively. These results showed dramatic reductions in the activity of complex I-V by approximately 30–90% compared with the control groups.

Variation tendency of strain 5–4 antimicrobial extracts during fermentation culture determined by HPLC-MS

To detect any antimicrobial activity of strain 5–4 extracts, a filter paper diffusion method was employed at different time points: 2, 4, 6, 7, and 8 days post-inoculated cultured (dpi). The antimicrobial activity of the extracts was 16.5, 20.64, 37.37, 41.26, and 33.79%, respectively (Supplementary Figure S2A). With the progression of fermentation cultures, the antimicrobial activity increased, and the antimicrobial activity of 7 dpi was the highest, which was significantly higher than that of 2 dpi. To investigate the metabolome changes of strain 5–4 extracts during fermentation culture, the metabolites of extracts obtained at 2 dpi, 4 dpi, and 7 dpi were analyzed using HPLC-MS.

Principal component analysis (PCA) was used to analyze the discrete intra- and inter-group differences in metabolites (Gao et al., 2022). In the PCA score plots, the principal components in ESI+ (Supplementary Figure S2B) and ESI- (Supplementary Figure S2C) modes accounted for 62.60% and 60.30% of the variation, respectively. The samples for the intra-group comparison were gathered, which showed the reliability and repeatability of the experiment.

In total, 647 metabolites in strain 5–4 extracts were identified at three fermentation periods, including "amino acids, peptides, and analogs" (156 metabolites, 26.62%); "carbohydrate metabolomics" (33 metabolites, 5.63%); "fatty acids and conjugates" (31 metabolites, 5.29%); "terpene glycosides" (21 metabolites, 3.58%); "glycerophosphoethanolamines" (17 metabolites, 2.90%); "triterpenoids" (13 metabolites, 2.22%); "sesquiterpenoids" (12 metabolites, 2.05%); "fatty acyl glycosides" (11 metabolites, 1.88%), and "terpene lactones" (11 metabolites, 1.88%) (Figure 4A). The results showed that the primary metabolites in strain 5–4 extracts were rich and diverse. Abundant terpenoids and amino acid compounds were observed in the secondary metabolites, with a few compounds being "steroidal glycosides" and "glycosphingolipids." In addition, several hormones and hormone analogs were identified, such as flavonoid glycosides and indoles (Table S1). The hierarchical cluster analysis showed that the metabolites were significantly separated at different fermentation periods (Figure 4B). These metabolites were divided into 12 subclusters

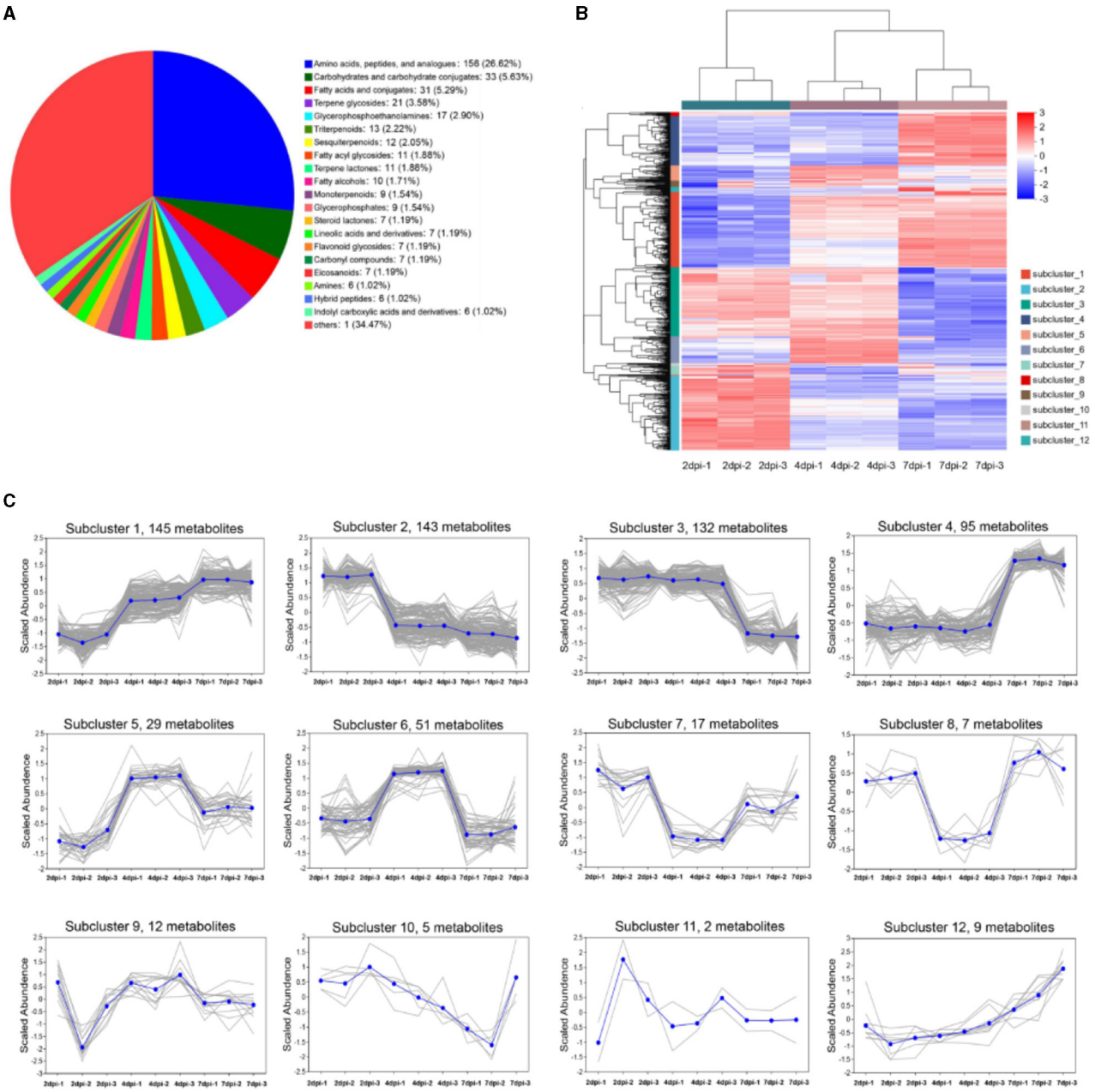

FIGURE 4
The metabolites of strain 5–4 were identified in three fermentation periods [2, 4, and 7 days post-inoculated cultured (dpi)]. Three biological replications were performed to analyze the metabolite profiling. **(A)** Pie chart of strain 5–4 metabolome data. A total of 647 metabolites were found in the subclass; **(B)** Hierarchical cluster analysis of metabolites in three fermentation periods. Divided into 12 subclusters; **(C)** K-MEAN analysis of metabolites. The subcluster 1 and 4 of metabolites were increased with fermentation periods.

using K-MEAN analysis (Figure 4C). Subcluster 1 contained the most metabolites (145), and subcluster 11 had only two metabolites. The relative contents of metabolites in the subcluster 1 and 4 increased with an extension of fermentation periods. Therefore, the key antimicrobial metabolites existed in subclusters 1 and 4.

Metabolites with VIP > 1 and FC=> 1.8 were defined as differentially accumulated metabolites (DAMs). As a result, the 37 DAMs (27 upregulated, 10 downregulated) were identified in groups of 7 dpi vs. 2 dpi, the 19 DAMs (12 upregulated, 7 downregulated) in groups of 7 dpi vs. 4 dpi, and the 16 DAMs (13 upregulated, 3 downregulated) in groups of 4 dpi vs. 2 dpi (Figure 5A). The upregulated metabolites of strain extracts at 7 dpi were mainly triterpenoids, amino acids, carbohydrates, and their derivatives (Supplementary Table S2). UpSet Venn diagram analysis revealed that the 18 specific DAMs were identified in groups of 7 dpi vs. 2 dpi. Ganoderenic acid D, pubescenol, tetrahydrofolic acid, hemorphin-4, vignatic acid B,

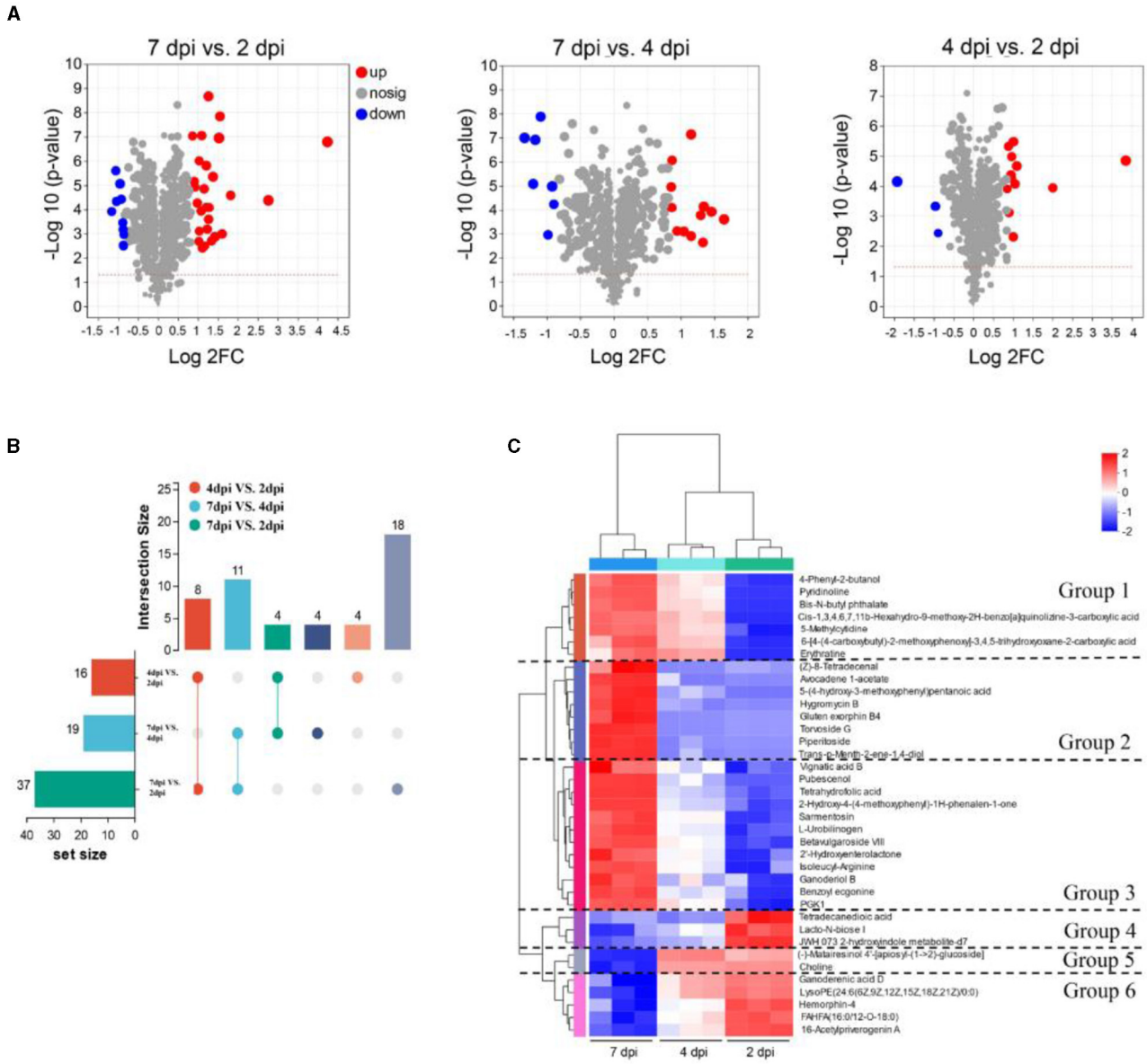

FIGURE 5
Analysis of the differentially accumulated metabolites (DAMs) at three fermentation periods. **(A)** Volcano plot of DAMs. Each dot represents a metabolite, blue dots are downregulated DEMs, red dots are upregulated DEMs, and gray dots are no significantly different metabolites. **(B)** The number of DAMs at three fermentation periods. The left column shows the number of DAMs in different pairwise comparisons, and the right column represents overlapping and specific DAMs in different comparisons. **(C)** The heatmap analysis of 37 DAMs in the three fermentation periods. The metabolites are generated in six groups.

and L-urobilinogen belonged to the class. The groups of 7 dpi vs. 4 dpi and 7 dpi vs. 2 dpi shared 11 DAMs and contained hygromycin B and piperitoside (Figure 5B). A heat map cluster analysis was conducted on the DAMs, and six groups were generated. The DAMs of groups 2 and 3 may be the key antimicrobial metabolites, which are only significantly enriched at 7dpi (Figure 5C). To explore the physiological processes of these DAMs, KEGG annotation, and analysis were conducted. A total of 100 pathways were involved in the DAMs (Supplementary Table S3). The pathways of "biosynthesis of plant secondary metabolites" and "protein digestion and absorption" were associated with the most DAMs. The KEGG pathway enrichment analysis showed that "novobiocin biosynthesis," "biosynthesis of vancomycin group antibiotics," "zeatin biosynthesis," "carbapenem biosynthesis," "bacterial chemotaxis," "biosynthesis of plant hormones," "tropane, piperidine, and pyridine alkaloid biosynthesis," and "Staurosporine biosynthesis" were significantly enriched ($p < 0.05$) (Supplementary Figure S3A). To identify unique DAMs at 7 dpi, VIP values, and p-values were used for screening. The predictive performance of these DAMs were evaluated using receiver operating characteristic curves (ROC). DAMs with an AUC of 1 were considered to have good predictive performance and were

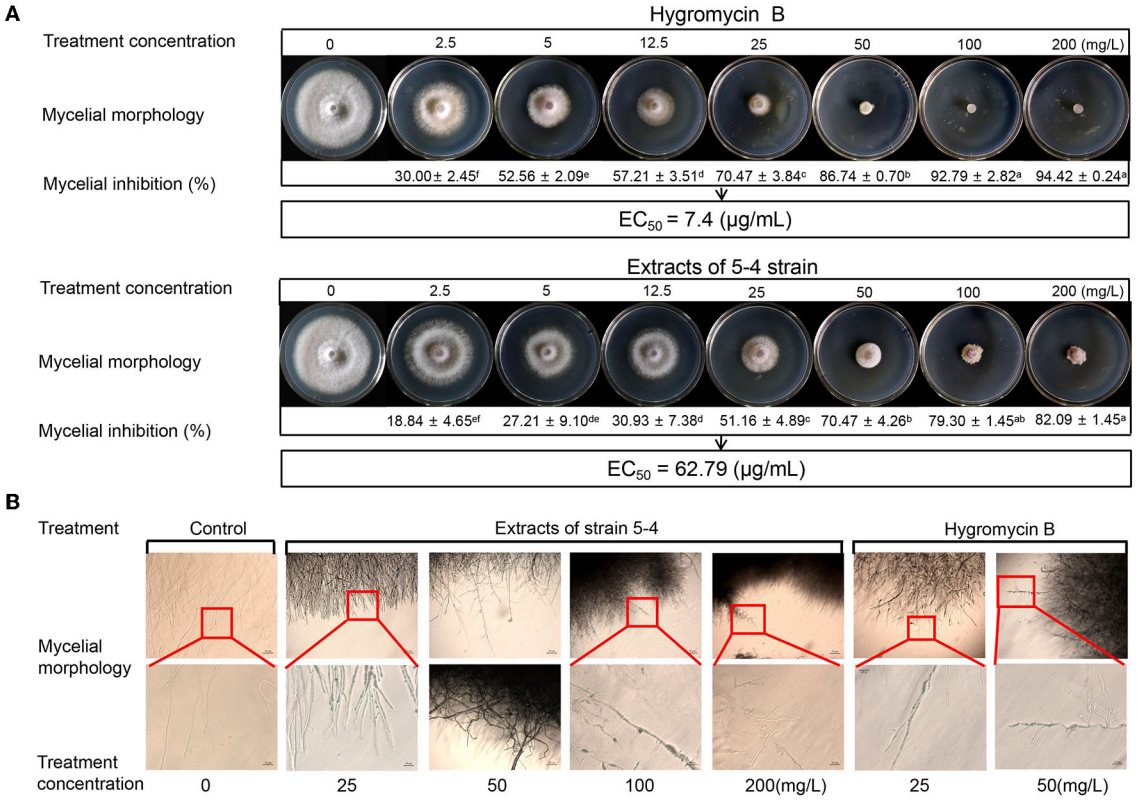

FIGURE 6
Antagonistic effects of hygromycin B and strain 5–4 extracts against Foc TR4 *in vitro*. **(A)** Antimicrobial activity of Foc TR4 treated with various concentrations of hygromycin B and strain 5–4 extracts (0, 2.5, 5, 12.5, 25, 50, 100, and 200 mg/L). Different letters were used to indicate significant differences ($p < 0.05$). **(B)** The hindered growth of Foc TR4 mycelium in the presence of hygromycin B and strain 5–4 extracts with microscopic observation.

selected as potential biomarkers. Notable DAMs with an AUC of 1 included hygromycin B, gluten exorphin B4, tetrahydrofolic acid, torvoside G, and pipertoside (Supplementary Figure S3B). An abundance of hygromycin B at 7 dpi was significantly higher compared to that at 2 dpi (Supplementary Figure S3C). This suggests that hygromycin B may be a key metabolite responsible for the antimicrobial activity exhibited by strain 5–4.

Comparative analysis of antimicrobial activity between hygromycin B and strain 5–4 extracts

The inhibitory effects of various concentrations of hygromycin B and extracts on the growth of Foc TR4 mycelium are depicted in Figure 6. In the control group, colony growth continued to increase during cultivation at 28°C. However, hygromycin B and extracts (2.5–200 μg/mL) demonstrated antifungal activity against Foc TR4, exhibiting a dose-dependent effect. Compared to the control group, the antifungal activity of extracts and hygromycin B at a concentration of 200 μg/mL was 82.09 ± 1.45% and 94.42 ± 0.24%, respectively (Figure 6A). Using the log-transformation analysis calculation, we found that the EC_{50} value of hygromycin B against Foc TR4 was 7.4 μg/mL. Moreover, when Foc TR4 mycelium was treated with hygromycin B and extracts from strain 5–4, noticeable coarseness and abnormal branching of the mycelium were observed (Figure 6B). These results indicated that both hygromycin B and strain 5–4 extracts had similar effects on the morphology of Foc TR4 mycelium.

Discussion

Bananas are an important fruit for production and global trade (Dita et al., 2018). However, the prevalence, severity, and rapid spread of FWB have led to substantial economic losses for banana farmers and posed a serious threat to the development of the banana industry (Dusunceli, 2017). Compared with traditional chemical pesticides, biological control has advantages such as eco-friendly nature and low investment costs. Biocontrol has shown promising effectiveness in managing FWB (Bubici et al., 2019). The effects of different bioagents can vary depending on the specific microbes and the complex natural environment. Therefore, it remains crucial to adapt to local environments and develop safer and more effective biological agents. This approach holds significant guiding importance for practical production purposes. Endophytic actinomycetes have been reported as a major source of bioactive metabolites and are considered a promising strategy for the biocontrol of plant pathogens (Xu et al., 2020).

Strain 5–4 were isolated from the roots of *Piper austrosinense* and exhibited high antimicrobial activity against Foc TR4. In our present study, the antifungal mechanisms and metabolomic profile of strain 5–4 were analyzed. Consistent with the results of previous studies, the morphological changes in Foc TR4 mycelium were observed through microscopic analysis. Therefore, comparing the potency of the mycelium's growth can provide information on its mode of antimicrobial action. The results showed that strain 5–4 exhibited inhibitory effects on the mycelia development of Foc TR4, leading to malformation and dissolution of the mycelium. We believed that the antifungal mechanism of strain 5–4 extracts involved the disruption of the cell membrane and cell wall of Foc TR4, ultimately resulting in the death of the Foc TR4 cells. In our previous study, strain 5–4 destroyed the cell wall of Foc TR4 through the action of cell wall-degrading enzymes. Therefore, the secretion of β-1,3-glucanase and chitinase by strain 5–4 may be considered one of its antifungal mechanisms (Yun et al., 2022).

In addition to the cell wall, the cell membrane is the mechanical and osmotic barrier between the cell and the external environment and is a common action site of fungicides against pathogenic fungi (Kim and Lee, 2019; Xu et al., 2019; Zhang et al., 2021). Light microscope analysis showed that strain 5–4 caused the abnormally lysed Foc TR4 mycelium. Moreover, TEM analysis revealed that the cell membrane of Foc TR4 was damaged, and organelles were degraded. The changes in Foc TR4 mycelium may be related to an increase in cell permeabilization (Wang et al., 2019). Thus, the antifungal mechanism of strain 5–4 extracts against Foc TR4 might be dependent on cell membrane destruction, leading to the death of the cell (Wang et al., 2019; Chen et al., 2022a). The integrity of the cell membrane is essential for the growth of Foc TR4. Soluble proteins and reducing sugars are critical cellular components and fundamental to the function of fungi (Chen et al., 2020). The impact of strain 5–4 on the integrity of the Foc TR4 cell was evaluated by determining the contents of soluble proteins and reducing sugar. The results showed a significant decrease in the content of SP after exposure to different concentrations of extracts, suggesting leakage of endoplasmic reticulum (ER) stress in the Foc TR4 cells compared to the control groups. NADH oxidase activity is a vital antioxidant function that is the main defense barrier for protecting cellular metabolism (Li et al., 2022). The content of MDA is commonly used as an indicator of oxidative damage to the cell membrane (Liu et al., 2021). The results indicated a dose-dependent decrease in NADH oxidase activities of Foc TR4, along with a dose-dependent increase in MDA activities, following treatment with extracts of strain 5–4. These results confirmed that the cell membrane of Foc TR4 was irreversibly damaged after being exposed to the extracts of strain 5–4. This suggests that the metabolites produced by strain 5–4 may directly interact with the components of the cell membrane.

Mitochondria are one of the earliest and most sensitive organelles for sensing biological damage (Zhang et al., 2021). The decreased fluorescence intensity of rhodamine-123 indicates that the extract of strain 5–4 caused a reduction in the mitochondrial membrane potential of Foc TR4. The activity of the mitochondrial respiratory chain complex enzymes plays an essential role in organisms, with complex II being one of the most important targets in the field of pesticide research, especially for the discovery of novel fungicides (Zhang and Chen, 2017; Zhao et al., 2020). The activity of complex I-V was observed to decrease after treatment with the extracts, displaying a negative correlation with the dose and a significant difference. Inhibition of electron transport in the mitochondrial respiratory chain disrupts the normal flow of electrons, leading to the accumulation of free radicals. The extent of free radical formation depends on the level of panthenol (Chen et al., 2022b). The accumulation of free radicals caused by the altered activity of complex III may be responsible for the extract-induced production of ROS in Foc TR4.

When the cell membrane of fungi is destroyed, it causes the efflux of cellular contents such as cytoplasm and proteins. This disruption also leads to the disorder of transmembrane potential, causing irreversible damage to the cell membrane and inhibiting the growth of fungal mycelium (Lin et al., 2018; Zhang et al., 2021). By targeting multiple sites and exerting various kinds of pressure, the stress response of the fungus becomes overwhelmed and unable to sustain growth. In the case of strain 5–4, it appears that the cell membrane of Foc TR4 is the main target, indicating its significant impact on fungal growth and development.

Metabolic profiles of strain 5–4 were performed using LC-MS analysis at different time periods, revealing changes in metabolite composition over the course of fermentation. The LC-MS analysis identified a total of 876 DAMs that exhibited an increase in the group of 7 dpi, including several unknown metabolites. These findings indicate the ability of strain 5–4 to produce a diverse range of metabolites during the fermentation process. Among the secondary metabolites, an abundance of terpene and amino acid compounds was observed, along with a few steroidal glycosides and glycosphingolipids. Notably, 27 upregulated DAMs were identified in the group of 7 dpi compared to 2 dpi. Furthermore, a high abundance of DAMs was involved in 100 pathways, with significant clustering in the biosynthesis of plant secondary metabolites and the protein digestion and absorption pathway. The KEGG pathway enrichment analysis showed significant enrichment of pathways such as novobiocin biosynthesis, biosynthesis of vancomycin group antibiotics, and zeatin biosynthesis. Among the unique DAMs identified in the 7dpi group based on VIP and p-value values, hygromycin B, gluten exorphin B4, tetrahydrofolic acid, torvoside G, and pipertoside were prominent. Comparative analysis of the antimicrobial activity between hygromycin B and strain 5–4 extracts showed similar inhibitory effects on the mycelium of Foc TR4. Hygromycin B belongs to the aminoglycoside antibiotic family and is known to inhibit protein synthesis in both prokaryotes and eukaryotes (Li et al., 2020). Therefore, we speculate that hygromycin B could target the cell membrane for Foc TR4, thereby inhibiting its growth. Hence, hygromycin B may be considered the key metabolite responsible for the antimicrobial activity of strain 5–4.

Based on the results of this study, it was determined that the antifungal mechanism of strain 5–4 inhibited the mycelium of Foc TR4 mycelium growth through the damage inflicted on the cell membrane and the suppression of mitochondrial respiratory chain complex enzyme activity. In future studies, we should explore the specific mechanisms of action of hygromycin B and determine the presence of other potentially significant antifungal metabolites. Such research will pave the way for the

development of highly potent fungicides with enhanced efficacy for agricultural applications.

Conclusion

Streptomyces hygroscopicus subsp. *hygroscopicus* 5–4 exhibited strong antifungal activity against Foc TR4. The most possible antimicrobial mechanism of strain 5–4 was explored using metabolomics, light microscopy imaging, and TEM. The morphological alterations in Foc TR4 mycelium cleared, indicating rupture and damage. Moreover, the organelles of Foc TR4 exhibited significant damage and hydrolysis. Strain 5–4 effectively inhibited the growth of Foc TR4 by targeting the cell membrane, resulting in the release of cellular contents and impairment of mitochondrial function. These multifaceted pressures on different cellular sites overwhelmed the fungal stress response system, leading to the inability to sustain growth. These results indicate that the direct antagonistic activity of strain 5–4 against Foc TR4 might be due to its damage to the integrity of the Foc TR4 cell membrane. Strain 5–4 produced an abundance of metabolites during the fermentation period and exhibited strong antifungal activity against Foc TR4 at 7 dpi. VIP and *p*-value values, the unique DAMs identified at 7 dpi included hygromycin B, gluten exorphin B4, tetrahydrofolic acid, torvoside G, and pipertoside. Hygromycin B and strain 5–4 extracts exhibited similar inhibitory effects on the mycelium morphology of Foc TR4. Hence, hygromycin B may be the key antimicrobial metabolite of strain 5–4.

Data availability statement

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

Author contributions

TY and TJ conceived the research. JX supervised the research. KL, YZ, and XZ performed some experiments. TY and DZ analyzed the data. TY and WW prepared 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.

The reviewer JP declared a shared affiliation with the authors to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1159534/full#supplementary-material

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Banana disease-suppressive soil drives *Bacillus* assembled to defense Fusarium wilt of banana

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Fusarium wilt of banana (FWB) caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc* TR4), poses a serious problem for sustainable banana production. Biological control is one of the effective measures to control this destructive disease. High-throughput sequencing of soil microorganisms could significantly improve the efficiency and accuracy of biocontrol strain screening. In this study, the soil microbial diversity of six main banana-producing areas in Yunnan was sequenced by Illumina Miseq platform. The outcome of this study showed the genus of *Chujaibacter*, *Bacillus,* and *Sphingomonas* were significantly enriched in microorganism community composition. Further correlation analysis with soil pathogen (*Foc* TR4) content showed that *Bacillus* was significantly negatively correlated with pathogen content. Therefore, we isolated and identified *Bacillus* from the disease-suppressive soils, and obtained a *B. velezensis* strain YN1910. *In vitro* and pot experiments showed that YN1910 had a significant control effect (78.43–81.76%) on banana Fusarium wilt and had a significant growth promotion effect on banana plants.

KEYWORDS

Fusarium wilt of banana, *Foc* TR4, soil microorganism diversity, *Bacillus* spp., biological control

1. Introduction

Banana is a staple fruit/crop in tropical and subtropical regions of the world, and are planted in more than 130 countries worldwide (D'Hont et al., 2012). China is the second largest producer of banana which is planted mainly in the south and southwest (Li et al., 2019). Yunnan is one of the origin places of banana planting, and banana is also an important economic industry in Yunnan's tropical and border areas with Laos, Myanmar, and Vietnam (Fan et al., 2021). The banana planting area in Yunnan mainly includes mountain, basin, razed land, river valley, and other landforms, with an average altitude of about 400 m (Yin et al., 2022). The unique ecological conditions make the "highland bananas" popular among consumers (Huang and Xiaojun, 2021).

Fusarium wilt of banana (FWB) caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc* TR4), elicits a serious problem for sustainable banana production (Butler, 2013). At present, all the banana main producing areas have suffered from this disease in China (Li

TABLE 1 The background information of sampling from different ecological banana plantations.

Sampling sites	Sampling code	Latitude/longitude	Altitude
Yuxi	YJI1, YJI2,YJI3,YJI4,YJI5 YJH1,YJH2,YJH3,YJH4,YJH5	23°35′19″N 101°59′29″E	380 m
Gejiu	FKI1,FKI2,FKI3,FKI4,FKI5 FKH1,FKH2,FKH3,FKH4,FKH5	23°12′19″N 102°56′33″E	480 m
Yuanyang	PJI1,PJI2,PJI3,PJI4,PJI5 PJH1,PJH2,PJH3,PJH4,PJH5	23°13′25″N 102°45′47″E	480 m
Wenshan	LQI1,LQI2,LQI3,LQI4,LQI5 LQH1,BNH2,BNH3,BNH4,BNH5	22°48′3″N 103°53′13″E,	410 m
Dehong	RLI1,RLI2,RLI3,RLI4,RLI5 RLH1,RLH2,RLH3,RLH4,RLH5	23°57′33.05″N 97°45′55.31″E,	775 m
Xishuangbanna	BNI1,BNI2,BNI3,BNI4,BNI5 BNH1,BNH2,BNH3,BNH4,BNH5	21°38′6″N 100°43′2″E,	620 m

et al., 2019). The banana industry has been seriously threatened by this pathogen, and it severely restricts banana production in China (He et al., 2021; Li et al., 2021). As a perennial crop irrigated with large amounts of water and fertilizer, the reduction of organic matter and acidification of the soil has gradually become serious, which changed the soil microbial diversity (Wang et al., 2015; Fu et al., 2016; Shen et al., 2019). The community structure composition change of microbes in the soil directly affects the functional diversity and the occurrence and development of soil-borne diseases (Wan et al., 2017; Kwak et al., 2018; Syed Ab Rahman et al., 2018; Carrión et al., 2019). At present, there has been a lot of research on the application of beneficial microorganisms to control the banana fusarium wilt (Damodaran et al., 2020; Jing et al., 2020; Li et al., 2020; Wei et al., 2020). This soil-borne fungal disease could be easily affected by soil microorganisms (Shen et al., 2019). The application of an antagonistic bacteria agent is an effective way to inhibit the occurrence of banana fusarium wilt (Koberl et al., 2017; Wan et al., 2017; Syed Ab Rahman et al., 2018).

The screening methods of beneficial microbes are bent to the direct isolation and culture of microorganisms from soil or plants (Bubici et al., 2019; Chen et al., 2020; Fan et al., 2021). Also, there are other methods such as phospholipid fatty acid (PLFA) map analysis method (Bååth and Anderson, 2003), Biolog ECO-plate detection (Zhang et al., 2009), and others (Bubici et al., 2019), but these methods are time-consuming and easy to obtain miscellaneous microbes. Therefore, the second-generation high-throughput sequencing method can be used to directly detect microbial genes without culture, and obtain detailed classification information of microorganisms, which would greatly improve the screening efficiency of beneficial microorganisms (Huang et al., 2015). There are some in-depth studies in other crops utilizing this method for beneficial microorganism screening, but the application in banana has not successful reports yet.

In this study, six ecological banana plantations representative of the main banana-planting regions in Yunnan Province were selected, based on high-throughput sequencing technology, bulk soil bacteria community structure diversity and difference, real-time fluorescence quantitative PCR, and separation of culturable microorganisms were analyzed of infected/healthy banana bulk soils, verifying the soil biological function of the major bacteria groups through the separation of culturable microorganisms and functional bacteria screening, exploring its biocontrol mechanism of FWB. The screened antagonistic bacterium from bulk soil from this study could provide new approaches for the subsequent ecological control of FWB.

2. Materials and methods

2.1. Sample collection

Bulk soil samples from 6 banana main producing areas (representative areas of the main banana-planting regions in Yunnan Province, and they have different longitudinal distributions and different climatic conditions.) were collected in Yunnan in 2018, where the disease incidence of FWB was 5–8%. The 5-point sampling method was used to randomly collect bulk soil samples from infected (I) and healthy (H) banana cultivar (Brazilian) in banana fields. Five replicates were collected in each sampling location. The 10 cm-deep soil was collected with a soil collector, and the soil around the roots was collected as the bulk soil. Impurities were removed from the samples such as roots and stones and then sealed with a sterile polyethylene bag and placed in an ice box, transferred to the laboratory to store immediately. The background information of soil samples was shown in Table 1.

2.2. TR4 determination in banana bulk soil by RT-qPCR

TR4 content was determined according to the method described by Bai et al. (2019). Primer FocSc-1 (5′- CAGGGGATG TATGAGGAGGCTAGGCTA-3′)/ FocSc-2 (5′- GTGACAGCGTC GTCTAGTTCCTTGGAG-3′) were used for fluorescence quantitative PCR to detect TR4 content (Lin et al., 2013). The fragment size of the amplification is 242 bp. PCR amplification was performed using Takara SYBR Premix Ex TaqTM (Tli RHaseH Plus) reagent box (Code No. RR820). The standard curve was established by using a recombinant plasmid PMD18T158 containing 158 bp target fragment to determine the concentration of extracted plasmid DNA. The requirements of standard curves were $R^2 > 0.99$, $90 < \text{Eff\%} < 110$.

2.3. High-throughput sequencing analysis of soil microorganisms

The total DNA of soil microorganisms was extracted according to the instruction book of Fast DNA SPIN Kit for Soil Kit for soil (MP Biomedicals, United States). The total DNA of the samples was stored at −20°C. Primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5'-GGACT ACCAGGGTATCTAAT-3′) were used to amplify the V3-V4 region of bacterial 16S rRNA. The amplified products were sent to Shanghai Personalbio Co., Ltd. and Shanghai Magi Biotechnology Co., Ltd. for high-throughput sequencing on the Illumina Miseq platform.

QIIME (Uparse) 8.0 software was used to process the raw data of Illumina Miseq sequencing. OTUs (Operational taxonomic units) classification was performed at the 97% similarity level. Principal coordinate analysis (PCoA) was conducted according to the Unifrac distance matrix between groups. The heatmap was drawn by the 'pheatmap package' of R (V.3.2.5). Multivariate analysis of variance (PERMANOVA) was used to test differences between groups by means of the package (V.2.3–5) in R (V.3.2.5). T-test was used to test the significance between groups, and one-way analysis of variance (ANOVA) was used to obtain the significance of multiple groups. Lefse analysis was performed on differences in bacterial community composition at the phylum and genus levels using Mothur software ($p < 0.05$). R was used to analyze the OTU number of each group, and a Venn diagram was used to show the OTU proportion. The sequence data (accession number PRJNA949429 (16S rRNA)) were downloaded from the NCBI database.

2.4. Isolation and antagonist bacteria screening of banana bulk soil

The bacteria isolation was performed according to the following methods: 2 g of soil sample was added into a centrifuge tube filled with 18 mL sterile water. The mixture was oscillated and mixed with a vortex oscillator to obtain the soil suspension, which was then diluted to 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} successively by a 10-fold gradient dilution method. One hundred micro liter soil dilutions were inoculated on NA medium (beef extract 3 g, peptone 10 g, agar 15 g, sodium chloride 5 g, for 1 L) for bacteria culture. After 28°C for 24 h culturing, single colonies with different morphology were selected. The bacteria selected were then stored in 50% sterile glycerol at −80°C for later use.

The pathogen *Foc* TR4 (Zhang et al., 2018) was active at PDA medium (potato 200 g, agar 15 ~ 20 g, glucose 20 g for 1 L) and cultured at 28°C for 7 days. Five diameter colony section was transferred to the center of PDA medium with a sterilized punch. The isolated strains were evenly inoculated at four points 25 mm from the center. After 7 days of incubation at 28°C, the inhibitory effect of isolated strains on TR4 was investigated and recorded. PDA plate inoculated only with TR4 was used as the control. The TR4 diameter measurement and inhibition rate were calculated according to Li et al. (2021).

2.5. Identification of antagonistic strains

2.5.1. Morphological observation

The isolated strains were cultured on an NA medium and incubated at 30°C for 24 h. Strain characteristics (including morphology, transparency, and color) were carefully recorded, and the micro-morphology character of bacteria was observed under a scanning electron microscope (ZEISS Sigma 300, Germany).

2.5.2. Molecular identification

The isolated strains were inoculated in NB medium and cultured in a shaking incubator at 37°C at 180 rpm for 18 h. Then 1 mL bacterial suspension was centrifuged at 12,000 rpm for 1 min and the bacteria cells were collected. Bacteria DNA was extracted using the Ezup column bacterial genomic DNA Extraction Kit (Sangon Bioengineering Co., Ltd). Primers 27F/1492R (5′-AGAGTTTGATCCTGGCTCAG3′/5′-GGTTACCTTGTTACG ACTT-3′) was used to amplify the 16S rDNA gene sequence and Sanger sequencing of bacteria was conducted by Shanghai Personalbio Biotechnology Co, Ltd. The reaction system was as follows: 5 μL 10× buffer (containing 2.5 mM Mg^{2+}), 1 μL Taq polymerase (5 u/μL), 1 μL dNTP (10 mM), 39 μL ddH2O, 1 μL DNA template, 1.5 μL upstream primer and downstream primer; Reaction conditions was 95°C, 5 min; 95°C for 30s, 58°C for 30s, 72°C for 1 min 30s, 35 cycles; 72°C for 7 min. The BLAST procedure of the NCBI database was used for sequence alignment analysis, and the phylogenetic tree was constructed using MEGA 7.0 software.

2.5.3. Preparation of biocontrol strain and pathogen fermentation broth

The bacteria were activated at NA medium at 37°C for 24 h. After single colonies were selected and inoculated in NB medium (beef extract 3 g, peptone 10 g, sodium chloride 5 g for 1 liter) at 180 rpm for 48 h at 37°C to obtain fermentation broth. The concentration of strain was diluted by sterile water to 1×10^8 cfu/mL and 1×10^7 cfu/mL, respectively. The activated TR4 strain (Zhang et al., 2018) was inoculated into PDB medium (potato 200 g, glucose 20 g for 1 liter) at 28°C and 150 rpm for 72 h. The suspension was filtered with 4-layer sterile gauze to obtain the spore suspension of TR4 (1×10^6 cfu/mL).

2.5.4. Preparation of pot experiment

The experiment was implemented in the glasshouse at Agricultural Environment and Resources Institute, Yunnan Academy of Agricultural Sciences in April 2021. Banana plants (Brazilian, Cavendish, AAA) grown in quartz stones with 3–4 leaves were transplanted into plastic pots (11 cm × 12 cm) with seedling substrates. Every pot was planted with one banana plant. Until the plant grows to 5–6 leaves, proceed to the next step of the experiment.

2.5.5. Experiment design

Six treatments were set in the pot experiment, which are shown in Table 2. The treatment inoculated with biocontrol strain fermentation broth only was marked as DC1 and DC2, the treatment inoculated with pathogen only was marked as TR4, and

TABLE 2 The treatments of pot experiment in greenhouse.

Code	Treatments
DC1	Drenched biocontrol strain fermentation broth (1×10^8 cfu/mL) and blank PDB medium
DC2	Drenched biocontrol strain fermentation broth (1×10^7 cfu/mL) and blank PDB medium
CK	Drenched blank NB medium and blank PDB medium
DC1 + TR4	Drenched biocontrol strain fermentation broth (1×10^8 cfu/mL) and TR4 spore suspension
DC2 + TR4	Drenched biocontrol strain fermentation broth (1×10^7 cfu/mL) and TR4 spore suspension
TR4	Drenched blank NB medium and TR4 spore suspension

the treatment inoculated blank culture medium broth was marked as CK. Among them, treatments I-III were inoculated without TR4, and IV-VI were TR4-inoculation treatments. The pot experiment was set up in three replicates, with 12 plantlets in each treatment.

When the banana plants grew to 6 leaves, 40 mL biocontrol strain fermentation broth (DC1, 1×10^8 cfu/mL) and 10-fold dilution (DC2, 1×10^7 cfu/mL) were drenched on the roots of every potted banana plant, and the control treatment was drenched with 40 mL blank NB culture medium. The spore suspension of TR4 (40 mL, 1×10^6 cfu/mL) was drenched onto the root of banana plants after 7 days of inoculation of biocontrol strain. CK treatment was drenched with a blank PDB culture medium.

2.5.6. Disease and control effect investigation

Disease investigation (leaf and corm symptoms survey) was conducted after 40 days of TR4 inoculation. The disease severity index (DSI) was classified according to Fan et al. (2021). In order to explore the growth promotion effect of biocontrol bacteria, banana plant height, pseudostem thickness, and leaf number were measured 20 days and 40 days after TR4 inoculation referring to the methods of Fan et al. (2021).

2.6. Data analysis

SPSS 18.0 software was used for statistical analysis. Duncan's new complex range method was applied and independent sample T-test were used to analyze the significance of differences among treatments. Means are shown as "Mean ± standard error (S.E.)."

3. Results

3.1. High-throughput sequencing and OTU cluster analysis

The V3-V4 region of 16S rRNA gene was amplified and sequenced in bulk soil samples of infected (I) and healthy (H) plants from six banana producing areas in Yunnan. The composition ratio of OTUs number of rhizosphere soil samples was shown by a Venn diagram (Supplementary Figure S1). There were 112,365 OTUs in total, 44,077 OTUs were unique in the infected plants' soil, and 48,859 OTUs were unique in the healthy plants' soil. Bacterial dilution curves constructed by the number of randomly sampled sequences and the corresponding number of OTUs were flat (Supplementary Figure S2), indicating that bulk soil samples collected from infected and healthy banana plants were reasonably sampled and the bacterial communities' difference in rhizosphere soil samples could be reflected.

3.2. Alpha diversity of soil bacterial communities

The bacterial community of alpha diversity analysis was used to compare the differences of bulk soil bacterial diversity between infected and healthy banana plants in the regional climate types of different producing areas. The results showed that the Ace index and Chao1 index of healthy banana plants were higher than those of infected plants, and the bacterial community diversity in the bulk soil of healthy plants was richer. The bacterial α diversity of infected plants and healthy plants was significantly different ($p < 0.05$). The Shannon index and Simpson index of healthy banana plants were higher than those infested with TR4, but there was no significant difference in the Simpson index (Table 3).

3.3. Bacterial community structure in banana bulk soil

QIIME software was used to analyze the bacteria community structure of the bulk soil samples from six main banana producing areas. The results showed that there were 10 bacteria species enriched at the phylum level (Figure 1A). Among them, Proteobacteria is the dominant species, accounting for 38.2% (H) and 39.8% (I) of the total number of bacteria. Other dominant bacterial groups were Actinobacteria (21.2% (H), 20.4% (I)), Acidobacteria (10.0% (H), 10.6% (I)), etc. Among the dominant phyla, the abundance of Proteobacteria and Acidobacteria was higher in infected soil (I) than in healthy soil (H), and the abundance of Actinobacteria, Firmicutes, Chloroflexi was higher in healthy soil (H) than in infected soil (I). There were significant differences in bacterial community structure and abundance groups in infected soils (I) and healthy soils (H) at the genus level. The dominant genera were *Chujaibacter* [3.7% (H), 4.2% (I)], *Bacillus* [3.9% (H), 3.2% (I)], *Sphingomonas* [2.3% (H), 2.0% (I)], and *Saccharimonadales* [2.0% (H), 1.7% (I)], etc. (Figure 1B). Among the dominant genera, the abundance of *Bacillus* in healthy soil was significantly higher than that of fusarium wilt-infected soil, which was in accordance with the trend with the Firmicutes.

TABLE 3 Diversity of bacterial community in 6 banana producing areas from soil samples derived from Fusarium wilt of banana infected and healthy plants.

Status of plant	Sampling code	OTUs	Chao1	Shannon	Simpson	Coverage (%)
	YJI	1993.42	2069.75	9.87	0.997	0.99
	FKI	3729.78	4143.66	9.73	0.994	0.99
I	PJI	5425.88	5776.17	11.07	0.999	0.98
	LQI	3822.34	4256.56	9.87	0.992	0.99
	RLI	4027.44	4408.29	10.29	0.997	0.99
	BNI	3389.86	3508.97	9.63	0.992	0.99
	Average	3731.45	4027.23	10.08	0.995	0.988
	YJH	1498.2	1562.22	9.03	0.994	0.995
	FKH	4651.9	5149.14	10.58	0.997	0.98
H	PJH	5446.7	6111.04	11.13	0.999	0.98
	LQH	4211.72	4677.63	10.26	0.995	0.99
	RLH	4020.18	4382.60	10.21	0.995	0.99
	BNH	4437.88	4620.12	10.43	0.997	0.99
	Average	4044.43	4417.3	10.27	0.996	0.988

* indicated significant differences in the 0.05 level, ** indicated significant differences in the 0.01 level. YJI indicated the infected soils in Yuanjiang in Yuxi city, FKI indicated the infected soils in Fengkou in Gejiu city, PJI indicated the infected soils in Dapijia in Yuanyang city, LQI indicated the infected soils in Maguan in Wenshan city, RLI indicated the infected soils in Ruili in Dehong city, BNI indicated the infected soils in Xishuangbanna city, Yunnan, China. YJH indicated the healthy (suppressed) soils in Yuanjiang in Yuxi city, FKH indicated the healthy (suppressed) soils in Fengkou in Gejiu city, PJH indicated the healthy (suppressed) soils in Dapijia in Yuanyang city, LQH indicated the healthy (suppressed) soils in Maguan in Wenshan city, RLH indicated the healthy (suppressed) soils in Ruili in Dehong city, BNH indicated the healthy (suppressed) soils in Xishuangbanna city, Yunnan, China.

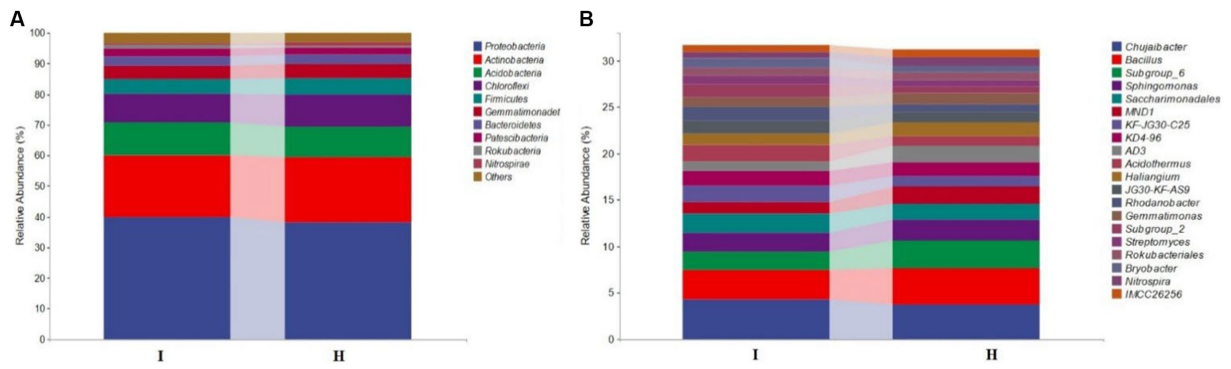

FIGURE 1
Bacterial community structure of banana bulk soils. Relative abundance of species on phylum level **(A)** and genus level **(B)** of bacteria community in banana infected (I) and healthy (H) soil samples of from 6 banana producing areas.

3.4. Cluster analysis of bacterial community composition

At the genus level, hierarchical clustering heatmap analysis was performed using R software for 19 bacterial microbiotas with high relative abundance (> 1%) in bulk soil samples from six main banana producing areas. The bacterial community composition of 60 samples was clustered into two large branches, and 30 samples of infected soil (I) and 30 samples of healthy soil (H) were basically one branch, indicating that their bacterial community composition structure was significantly different (Figure 2A). *Bacillus* was the dominant bacteria in both types of bulk soil and the abundance of *Bacillus* in healthy soil (H) was higher than that of infected soil (I). *Chujaibacter* was also the dominant bacteria in the healthy bulk soil, and *Gemmatimonadaceae* was the dominant bacteria in the infected bulk soil.

3.5. Correlation analysis of dominant population microorganisms and pathogen content

Firstly, we detected the content of soil pathogens (TR4) in six main banana producing areas by qPCR. The results showed that the content of TR4 in infected soil was significantly higher than that in healthy soil. Among them, the content of pathogenic bacteria in the Ruili area (RL) was the highest, and the content of TR4 in the Maguan (LQ) and Yuanjiang (YJ) areas was lower (Figure 2B). Correlation analysis was conducted between pathogen TR4 content and soil dominant microorganism's genus. In the top 20 dominant bacteria genera, the relative abundance of *Saccharimonadales, Gemmatinomas,* and *JG3—KF-AS9* was significantly positively correlated with the TR4 content. *Bacillus*, *Subgroup_6*, *Streptomyces*, *Rokubacteriales,* and

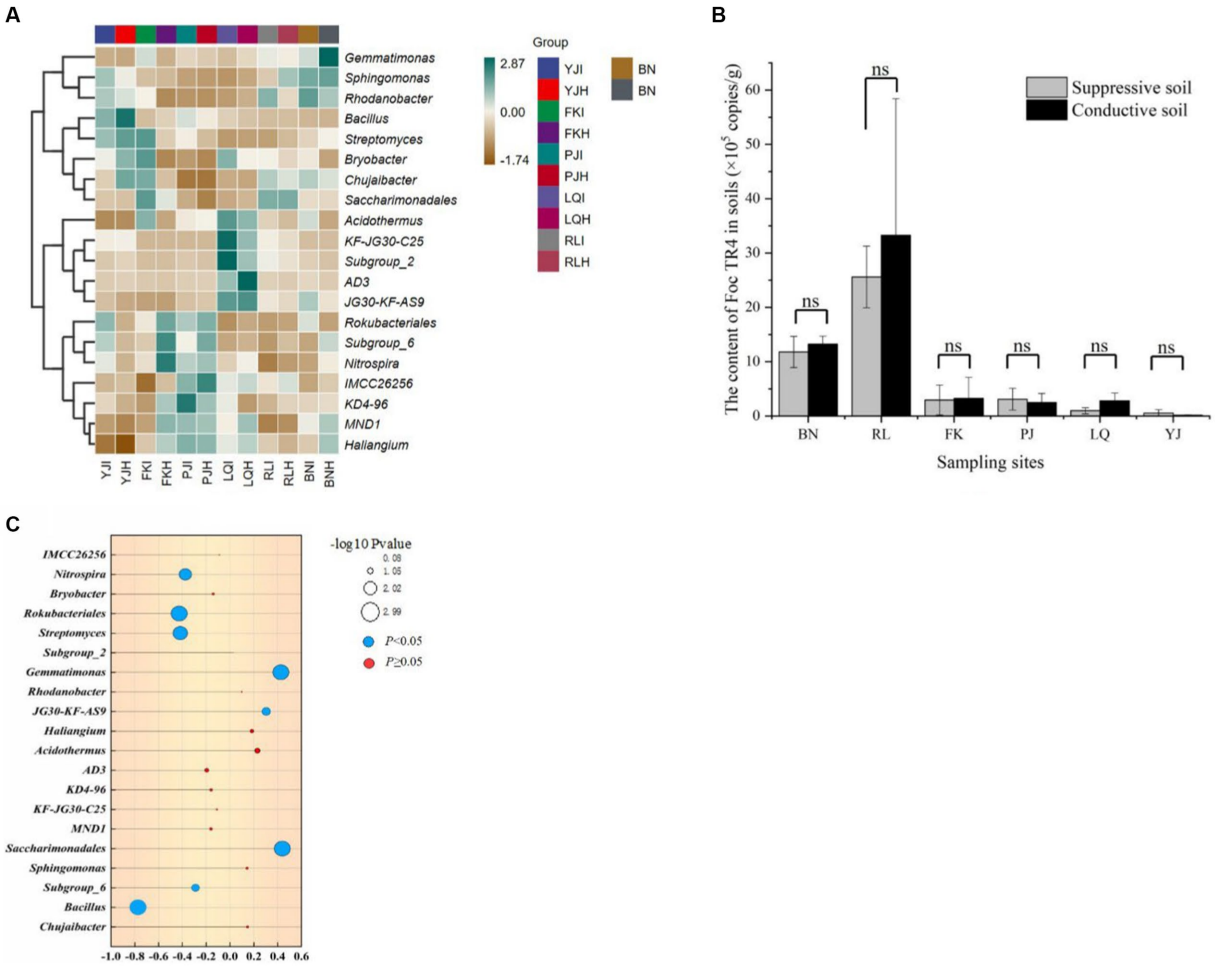

FIGURE 2
(A) Cluster heatmap of main bacteria community in healthy (H) and infected soil (I) samples from 6 banana producing areas. YJ indicated Yuanjiang in Yuxi city, FK indicated Fengkou in Gejiu city, PJ indicated Dapijia in Yuanyang city, LQ indicated Maguan in Wenshan city, RL indicated Ruili in Dehong city, BN indicated Xishuangbanna city, Yunnan, China. **(B)** The content of *Foc* TR4 in healthy (Suppressive) and infected (Conductive) soil samples from 6 banana producing areas. **(C)** Correlation of dominant genus and pathogen content. The X-axis represents the correlation coefficient based on Spearman. The correlation increases with the increase in bubble size. Blue bubbles represent *p* values less than 0.05, Red bubbles represent *p* values greater than 0.05.

Nietrospira were negatively correlated with the TR4 content (Figure 2C). We noticed that *Bacillus* not only had significant differences between infected and healthy plants, but also had a significant negative correlation with the pathogen content. Therefore, in order to verify whether *Bacillus* played a key role in the process of induced banana resistance, we carried out the isolation experiment of *Bacillus* genus.

3.6. Isolation of *Bacillus* from banana bulk soil

One thousand and one hundred twenty five bacterial strains were isolated from 60 bulk soil in 6 different banana-producing areas in Yunnan. Among these, 594 strains were isolated from the TR4-infected bulk soil, and 531 strains were isolated from healthy soil (Supplementary Table S1). After primary screening, seven *Bacillus* antagonistic strains were screened from infected soil and five *Bacillus* strains were screened from the healthy soil. After dual-culture for secondary screening, one *Bacillus* with the most antagonistic activity against TR4 labeled YN1910 was obtained (Figures 3A,B; Supplementary Table S1). We then observed the colony and microscopic morphology of YN1910. After cultured for 24 h in NA medium, the colony of YN1910 appeared milky-white and convex surface, the edge was irregular and the inside colony was smooth and moist (Figure 3A). Under the scan electron microscopy (SCM), the bacteria looked rod-shaped and rounded-shape at both ends (Figure 3B). To further observe the inhibitory effect of YN1910 on TR4, we conducted the dual-culture for further detection, the results showed that the average diameter of the inhibition zone and inhibition rate of YN1910 against TR4 was 1.63 cm and 81.78%, respectively (Figures 4A,B; Supplementary Table S2). The 'normal' TR4 mycelia were smooth and uniform (Figure 4C) under SCM. TR4 mycelium dual-cultured for 7 days culture with YN1910 were also observed under SCM, the results showed that TR4 mycelia were swollen and deformed, the mycelia gradually adhere and begin to dissolve (Figure 4D).

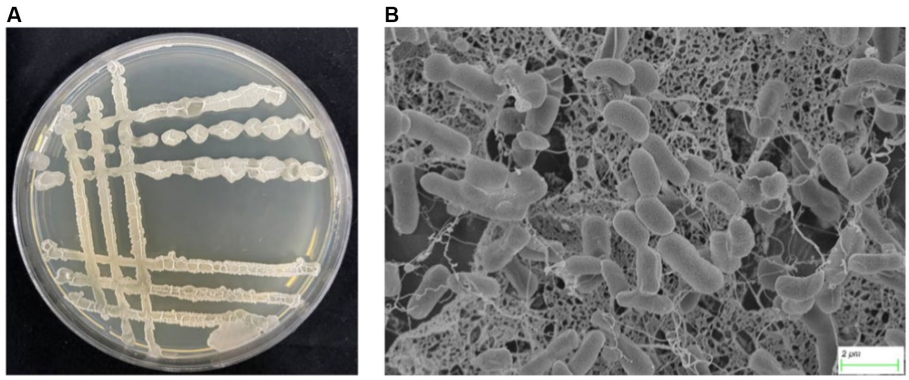

FIGURE 3
Colony morphology and mycelial morphology of YN1910. **(A)** Colony morphology of YN1910 cultured on NA medium at 30°C for 24 h. **(B)** Scanning electron micrograph of YN1910.

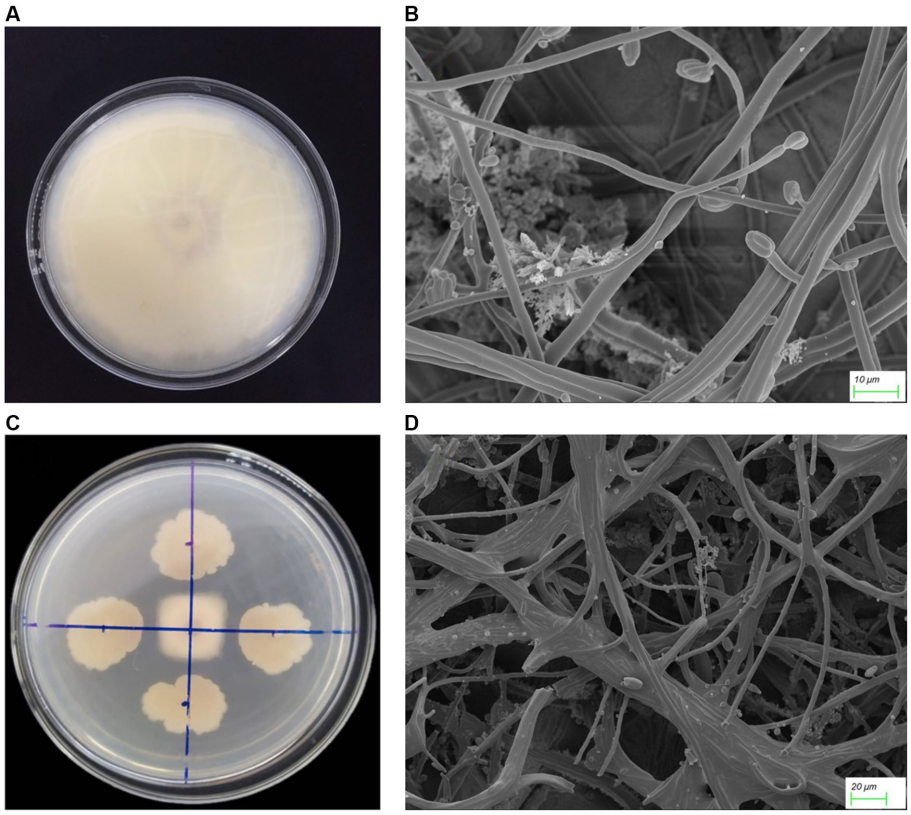

FIGURE 4
The antagonistic effect of strain YN1910 on TR4. **(A)** TR4 control. **(B)** The antagonistic effect of strain YN1910 on TR4. **(C)** Characteristics of mycelium morphology of control TR4. **(D)** Morphological characteristics of TR4 hyphae after being antagonized by YN1910.

3.7. Molecular identification of antagonistic bacteria

In order to determine the species of YN1910 accurately, we further sequenced the 16S region of YN1910. The 16S RNA sequences of strain YN1910 were compared in the GenBank database, and the results showed the highest similarity with *Bacillus velezensis*. Based on a phylogenetic tree of the sequences, YN1910 are related to *B. velezensis* (GenBank Accession No. MW647762) (Figure 5). Through the analysis of the above morphology, and molecular characteristics, the strain YN1910 was identified as a *Bacillus velezensis* (Figures 3–5).

3.8. YN1910 had significant control effect on TR4 in greenhouse pot experiment

To further verify the control effect of *B. velezensis* YN1910 on TR4, we conducted pot experiments. Forty days after inoculating TR4, the leaves of the control group (TR4) turned yellow and the banana

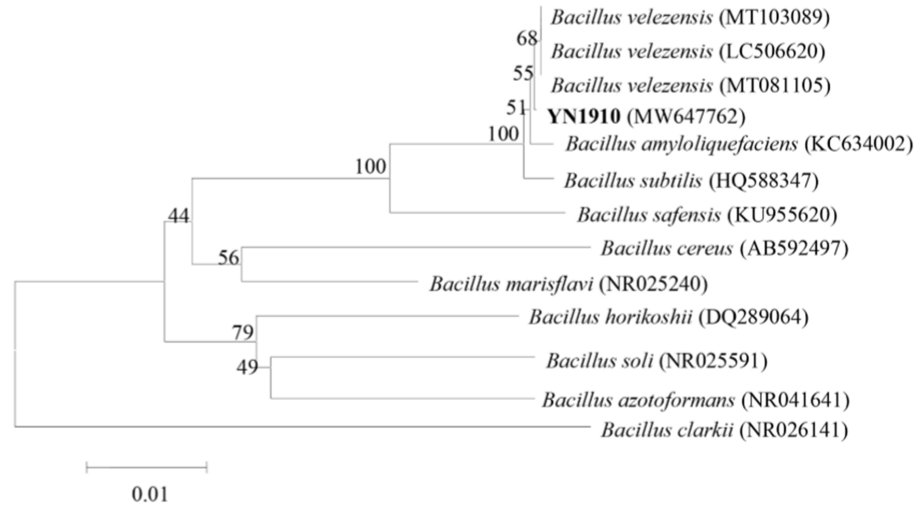

FIGURE 5
16S rRNA gene phylogenetic tree of YN1910.

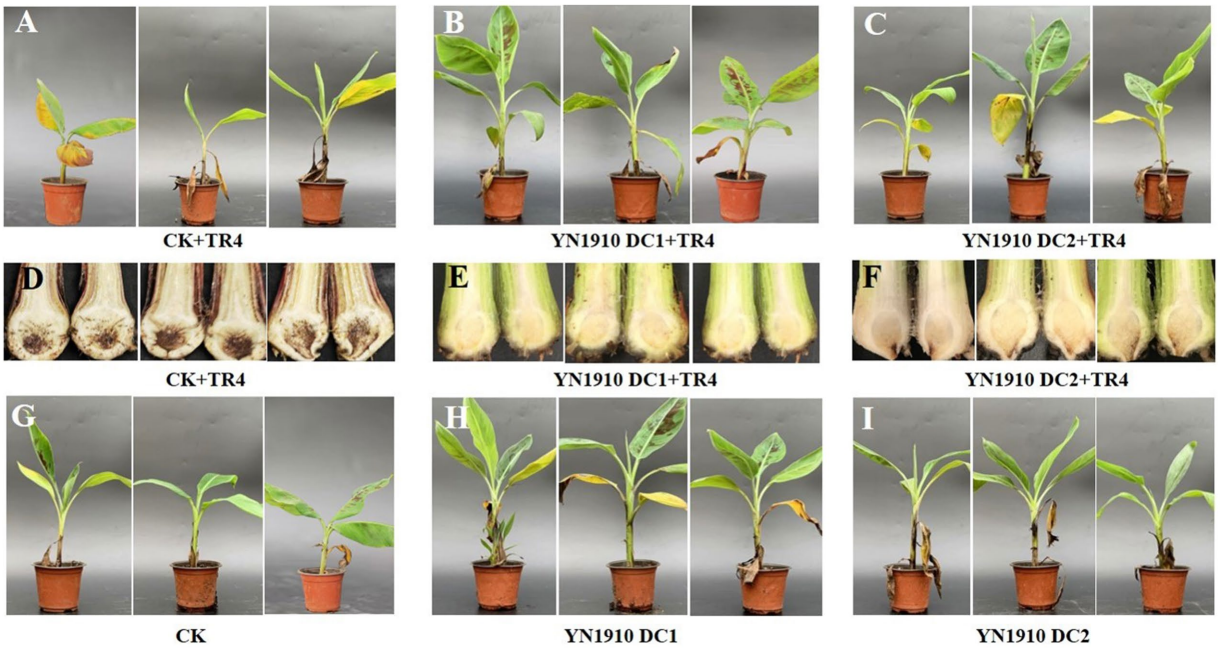

FIGURE 6
Control effect and growth promoting of antagonistic strain YN1910. **(A)** The banana leaf symptom after being inoculated with TR4. **(B)** The banana leaf symptom after being inoculated with YN1910 (DC1: 1×10^8 cfu/mL) and TR4. **(C)** The banana leaf symptom after being inoculated with YN1910 (DC2: 1×10^7 cfu/mL) and TR4. **(D)** The banana corm symptom after being inoculated with TR4. **(E)** The banana corm symptom after being inoculated with YN1910 (DC1: 1×10^8 cfu/mL) and TR4. **(F)** The banana corm symptom after being inoculated with YN1910 (DC2: 1×10^7 cfu/mL) and TR4. **(G)** The blank control without being inoculated with YN1910 or TR4. **(H)** Growth promoting effect after being inoculated with YN1910 (DC1: 1×10^8 cfu/mL). **(I)** Growth promoting effect after being inoculated with YN1910 (DC2: 1×10^7 cfu/mL).

plants began to wilt (Figure 6A), while the leaves of the treated group (DC1 + TR4, DC2 + TR4) remained green and healthy (Figures 6B,C). After cutting the corms, we observed that the corms in the control group (TR4) became brownish-black in color (Figure 6D), while the treatment corms (DC1 + TR4, DC2 + TR4) were healthy whitish (Figures 6E,F). The disease index on corms of the control group (TR4) was 58.33 ± 4.17, which was significantly higher than that of the treated groups (DC1 + TR4: 10.42 ± 2.08 and DC2 + TR4: 12.5 ± 3.61). The control effects of DC1 + TR4 and DC2 + TR4 were 81.67 and 79.17%, respectively (Supplementary Table S3), indicating that YN1910 had a significant effect on TR4. In addition, we also found that the control effect of DC1 + TR4 was higher than that of DC2 + TR4, indicating that within a certain range, the higher the concentration of biocontrol bacteria inoculated, the stronger the

control effect. However, our current evidence indicated insignificant in just two concentration gradients.

3.9. Promoting effect of YN1910 on banana growth

Except for the biological control effects on TR4, growth promoting effects were also an important indicator of *Bacillus*. We investigated the growth of banana plants at 0, 20, and 40 days after YN1910 inoculation (Figures 6G–I). The results showed that 20 days after inoculation, the plant height of DC1 (31.74 cm) was significantly higher than DC2 (26.26 cm) and CK (26.95 cm). After being inoculated for 40 days, the plant height of DC1 was 37.15 cm, which was significantly higher than that of DC2 (32.05 cm) and CK (30.33 cm). There was no significant difference of DC2 with CK (Supplementary Figure S3a). As for pseudostem growth, 20 days after inoculation, the pseudostem diameter of DC1 (10.30 mm) was significantly higher than DC2 (8.77 mm) and CK (9.14 mm). When inoculated for 40 days, the pseudostem diameter of DC1 was 11.36 mm, which was significantly higher than that of DC2 (10.26 mm) and CK (9.71 mm). And there was no significant difference between DC2 with CK (Supplementary Figure S3b). Bananas' leaf growth was also investigated, and the leaf number of DC1 (6.92) was significantly higher than DC2 (6.17) after 20 days of being inoculated YN1910. At 40 days after inoculation, the leaf number of DC1 (7.67) was significantly higher than DC2 (6.83) and CK (6.67), and the leaf number of DC2 and CK had no significant difference (Supplementary Figure S3c). It can be seen that YN1910 could significantly promote the growth of banana plants, and this effect is very significant under high concentrations.

4. Discussion

Microorganisms are the key driving force for the formation and transformation of soil contents, the engine of the ecological cycle, which is of great significance to the stability of the entire ecosystem (Veličković and Anderton, 2017; Syed Ab Rahman et al., 2018; Liu et al., 2019). There are many microbial resources with biocontrol potential in the soil, especially in suppressive soil (Xue et al., 2015; Perez-Jaramillo et al., 2016). Although their function is greatly affected by internal and external factors, the use of the characteristics of rapid microbial reproduction, and a large number of artificial reproductions after application into the soil, can regulate the root micro-ecological environment, limit the reproduction of soil-borne pathogens and inhibit the occurrence and development of soil-borne diseases, showing great application potential (Meena et al., 2017).

In the current study, most researchers are bent on the direct screening of antagonistic bacteria and indoor pot experiments for the prevention and control of FWB. Huang et al. (2010) sequenced the FWB-infected soils of banana orchards and normal plantations, they found that the bacterial diversity of soil in normal banana plantations was relatively abundant, among which *Proteobacteria*, *Firmicutes,* and *Acidobacteria* were the main bacterial groups. Deng et al. (2015) compared the diversity of bulk bacterial communities between healthy and diseased banana soils, the results showed that the bacterial diversity of diseased soils was less than that of healthy soils. Shen et al. (2015) and Fu et al. (2012) found that after 2 years of continuous application of microbial organic fertilizer, soil bacterial diversity, and microbial community structure were continuously enriched, thus reducing the incidence of FWB. In this study, *Proteobacteria, Actinobacteria,* and *Acidobacteria* were the dominant group in the infected soil, while *Firmicutes* accounted for more in the healthy soil (Figure 3). Further analysis at the genus level showed that *Bacillus* was the dominant genus in healthy soil that significantly negatively correlated with pathogen content (Figures 4, 5). It can be seen that the *Bacillus* in healthy soil is more abundant and which is why it can significantly resist pathogens, but we isolated more *Bacillus* species (seven strains were isolated from infected soil and five strains were isolated healthy soil) from infected soil, which might be due to the higher pathogen content could attracting more antagonistic microorganisms. Therefore, through high-throughput sequencing, we identified the genus *Bacillus* that is most significantly negatively correlated with *Foc* TR4. This has a good indication for the subsequent separation of biocontrol bacteria. According to this idea, we specially isolated and identified antagonistic *Bacillus* genus, and finally obtained a *B. velezensis* strain YN1910 with disease resistance potential in the infected soil of Yuxi.

B. velezensis is a heteromorphic species highly homologous to *B. amyloliquefaciens*, which is widely used due to its excellent ability to produce secondary metabolites and colonization in plants (Kang, 2019; Rabbee et al., 2019; Deng et al., 2020). Many strains of this species have been used as pathogen antagonists and plant growth promoters in agricultural production. For example, FZB42, which has high colonization ability in plants such as wheat, tomato, cucumber, and tobacco, has been successfully commercialized for disease control of multiple crops (Chen et al., 2007; Idris et al., 2007; Borriss et al., 2011; Fan et al., 2011; Chowdhury et al., 2013). Many *B. velezensis* have also been shown to have good disease control and growth-promoting effects, like NJN-6, B9601-Y2, CC09 et al. (Yuan et al., 2012; He, 2014; Kang, 2019). In this study, the isolated *B. velezensis* YN1910 was subjected to the controlled greenhouse pot experiment to determine its control effect on FWB. The results showed that YN1910 had a significant mitigation effect on the occurrence of FWB, and the effect was more obvious at high concentrations (Figure 6). This strain also significantly promoted the growth of bananas (Figure 6; Supplementary Figure S1). These results indicated that YN1910 is a successful biocontrol strain, and this is direct information obtained from the results of soil microbial diversity sequencing, which greatly reduces the workload of our isolation of biocontrol strains, and also improves the direction and accuracy of separation.

At present, the biocontrol mechanisms of *B. velezensis* are known through antibiosis, growth promotion, competition for nutrient uptake, and induced host systemic resistance. The biocontrol effects of this type of strain are often the results of the synergistic effect of multiple mechanisms (Bubici et al., 2019). Our research on the prevention and control of FWB by YN1910 is limited to the level of control effect and the specific mechanism is still in progress. And our experiment was carried out in a controlled greenhouse, the application of this biocontrol strain (YN1910) in the field is the next step in our research.

5. Conclusion

In this study, the 16S rRNA sequencing of soil microbial diversity showed that *Chujaibacter*, *Bacillus,* and *Sphingomonas* were significantly enriched in microorganism community composition. Correlation analysis with soil pathogen (*Foc* TR4) content showed that *Bacillus* was significantly negatively correlated with pathogen content. So, we isolated and identified a *B. velezensis* strain YN1910 from bulk soil. *In vitro* and pot experiments showed that YN1910 had significant prevention and control effects on FWB. It also had a significant promotion effect on banana growth.

Data availability statement

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

Author contributions

HF: conceptualization, designing, performing the experiment, analyzing the data, and writing the paper. PH: performing the experiment, analyzing the data, and preparing the manuscript. SX and SL: performing the pot experiment, and analyzing the data. YW: performing the morphological observation, and analyzing the data. WZ and XL: analyzing the data. HS: performing the physiology experiment. S-JZ and LZ: conceptualization and designing the experiment, reviewing and editing the manuscript, supervising the research, and providing funding support. 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.2023.1211301/full#supplementary-material

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A biological product of *Bacillus amyloliquefaciens* QST713 strain for promoting banana plant growth and modifying rhizosphere soil microbial diversity and community composition

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Introduction: Bananas are not only an important food crop for developing countries but also a major trading fruit for tropical and semitropical regions, maintaining a huge trade volume. Fusarium wilt of banana (FWB) caused by *Fusarium oxysporum* f. sp. *cubense* is becoming a serious challenge to the banana industry globally. Biological control has the potential to offer both effective and sustainable measures for this soil-borne disease.

Methods: In order to explore the biocontrol effects of the biological agent *Bacillus amyloliquefaciens* QST713 strain on banana plants, two cultivars, Brazilian and Yunjiao No. 1, with varied resistance to FWB, were used in greenhouse pot experiments.

Results: Results showed that the plant height and pseudostem diameter of banana-susceptible cultivar Brazilian increased by 11.68% and 11.94%, respectively, after QST713 application, while the plant height and pseudostem diameter of resistant cultivar Yunjiao No. 1 increased by 14.87% and 12.51%, respectively. The fresh weight of the two cultivars increased by 20.66% and 36.68%, respectively, indicating that this biological agent has potential effects on plant growth. Analysis of the rhizosphere soil microbial communities of two different cultivars of banana plants showed that TR4 infection and *B. amyloliquefaciens* QST713 strain application significantly affected the bacterial and fungal diversity of Yunjiao No. 1, but not in the cultivar Brazilian. In addition, TR4 infection and QST713 application changed the bacterial community composition of both banana cultivars, and the fungal community composition of Yunjiao No. 1 also changed significantly. Relevance analysis indicated that the relative richness of *Bacillus* and *Pseudomonas* in the rhizosphere of both cultivars increased significantly after QST713 application, which had a good positive correlation with plant height, pseudostem girth, aboveground fresh weight, leaf length, and leaf width.

Discussion: Therefore, the outcome of this study suggests that the biological agent QST713 strain has potential application in banana production for promoting plant growth and modification of soil microbial communities, particularly in the TR4-infected field.

KEYWORDS

banana, Fusarium wilt of banana, *Bacillus amyloliquefaciens* QST713, microbial diversity and composition, growth promotion

Introduction

Banana is a perennial monocotyledonous plant and is cultivated in more than 130 countries around the world. Bananas are not only a fruit crop but also a crop with significant global trade income. China has a long cultivation history. A survey by the World Food and Agriculture Organization (FAO) in 2022 found that banana planting in China ranks second in the world (FAO) (Zou and Fan, 2022).

Diseases are significant restrictive factors in banana production, resulting in severe loss of banana yield and quality (Yip, 2017). Fusarium wilt of banana is a soil-borne disease caused by *Fusarium oxysporum* f. sp. *cubense*, (*Foc*). Particularly with the spread of Tropical Race 4 (*Foc* TR4), no banana varieties are immune to its infection, and Fusarium wilt of banana (FWB) has become the most disruptive disease in banana production (Ghag et al., 2015; Li et al., 2019). *Foc* TR4 can be spread in various ways (irrigation water, flowing air, soil, farming tools, etc.), and pathogenic spores can survive in infected soil for over 30 years (Ghag et al., 2015; Li et al., 2019). At present, various control measures have been tested against this disease, such as chemical treatments, disease-resistant varieties, biological control agents, plant quarantine, and cultural controls. Disease resistance breeding is the most effective method, but there is difficulty in implementing this due to the low diversity of genetic background relating to the triploid nature of most commercial banana cultivars. Although crop rotation and intercropping in agricultural production can effectively reduce the damage caused by *Foc* TR4, it is not practical in large-scale implementation (Tao, 2020). The utilization of chemical fertilizers and pesticides can cause environmental issues (Tao, 2020). Therefore, biological control technologies with the characteristics of safety, non-toxicity, and environmental protection have advantages in disease prevention and control (Ploetz, 2006).

Beneficial microorganisms that form mutual interactions with plants are generally considered to have some potential for disease control or development as microbial agents (He et al., 2021). Jayamohan et al. (2020) and Saravanan et al. (2003) found that *Pseudomonas fluorescens* had a high inhibitory activity against Fusarium wilt, and it could also stimulate systemic resistance and raise the ability of bananas to resist diseases. Cheng et al. (2005) also isolated *P. fluorescens* with strong inhibitory activity from banana soil, and further confirmed the inhibitory effect of this bacterium on Fusarium wilt. Fishal et al. (2010) found that the isolated endophytic bacterium UPMP3 (*Pseudomonas* sp.) could induce resistance to Fusarium wilt in susceptible banana varieties, indicating that it is a promising biocontrol agent. Therefore, the development and application of biocontrol agents for diseases have broad prospects (Card et al., 2016; Bubici et al., 2019).

Direct or indirect application of these microorganisms to agricultural production has revealed many successful cases of disease control and/or increase in productivity. Serenade family products are brands of Bayer, based on the *B. amyloliquefaciens* QST713 strain. This strain can produce IAA and 2-3 butanediol to promote plant growth and can quickly colonize the roots, establishing a dense biofilm as a protective barrier. QST713 strain can also activate plant self-defenses to suppress diseases. Studies have shown that 3-indoleacetic acid (IAA) can promote bacterial survival and effective colonization, thereby inhibiting fungal infection to protect plants (Lahlali et al., 2013; Rotolo et al., 2018; Samaras et al., 2021; Clarke et al., 2022). It has been demonstrated that QST713 has played a positive role in promoting cucumber growth, and after application (Bubici et al., 2019), it can resist diseases such as wheat stripe rust (Reiss and Jørgensen, 2017), rice sheath blight (*Rhizoctonia solani*), and leaf spot (*Cochliobolus miyabeanus*) (Harish et al., 2008). However, it is unclear whether QST713 could play the same role in biocontrol and growth promotion of banana plants in the same way as the crops mentioned above.

The application of biological control agents can establish beneficial strains and change the relative dominance of components of the rhizosphere microflora/microbial community, thus reducing pathogen colonization in the banana rhizosphere (Shen et al., 2015; De Vrieze et al., 2018; Bubici et al., 2019). The use of 16S rRNA gene sequencing to evaluate the microbial flora in soils, and thus to find the dominant flora, is a reliable method for studying its suppression of Fusarium wilt in banana (Caporaso et al., 2011). Therefore, QST713 was used to explore the biocontrol effects and growth promotion mechanisms of a biocontrol microorganism in banana plants in this study. We found that the introduction of QST713 not only directly promoted plant growth but also significantly enriched *Pseudomonas* and *Bacillus* in the soil microflora. The aim of this study was to enrich biological growth promoter resources and provide a theoretical foundation for a set of comprehensive measures for sustainable production in the banana industry.

Materials and methods

Culture medium and substrate

Potato dextrose agar (PDA) medium (potato 200 g, glucose 15 g, agar 15 g for 1 L) was used for fungal culture and activation. Nutrient agar (NA) medium (yeast extract 5 g, peptone 10 g, sodium chloride 10 g, agar 15 g for 1 L) was used for bacteria culture and activation. MS medium (Coolaber Science & Technology Co., Ltd., Beijing, China) was used for banana tissue culture seedlings propagation. Banana seedling substrate and coconut bran were purchased from Yuxi Letu Technology Co., Yuxi, China.

Banana cultivars, microbial agents, and pathogens

Resistant cultivar Yunjiao No. 1 (AAA) and susceptible cultivar Brazilian (AAA) propagated by the Agricultural Environmental and Resources Institute, Yunnan Academy of Agricultural Sciences, were used in this study to determine the effects of QST713 on bananas (Zhou et al., 2023). The tissue culture plantlets cultured at 25°C with 16 h/8 h (light/dark) for 20 days were transplanted to the seedling substrate for cultivation. When the banana plantlets had grown with 2–3 more new leaves, they were transplanted into plastic pots

(25 cm × 18.3 cm) containing transplanting soil in which the seedling substrate (Banana Planting Substrate, Yunnan Yuxi Leshi Technology Co. Ltd., Yunnan, China) was mixed with fresh soil (native horticultural red soil Ferralosols, which bananas have never been planted in previously) at a ratio of 1:1. One banana plant was planted in each pot. There were 50 banana plantlets in each treatment, and each treatment was replicated three times (Table 1). Routine fertilizer and watering were applied in greenhouse management.

Bacillus amyloliquefaciens strain QST713 was provided by Bayer's Crop Science Department (Leverkusen, Germany). When banana plantlets were transplanted and produced 6 to 7 leaves, a 100 mL QST713 solution (5 mL QST713 original agent labeled as EZU 1631901*1.0 + 95 mL water) was provided to each plant, the roots around the banana plant being drenched. QST713 was applied every 4 weeks, three times in total.

Foc TR4 previously isolated by our laboratory in Xishuangbanna, Yunnan Province was used in this experiment (Zhang et al., 2018, 2020). TR4 spore suspension was prepared with 1×10^7 CFU/mL after culturing in PDB medium at 28°C for 48 h. Seven days after the second application of QST713, the soil around each banana plant was drenched with 100 mL TR4 spore suspension (BTR4 + QST713, YTR4 + QST713). The TR4 treatments of Brazilian and Yunjiao No. 1 cultivar was inoculated with TR4 at the same time (BTR4, YTR4). The control treatments of Brazilian and Yunjiao No. 1 cultivar were inoculated with normal tap water (BCK, YCK). There were six different treatments in the experiment, each replicated three times. Each treatment was a total of 150 banana plants, 50 banana plants/treatment/replication (Table 1).

Investigation on banana growth traits

The banana pot experiment was carried out in the greenhouse of the Agricultural Environment and Resources Institute, Yunnan Academy of Agricultural Sciences. The indoor average illumination time was about 12 h, the average indoor temperature was 32°C (day)/22°C (night), and the average humidity was about 50%–60%. The banana growth traits (plant height, pseudostem girth, leaf number, leaf length, and leaf width) were investigated at 0 day, 28 days, 56 days and 97 days after the first application of QST713. The measurement of plant height was the range from the base of the pseudostem to the intersection of the newest two leaves. Pseudostem girth was measured as the pseudostem diameter 1 cm from the base using vernier calipers. The leaf number was counted as all functional leaves, and the length and width of the first expanded leaf were measured (Fan et al., 2021). After 62 days of TR4 inoculation, the fresh weights of the above and below ground parts of the banana plants were weighed.

TABLE 1 Treatments of the pot experiments.

Code of treatments	Description
BCK	Control in Brazilian cultivar with normal tap water
BTR4	Brazilian inoculated with TR4 in pot soil
BTR4 + QST713	QST713 drenched to Brazilian and inoculated with TR4 in pot soil
YCK	Control in Yunjiao No. 1 cultivar with normal tap water
YTR4	Yunjiao No. 1 inoculated with TR4 in pot soil
YTR4 + QST713	QST713 drenched to Yunjiao No. 1 and inoculated with TR4 in pot soil

Investigation on disease index of banana

In order to evaluate the efficacy of the QST713 agent for the control of banana wilt in pot experiments, we investigated the incidence on each plant 62 days post-inoculation with TR4 according to the lesion characteristics of bananas. The disease severity index (DSI) standards from Zhang's et al. (2018) protocol were referred to. DSI was graded from 0 to 4 according to the necrosis degree of the banana corm ("0" represented healthy plants with no internal symptoms and "4" represented severe wilting or that the plant had died).

Collection of banana rhizosphere soil samples

At 9 weeks (62 days) after inoculation with TR4, the rhizosphere soils of 15 plants of each treatment (CK, TR4, TR4 + QST713) were collected with a sterilized soil shovel. After pulling out the banana plant from the pot and shaking the roots vigorously, soil attached to the surface of the root was regarded as rhizosphere soil and was collected. Before collecting soil samples, tweezers were used to remove residual root system and other sundries from the collected mixed rhizosphere soil. Soil samples were stored at −20°C before DNA extraction. In total, 15-pot-plant soils were sampled, and three pots of soil were mixed into one sample (thus five soil sample replicates) for sequencing. Approximately 20 g rhizosphere soil was collected per plant.

Soil microbial genomic DNA extraction and detection

Total genomic DNA was extracted from soil samples using the OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, United States) following the manufacturer's instructions, and the isolated DNA was stored at −20°C. The concentration of extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), and the quality was checked by agarose gel electrophoresis.

PCR amplification and high-throughput sequencing

PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR amplification of the fungal ITS1 region was performed using the forward primer ITS5F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and the reverse primer ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′) (Caporaso et al., 2011; Kozich et al., 2013). The PCR contained 5 μL of buffer (5×), 0.25 μL of Fast

pfu DNA Polymerase (5 U/μL), 2 μL (2.5 mM) of dNTPs, 1 μL (10 μM) of each forward and reverse primer, 1 μL of DNA template, and 15.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 5 min, followed by 25 cycles consisting of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, with a final extension of 5 min at 72°C. The amplification products were sent to Personalbio (Personal Biotechnology Co., Shanghai, China) for high-throughput sequencing on the Illumina Miseq platform. Average length of the sequences was 337.77 for 16S rRNA genes, and 269.12 for ITS1 region. QIIME2 2019.4 (Bolyen et al., 2019) was used to performed microbiome bioinformatics. We finally obtained 249,376 ASVs for bacterial communities, and 20,109 ASVs for fungal communities. The sequential data of all samples were loaded into NCBI with accession numbers PRJNA 949124 (16SrRNA) and PRJNA949129 (ITS).

Data analysis

Kruskal Wallis rank sum test was used to test the difference between Chao1 and Shannon indices. Principal coordinates analysis (PCoA) was used to analyze the visual difference of different community compositions. Permutational MANOVA (PERMANOVA) was used to analyze the differences between community compositions. Co-occurrence patterns were reconstructed by Hmisc package in R and Gephi 0.9.2. Gephi 0.9.2 was used to calculate the modular index. If the Spearman's correlation coefficient was >0.70 and $p < 0.05$, then co-occurrence was considered robust (Gao et al., 2021). Network stability was measured by the proportion and modularity of negative or positive correlations. The correlation between growth and relative abundance of bacterial genera was calculated by R and visualized with the ggpubr package. Wilcoxon rank sum test was used to analyze the relative abundance of dominant bacteria in the bacterial community between two different treatments. Excel 2019 and IBM SPSS Statistic 25.0 software was used for data processing and statistical analysis, and univariate analysis (LSD and Duncan) in SPSS Statistic 25.0 software was used for variance analysis.

Results

Effect of QST713 on promoting banana plant growth

The results of our study showed that the microbial products have a significant effect on promoting plant growth. At 28 days post application (dpa) of QST713 on cultivar Brazilian, there were no significant difference among the CK, TR4, and TR4 + QST713 treatments in plant growth. At 56 dpa, the plant height (36.57 ± 0.79 cm), pseudostem girth (26.48 ± 0.51 mm), leaf length (36.07 ± 0.87 cm), and leaf width (18.00 ± 0.46 cm) of TR4 + QST713 treatments were significantly higher than CK (32.26 ± 1.03 cm, 24.36 ± 0.31 mm, 32.3 ± 1.24 cm, 16.16 ± 0.64 cm) and TR4 (31.36 ± 0.17 cm, 24.07 ± 0.19 mm, 32.69 ± 0.39 cm, 15.67 ± 0.23 cm) treatments. There was no significant difference in leaf number. At 97 dpa, the plant height (42.37 ± 1.36 cm) and pseudostem girth (32.15 ± 0.57 mm) of TR4 + QST713 treatment were significantly higher than the corresponding traits in the TR4 treatment (36.40 ± 0.93 cm, 29.37 ± 0.22 mm) and CK (37.94 ± 1.25 cm, 28.72 ± 1.32 mm), and the leaf length (37.03 ± 1.52 cm) and leaf width (16.82 ± 0.93 cm) were higher than TR4 treatment (32.38 ± 0.98 cm, 13.77 ± 0.06 cm) but not significantly different from the control (36.33 ± 1.65 cm 16.36 ± 0.83 cm) (Figures 1A, 2; Supplementary Figures S1A–S4A).

As for the resistant cultivar Yunjiao No. 1, there was the same trend as Brazilian. Twenty-eight days after the first application of QST713, there were no significant differences in plant growth of QST713 + TR4, TR4, and CK. At 56 dpa, the plant height (37.27 ± 1.22 cm), pseudostem thickness (26.66 ± 0.75 mm), leaf length (39.79 ± 0.87 cm), and leaf width (18.58 ± 0.28 cm) of QST713 + TR4 treatment were significantly higher than those of TR4 treatments (31.97 ± 0.16 cm, 24.79 ± 0.27 mm, 35.43 ± 0.89 cm, 15.86 ± 0.36 cm). At 97 dpa, the plant height (41.24 ± 2.19 cm), pseudostem thickness (33.37 ± 0.52 mm), leaf length (41.5 ± 1.76 cm), and leaf width (17.91 ± 0.67 cm) of the QST713 + TR4 treatment were significantly higher than those of TR4 (34.97 ± 0.46 cm, 29.95 ± 0.41 mm, 34.40 ± 2.47 cm, 15.25 ± 0.39 cm) and the control (35.90 ± 0.53 cm, 29.66 ± 0.51 mm, 38.4 ± 0.34 cm, 15.76 ± 0.25 cm), respectively (Figures 1B, 2; Supplementary Figures S1B–S4B).

The fresh weight of aerial biomass in QST713 + TR4 (Brazilian: 0.292 ± 0.017 kg, Yunjiao No. 1: 0.313 ± 0.026 kg) treatment was significantly higher than that of TR4 (Brazilian: 0.237 ± 0.013 kg, Yunjiao No. 1: 0.234 ± 0.006 kg) and the control treatments both in two cultivars. In terms of fresh weight of underground biomass (roots), QST713 + TR4 treatment of Yunjiao No. 1 (0.079 ± 0.001 kg) was significantly higher than that of TR4 (0.067 ± 0.005 kg) but showed no statistically significant difference from CK (0.069 ± 0.001 kg). In this respect, there were also no statistically significant differences between any treatments on cultivar Brazilian (Figure 3).

Disease investigation indicated that QST713 did not decrease the incidence rate of disease of Fusarium wilt of banana. The disease index of BTR4 + QST713 (55.20 ± 3.79) was not significant reduced compared to that of BTR4 (54.17 ± 0.52). The disease index of TTR4 + QST713 (30.73 ± 5.29) was not significant reduced compared to that of TTR4 (26.64 ± 2.61). It was noticed that the disease incidence on Yunjiao No. 1 was prominently lower than Brazilian, which reflects the differences in disease-resistant characteristics of these two cultivars (Supplementary Figures S5, S6).

Effect of bacterial and fungal community diversity

In order to determine the effect of QST713 on the soil microbial diversity (Chao1 and Shannon index) of two different banana cultivars (Brazilian and Yunjiao No. 1), we compared the alpha diversity of bacterial and fungal communities in rhizosphere soils. The results showed that there were no statistically significant differences in bacterial community richness (Chao1 index) between BCK, BTR4, and BTR4 + QST713 treatments in the Brazilian cultivar (Figure 4A and Supplementary Table S1). However, there was a statistical significance in bacterial community richness from YTR4 to YTR4 + QST713 treatments in Yunjiao No. 1 (Figure 4C and Supplementary Table S1). The Chao1 index of YTR4 + QST713 was significantly lower than YTR4. In addition, the Chao1 index of YTR4 + QST713 was significantly lower than that of YCK treatment (Figure 4C and Supplementary Table S1). However, there was no statistical significance in the diversity of the bacterial communities in either cultivar.

From the perspective of alpha diversity of fungal community, there was no significant difference in fungal community richness between BCK, BTR4, and BTR4 + QST713 in Brazilian cultivar

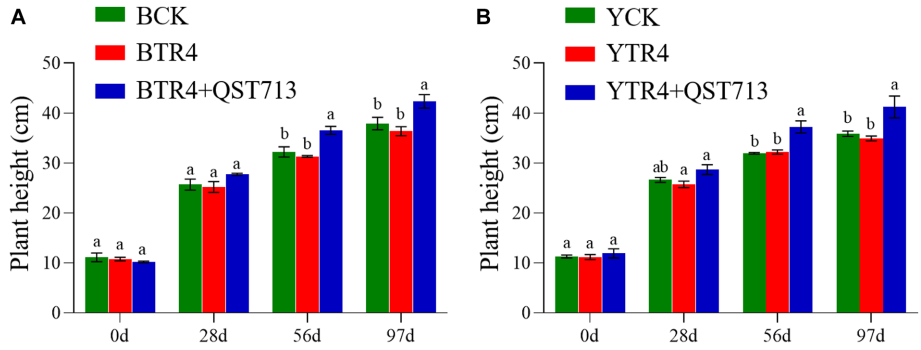

FIGURE 1
Growth promoting effect of QST713 on banana plant height. **(A)**The plant height of Brazilian. **(B)** The plant height of Yunjiao No. 1. Data are expressed as mean ± standard error. Data for different lowercase letters indicate a significant difference at the 0.05 level.

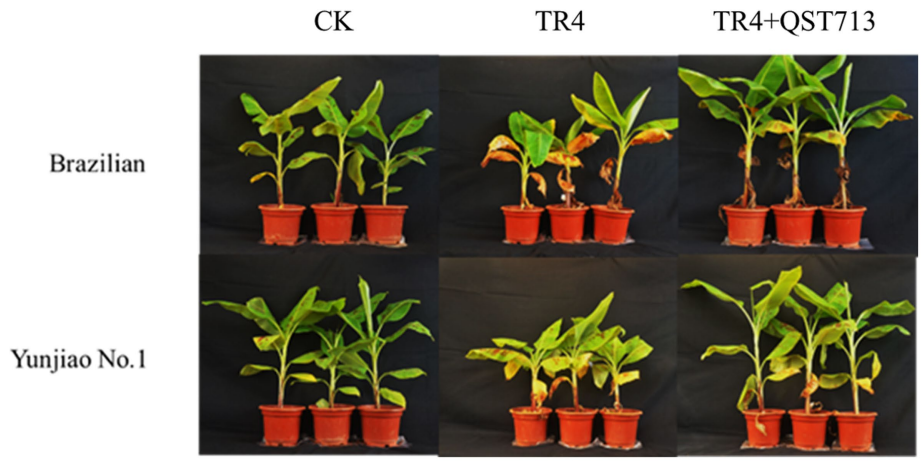

FIGURE 2
Effect of QST713 on promoting banana plant growth, 97 days after the first application of QST713.

(Figure 4B and Supplementary Table S1). However, in Yunjiao No. 1, compared with YCK treatment, YTR4 + QST713 and YTR4 treatments showed significantly higher fungal community richness (Chao1 index) (Figure 4D and Supplementary Table S1).

The results showed that there were no statistically significant differences in bacterial and fungal community diversity and richness (Chao1 and Shannon index) between BCK, BTR4, and BTR4 + QST713 treatments, and there were also no statistically significant differences in bacterial and fungal community diversity (Shannon index) between YCK, YTR4, and YTR4 + QST713 treatments. However, there were significantly differences in the richness of bacterial and fungal communities (Chao1 index) between YTR4 and YTR4 + QST713 treatments. The Chao1 index of YTR4 + QST713 was significantly lower than YTR4 and YCK in the bacterial community, and the Chao1 index of YCK was dramatically lower than YTR4 + QST713 and YTR4 in the fungal community.

Effect of bacterial and fungal community composition

PCoA and PERMANOVA were used to analyze the effects of QST713 application and pathogen TR4 on the bacterial and fungal communities of banana rhizosphere microbial. From the perspective of bacterial community composition in Brazilian, compared with BCK treatment, the composition of bacterial communities treated with BTR4 was statistically significant ($R^2 = 0.04$, $p < 0.05$) (Figure 5A). In addition, there were significant differences between BTR4 + QST713 and BCK treatments in bacterial communities ($R^2 = 0.06$, $p < 0.001$) (Figure 5C). BTR4 + QST713 treatment was also significantly different from BTR4 treatment ($R^2 = 0.06$, $p < 0.001$) (Figure 5B). Therefore, the application of QST713 changed the composition of the bacterial community additionally to the changes brought about by TR4 alone. These changes had the same tendency in resistant cultivar Yunjiao No. 1 (Figures 5D–F). The results showed that the application of QST713 and inoculation of TR4 could significantly change the rhizosphere soil bacterial community of banana plants.

From the point of view of fungal community composition, there were no statistically significant differences between the compositions of fungal communities for the Brazilian BTR4 vs. BCK treatments (Figure 6A, $p > 0.05$) or BTR4 vs. BTR4 + QST713 treatments (Figure 6B, $p > 0.05$). Compared with BCK treatment, there was a significant difference in BTR4 + QST713 treatment in the composition of fungal communities (Figure 6C, $R^2 = 0.08$, $p < 0.05$). As for Yunjiao No. 1, there were significant differences

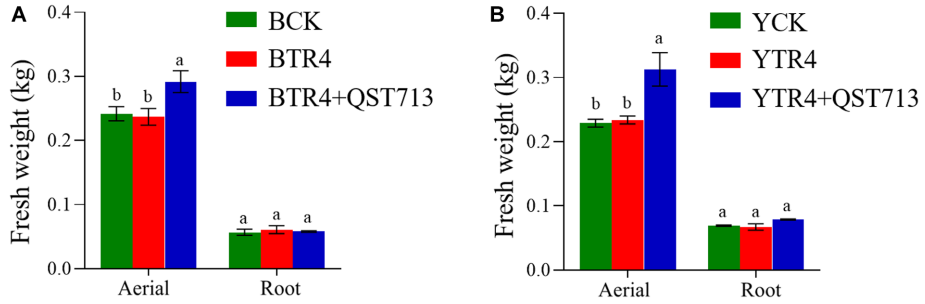

FIGURE 3
Growth-promoting effects of QST713 on banana plant fresh weight. **(A)** The fresh weight of Brazilian. **(B)** The fresh weight of Yunjiao No. 1. Data are expressed as mean ± standard error. Data for different lowercase letters indicate a significant difference at the 0.05 level.

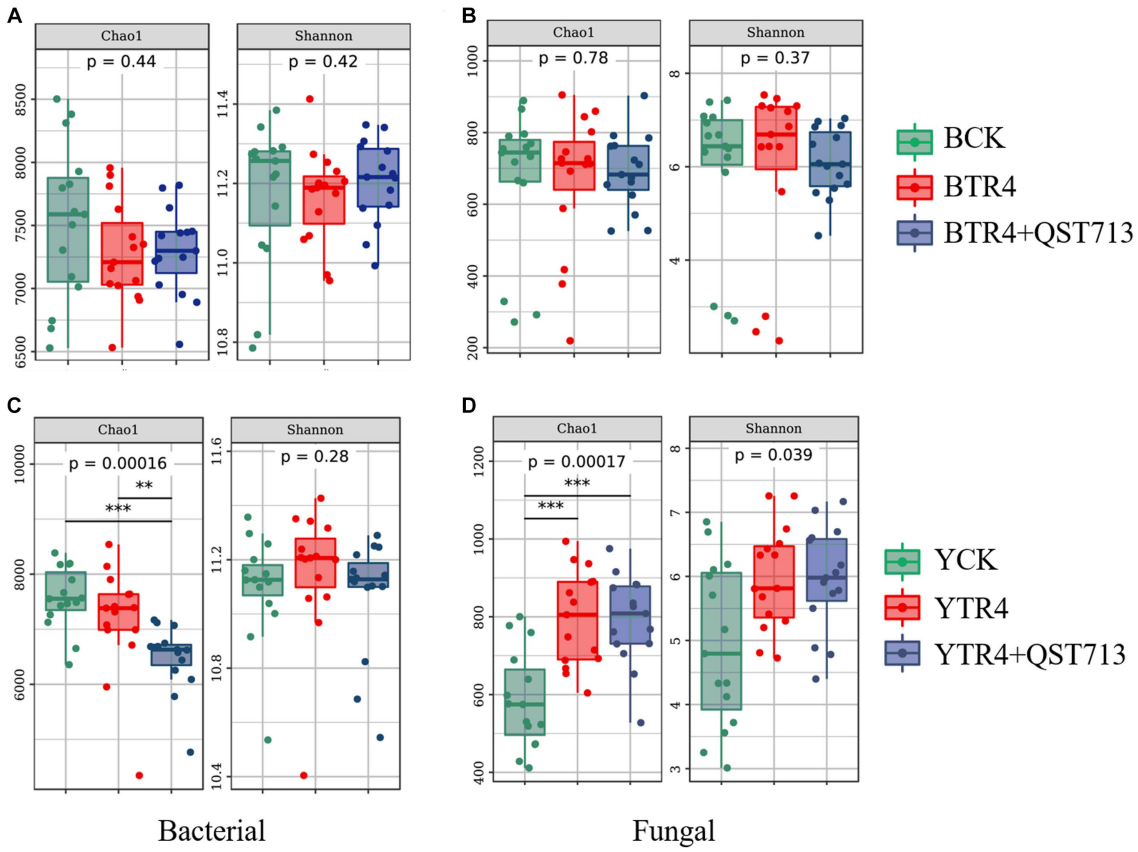

FIGURE 4
Alpha diversity analysis of microbial bacteria and fungi in banana rhizosphere soil. Alpha diversity in rhizosphere soil bacterial community of Brazilian **(A)** and Yunjiao No. 1 **(C)**. Alpha diversity in rhizosphere soil fungal communities of Brazilian **(B)** and Yunjiao No. 1 **(D)**. BCK represents the control in Brazilian cultivar. BTR4 represents the Brazilian inoculated with TR4. BTR4 + QST713 shows that QST713 was applied to Brazilian and inoculated with TR4. YCK represents the control in Yunjiao No. 1 cultivar. YTR4 represents Yunjiao No. 1 inoculated with TR4. YTR4 + QST713 represents QST713 applied to Yunjiao No. 1 and inoculated with TR4.

between YTR4 vs. YCK (Figure 6D, $R^2 = 0.08$, $p < 0.01$), YTR4 vs. YTR4 + QST713 (Figure 6E, $R^2 = 0.08$, $p < 0.001$), and YTR4 + QST713 vs. YCK (Figure 6F, $R^2 = 0.17$, $p < 0.001$). It can be seen that TR4 infection and QST713 application could both change the bacterial community composition of two banana cultivars, and the fungal community composition of Yunjiao No. 1 also changed significantly.

Effect of QST713 on maintaining the stability of rhizosphere soil microorganism community composition

We further used the negative correlation edges and modularity index to determine the stability of community network relationships (Figure 7; Supplementary Figure S7). The higher the ratio of

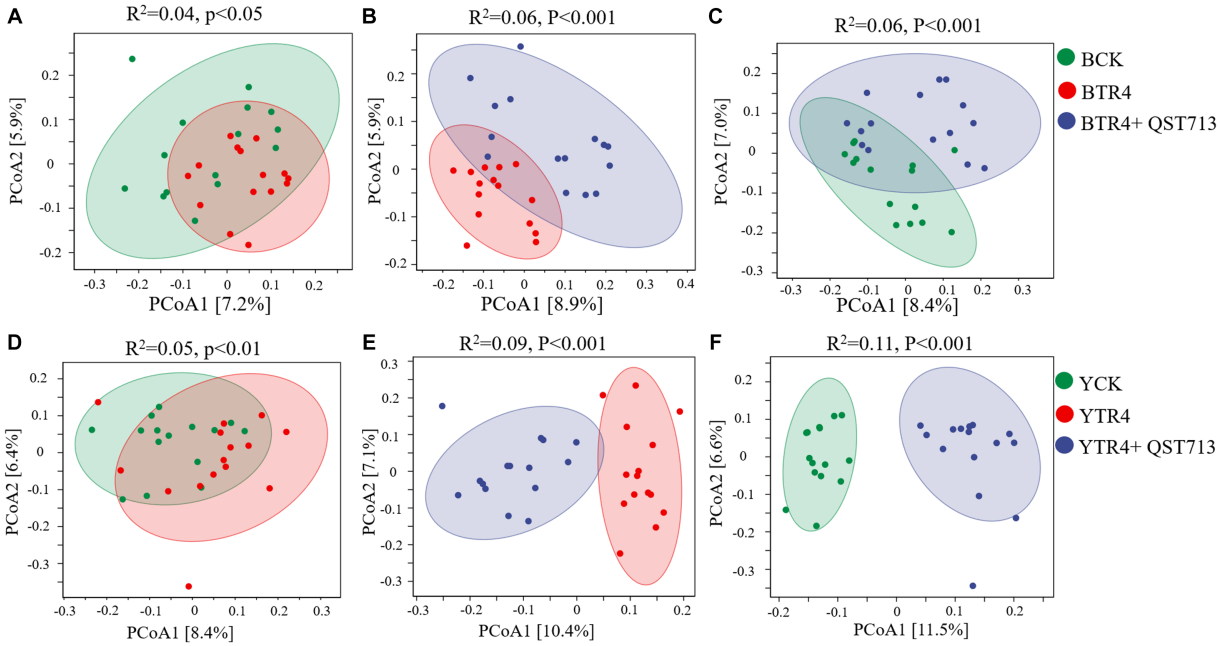

FIGURE 5
Principal coordinate analysis (PCoA) of bacterial community structure based on the Bray–Curtis distance metric among all soil samples. **(A)** Difference between CK vs. TR4 in bacterial communities' composition of Brazilian's soil rhizosphere. **(B)** Difference between TR4 vs. TR4 + QST713 in bacterial communities' composition of Brazilian's soil rhizosphere. **(C)** Difference between CK vs. TR4 + QST713 in bacterial communities' composition of Brazilian's soil rhizosphere. **(D)** Difference between CK vs. TR4 in bacterial communities' composition in Yunjiao No. 1's soil rhizosphere. **(E)** Difference between TR4 vs. TR4 + QST713 in bacterial communities' composition in Yunjiao No. 1's soil rhizosphere. **(F)** Difference between CK vs. TR4 + QST713 in bacterial communities' composition in Yunjiao No. 1's soil rhizosphere. R^2 represents differential explanation rate, *P* represents significance by Permutational MANOVA. Percentages in parentheses represent the variance explained by the respective axis.

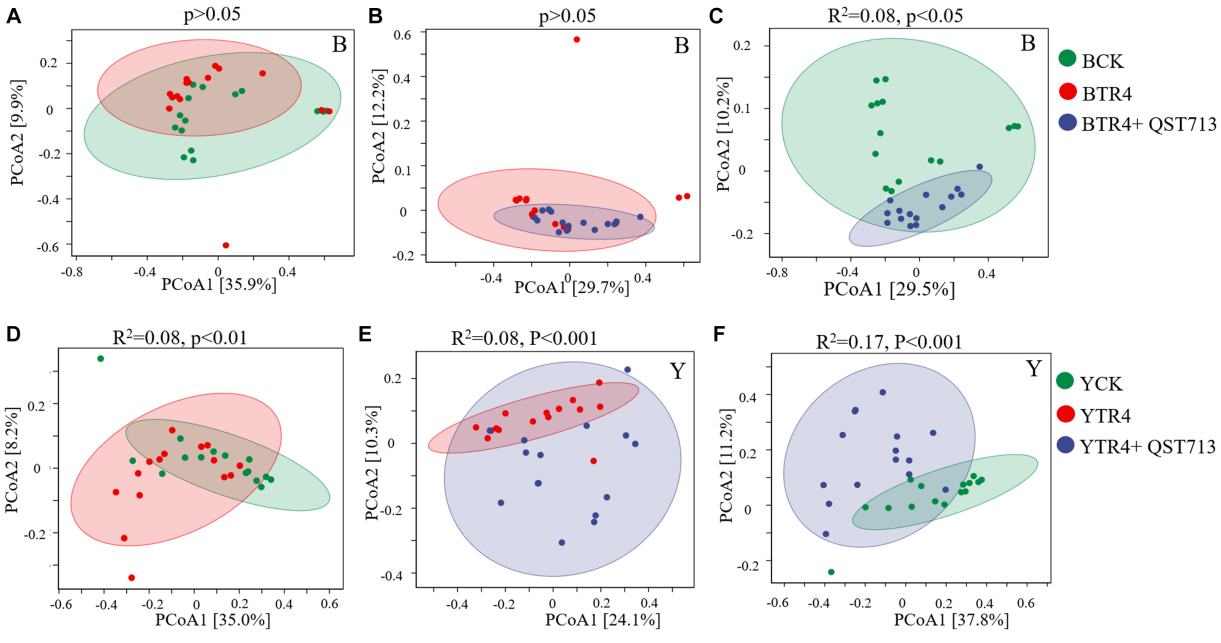

FIGURE 6
Fungal communities' composition of banana soil rhizosphere. **(A)** Difference between CK vs. TR4 in fungal communities' composition in Brazilian's soil rhizosphere. **(B)** Difference between TR4 vs. TR4 + QST713 in fungal communities' composition in Brazilian's soil rhizosphere. **(C)** Difference between CK vs. TR4 + QST713 in fungal communities' composition in Brazilian's soil rhizosphere. **(D)** Difference between CK vs. TR4 in fungal communities' composition in Yunjiao No. 1's soil rhizosphere. **(E)** Difference between TR4 vs. TR4 + QST713 in fungal communities' composition in Yunjiao No. 1's soil rhizosphere. **(F)** Difference between CK vs. TR4 + QST713 in fungal communities' composition in Yunjiao No. 1's soil rhizosphere. R^2 represents differential explanation rate by Permutational MANOVA, *P* represents significance. Percentages in parentheses represent the variance explained by the respective axis.

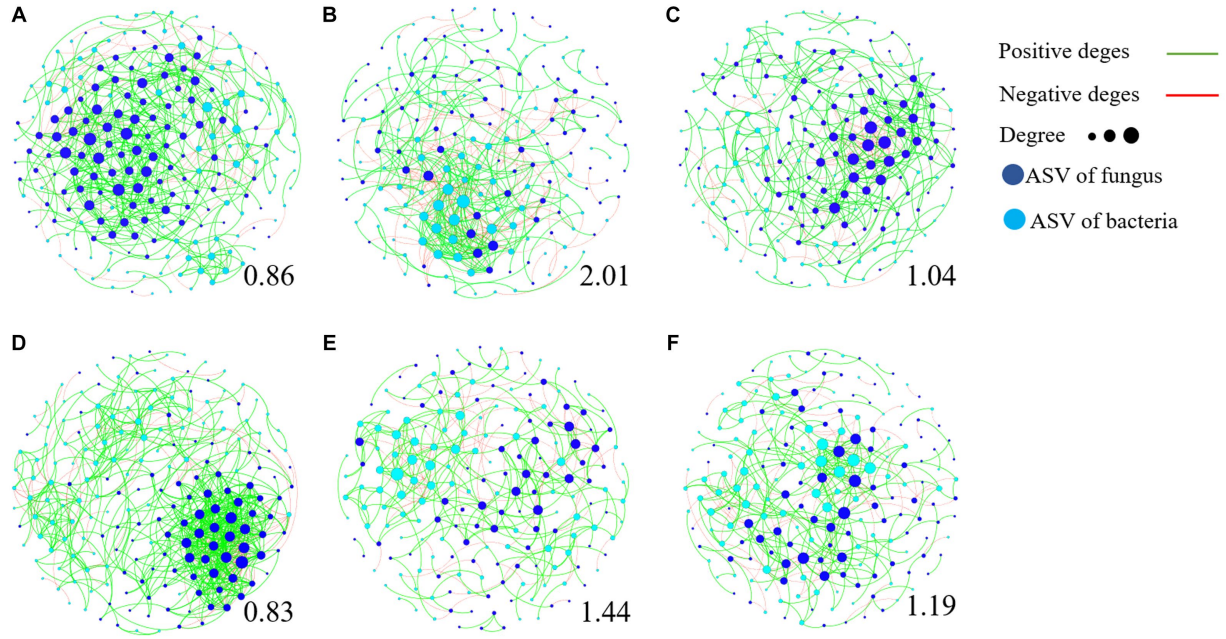

FIGURE 7
Transboundary network diagram of dominant amplicon sequence variant (ASV, relative abundance >0.1%). The dots represent ASVs, and the lines represent the relationship between ASVs. The red edges are negatively correlated, while the green edges are positively correlated. The dark blue dots are fungal ASV, while the light blue dots are bacterial ASV. The larger the dots are, the more ASV is associated with them, and the greater degree of connected edges. The number below each graph represents its modularity index. The higher the modularity index, the more stable the community structure. **(A)** BCK. **(B)** BTR4. **(C)** BTR4 + QST713. **(D)** YCK. **(E)** YTR4. **(F)** YTR4 + QST713.

negative correlation edges and modularity index, the more stable the community structure. The results showed that the ratio of modularity index and negative correlation edge of TR4 inoculation (Figures 7B,E) were significantly higher than that of CK treatment (Figures 7A,D), while the influence of TR4 + QST713 treatment lay between them (Figures 7C,F). This shows that ST713 has a significant effect on maintaining the stability of rhizosphere soil microorganism community composition in the presence of TR4.

Effect of QST713 on the specific genus of the rhizosphere soil microorganism community

The above diversity analysis showed that the bacterial community composition was significantly different in different treatments, but the fungal communities were significantly different between the two cultivars. The change of bacterial community could be the potential reason for the growth of the two cultivars. Therefore, Wilcoxon rank sum test of variance was used to analyze the relative abundance of dominant bacteria in the bacterial community between different treatments of the two cultivars, so as to identify the dominant bacteria with specific changes. The outcome of this study indicated that compared to the TR4 treatments, the bacteria of *Pseudomonas* and *Bacillus* were significantly up-regulated in TR4 + QST713 treatment (Figure 8), which may provide a key insight into the observed growth promotion effect on banana plants.

Linking the dominant bacteria with banana plant growth

In order to clarify the correlation between dominant bacteria (*Pseudomonas* and *Bacillus*) and plant growth effect, the correlation analysis of the relative abundance of dominant bacteria and banana growth parameters were conducted. The outcome of this study indicated that that the relative abundance of the two genera had a significant positive correlation with plant height, pseudostem girth, and above-ground fresh weight (Figure 9). It was the same for leaf length and leaf width but had no correlation with leaf number (Supplementary Figure S8). There was a low positive correlation between *Bacillus* and underground fresh weight (Supplementary Figure S8). Finally, we conclude that QST713 can significantly promote the growth of banana plants by altering the microbial community of bananas, especially by increasing the relative abundance of *Pseudomonas* and *Bacillus*.

Discussion

Plant rhizosphere growth promoting bacteria (PGPR) is an important component of biocontrol factors, which can effectively promote plant growth (Hu et al., 2004). Zheng et al. (2013) found that the application of biocontrol agents could significantly increase the number of plant roots and improve the microbial diversity of rhizosphere soil. Dan et al. (2018) showed that biocontrol bacteria can effectively promote the growth of melon seedlings and can significantly resist the growth of the pathogen of melon vine blight.

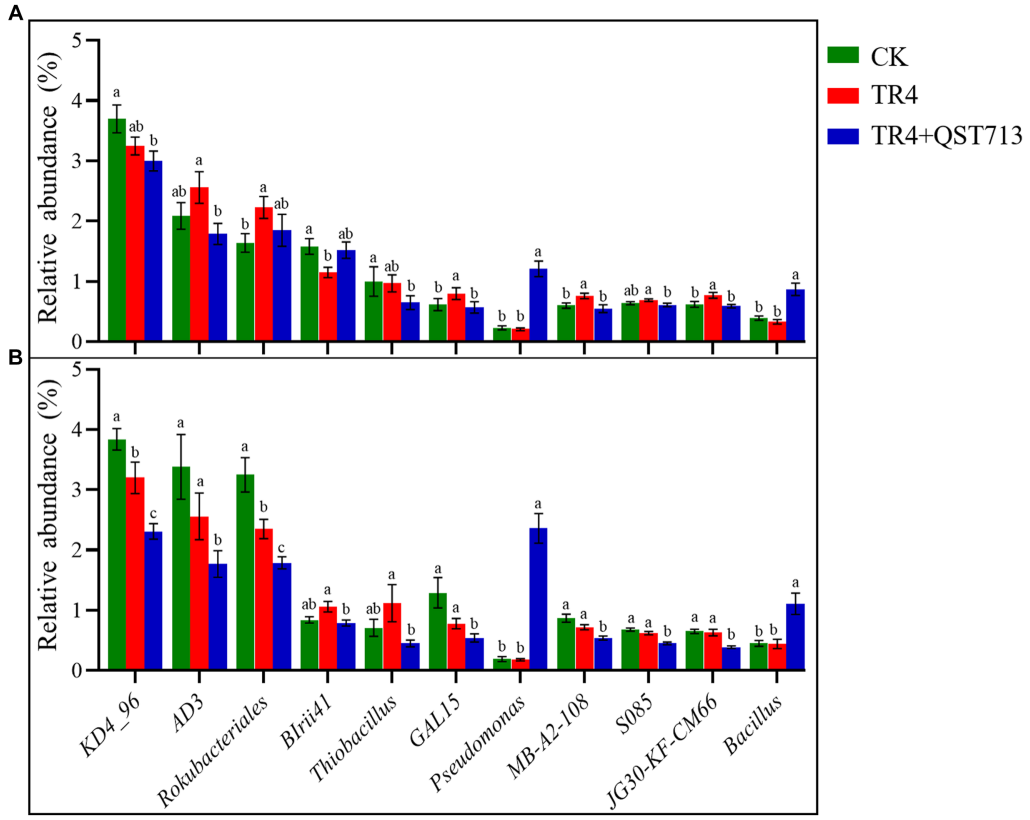

FIGURE 8
Significantly dominant bacterial genera (relative abundance >0.5%). **(A)** Significant genus of bacteria in banana rhizosphere in Brazilian cultivar. **(B)** Significant genus of bacteria in banana rhizosphere in Yunjiao No. 1 cultivar. Data with different lowercase letters indicate a significant difference at the 0.05 level.

B. amyloliquefaciens W19 was found to observably decrease the incidence of FWB and promote the growth of banana plants (Wang et al., 2013). Our study showed that the biocontrol agent QST713 could significantly promote the growth of bananas and has a significant effect on plant height, pseudostem girth, fresh weight, and other traits of bananas (Figures 1–3; Supplementary Figures S1–S4). However, through the symptom observation of banana corms, we found that QST713 had no clear control effect on TR4 under the current test conditions although there were clear differences in the level of resistance between the two cultivars, which was confirmed by Zhou et al. (2023) (Supplementary Figures S5, S6). Why did QST713 not suppress the disease in the current study? This could be due to the low dosage application of QST713 in this study. Why QST713 significantly promoted banana growth while there was no clear TR4 suppression in the current dosage application needs further investigation, with various dosage level applications, in the next step of our research. QST713 has also been shown to significantly induce disease resistance in canola, bean, grape, mushroom, and rice (Lahlali et al., 2013; Rotolo et al., 2018; Samaras et al., 2021; Clarke et al., 2022). The application of QST713 improved the "tolerance" of disease in bananas as evidenced by the better growth of leaves and pseudostems (Supplementary Figures S1–S4). Unfortunately, we did not find that QST713 had significant disease resistance against Fusarium wilt in banana with the applied dosage, and we need to monitor its performance at different dosage levels in banana plants in the next step of our research. We also need field trials to further study open field conditions. For the effect caused by QST713 application that triggered the dynamics of soil microorganisms, we measured the soil microbial community changes in bananas.

The soil microbial community is the most important functional component of soil biota. Microorganisms not only provide nutrients for the soil but also help plants adapt to the environment. They respond quickly to changes in soil microecological environment and stress, resulting in changes in microbial diversity and community structure (Chen et al., 2019). The role of biocontrol agents in field soil is greatly affected by the soil environment. It is of great important that new and effective biocontrol resources from different habitats are obtained for the control of Fusarium wilt in banana. There are interactions, such as antagonism, promotion, and coexistence, between soil microorganisms. Adding specific microorganisms to soil artificially would cause significant changes to the soil microbial community (Fan et al., 2021).

In this study, 16S rRNA of bacteria and ITS of fungi were sequenced through the Illumina platform to quantitatively analyze the diversity of soil bacterial and fungal communities. The results showed that the biocontrol agent (QST713 ASO) increased the relative abundance of certain microorganisms in soil bacterial and fungal communities, and significantly increased/the diversity of fungal communities, indicating that the application of this biocontrol agent led to the increase of soil bacterial and fungal species, and improved the diversity of bacterial and fungal communities (Figures 4–6). Notably, the application of QST713

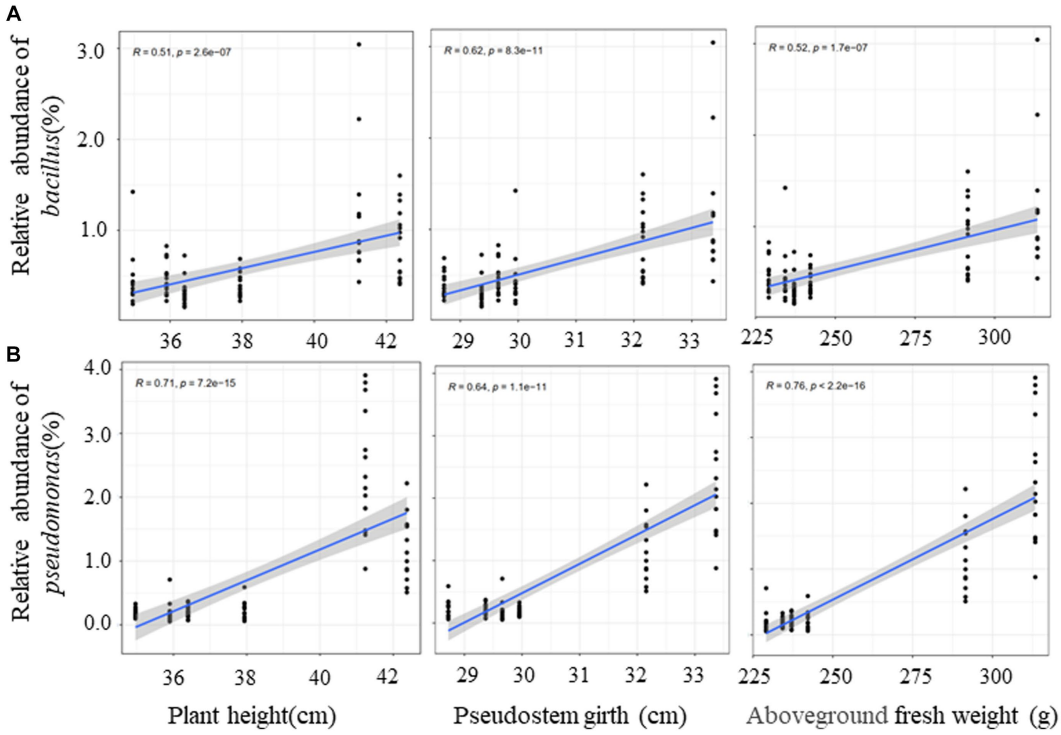

FIGURE 9
Correlation between dominant bacteria and growth traits of banana based on Spearman. The *y*-axis indicates the relative abundance of dominant bacteria, and the *x*-axis indicates the agronomic traits. Blue lines show linear regression. *R* represents differential explanation rate, *P* represents significance.

had varying effects on soil microorganisms depending on the banana cultivar. It can be seen that for the susceptible cultivar Brazilian, the inoculation/application of pathogen or QST713 did not affect the abundance and diversity of rhizosphere soil microorganisms (Figure 4), which may be related to its susceptible characteristics. After the application of QST713, the variation of microbial diversity of Brazilian rhizosphere was more stable (Figure 7). For the resistant cultivar Yunjiao No. 1 (Zhou et al., 2023), the inoculation of pathogens or QST713 application reduced the abundance of bacteria but increased the abundance of fungi (Figure 4), indicating that Yunjiao No. 1 somehow reacted more in the presence of a fungal pathogen, then influenced other fungi in the rhizosphere.

After application of the biocontrol agent, QST713, the composition of the soil microbial community changed significantly. The PERMANOVA analysis results showed that the bacterial and fungal communities of the control treatment were significantly different from those of the biocontrol agent QST713 application, indicating that the difference in soil microbial community structure might be one of the main factors regulating the banana plant growth. In addition, the PERMANOVA analysis results showed that the effect of inoculation of pathogens or QST713 application on the bacterial and fungal communities of resistant cultivar Yunjiao No. 1 is always greater than that on the susceptible cultivar Brazilian. This shows that the response of the microbiota of disease-resistant cultivars to interference is higher than that of susceptible cultivars, which indicates that disease-resistant cultivars can effectively adjust the response of the microbiota in the face of environmental changes. Our results clearly demonstrated that there is a significant interaction between host cultivar and pathogen inoculation or biocontrol agent application. Both cultivars showed the same trend in co-occurrence networks, possibly because TR4 inoculation disturbed the balance of the rhizosphere microbiome of the banana, and an increased microbe-mediated immune response disrupted the microenvironment required for normal growth. However, QST713 could reduce the disturbance caused by TR4 inoculation without changing the occurrence of disease and maintain a microbiome that was more conducive to the normal growth of banana.

The data from this study also indicate that after the application of QST713, the relative abundance of *Pseudomonas* and *Bacillus* increased significantly, and their abundance was positively correlated with the growth characteristics of bananas, indicating that the microbial abundance of these two genera (*Pseudomonas* and *Bacillus*) could be directly (or indirectly) connected to the observed enhanced growth of banana plants. Due to the fixed amount of QST713 for plant application and *Foc* TR4 for plant inoculation in this study, we did not quantify *Foc* TR4 and QST713 in pot soil. Therefore, we cannot analyze the correlation between *Foc* TR4 and *Pseudomonas* and *Bacillus*. However, *Pseudomonas* is widely found in nature and is an important part of soil micro-ecosystems. It participates in important metabolic activities such as carbon and nitrogen cycling in the environment and can biodegrade some environmental pollutants (Meng et al., 2022). Studies have shown that *Pseudomonas* species/strains can synthesize secondary metabolites such as siderophores and IAA to provide mineral nutrients for plants and regulate plant growth (Wang et al., 2012; Qian et al., 2022). Some researchers have also shown that it can induce systemic resistance and effective root colonization in plants (Luo, 2016; Qu et al., 2020; Tang, 2020). *Bacillus* species are also a very

important group of growth-promoting bacteria. We have isolated and identified one *Bacillus* strain, YN 1910, from banana-suppressive soil. *In vitro* and pot experiments from our study showed that this strain significantly suppressed pathogen and TR4 control effects (78.43%–81.76%) and also had a significant growth promotion effect on banana plants (Fan et al., 2023). At present, a number of "biological" products with a type of *Bacillus* as the main active ingredient have been commercialized. *B. amyloliquefaciens* products can be used as fungicides and plant growth promoters to control plant diseases and promote growth through root treatment. Auxin and similar metabolites such as cytokinin, zeatin, and abscisic acid have also been found in liquid culture (Idris et al., 2004). *B. amyloliquefaciens* BEB33 isolated by Yang et al. (2014) showed good biocontrol effect on wilt, and the strain could produce IAA and siderophore, which could promote the growth of banana plants and showed high biocontrol potential. In addition, we noticed that the relative abundance of *KD4-96* of the two cultivars decreased after inoculation with QST713. This kind of bacteria belongs to the *Chloroflexi*, which was reported to be positively correlated with plant growth (Chen et al., 2021), but there was little change compared with *Pseudomonas* and *Bacillus*.

The literature indicates that microbial species that normally promote plant growth may also play a role in plant disease resistance. In this study, the rhizosphere microbial diversity of banana soil was determined to identify species that changed significantly after the application of QST713. These species are considered important to biological control practice. This study also explained why QST713 can increase the tolerance of bananas to disease. QST713 not only directly promotes plant growth but also accumulates several beneficial microorganisms that may help in the long-term management of pathogenic bacteria or fungi. The mechanism behind this could be that the introduction of bacteria into the soil as key community members stimulates other potential growth-promoting species in indigenous beneficial microorganisms. These complex microbial communities could play a decisive role in promoting plant growth and health.

Conclusion

This study showed that the biocontrol agent *Bacillus amyloliquefaciens* QST713 strain could significantly promote the growth of banana in terms of plant height, pseudostem girth, and leaf traits of banana. Soil microbial diversity sequencing showed that the biocontrol agent *Bacillus amyloliquefaciens* QST713 could also significantly change the composition of the soil microbial community and significantly modify the diversity of the soil fungal community. Furthermore, the application of this biocontrol agent can significantly increase the abundance of *Pseudomonas* and *Bacillus*, and the abundance of these two genera is significantly and positively correlated with banana growth. The effect of QST713 on TR4 suppression with different applied dosages should be investigated, and we need to monitor its performance in banana plants with the application of different dosage levels in the next step of our research.

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

LT and PH: conceived, designed and performed the experiment, analyzed the data, and prepared the manuscript. S-JZ: conceived and designed the experiment, prepared and edited the manuscript, and supervised the research project and provided funding support. WZ, G-DZ, SL, YW, BY, TB, and HF: performed the experiment and analyzed the data. 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.2023.1216018/full#supplementary-material

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Secondary metabolite induced tolerance to *Fusarium oxysporum* f.sp. *cubense* TR4 in banana cv. Grand Naine through *in vitro* bio-immunization: a prospective research translation from induction to field tolerance

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An innovative tissue culture mediated incorporation of metabolite-based biomolecule (Bio-immune) at *in vitro* stage itself in banana cv. Grand Naine was developed and validated for the production of *Fusarium oxysporum* f.sp. *cubense* TR4 tolerant plantlets. The novel bio-immune formulation developed by us, exhibited a significant antifungal potency against *Foc* TR4 with a high percent inhibition (100%) at a 2.5% concentration of bio-immune on the 5th, 7th, and 9th DAI. Bio-immune integrated during *in vitro* shoot proliferation stage in banana cv. Grand Naine recorded significant enhancement in the growth of roots and shoots. Bio-immune (0.5%) fortified media produced 12.67 shoots per clump whereas control registered only 9.67 shoots per clump. Similarly, maximum root numbers (7.67) were observed in bio-immune plants which were significantly higher over control (5.0). The bio-immunized banana transplants recorded a higher survival rate (97.57%) during acclimatization as compared to the control (94.53%). Furthermore, evaluation of the bio-immunized plants in pot experiments revealed that unimmunized plants treated with *Foc*TR4 (TF) exhibited mortality between 60 and 90 days. On the 90th day after planting, a high mean disease severity index (DSI) of 3.45 was observed with unimmunized plantlets while the bio-immunized plants (TFBI) and ICAR-FUSICONT treated plants (TFTR) showed substantially reduced DSI (0.20 and 1.00) compared to *Foc*TR4 treated control (TF). Significant increases in polyphenol oxidase (PPO), peroxidase (POD), β-1,3-glucanase, phenylalanine ammonia-lyase (PAL), chitinase activities, and enhanced phenol contents were recorded in bio-immunized plants compared to unimmunized plants. Field experiments at two different locations in Bihar, India revealed that bunch weight, no. of hands/bunch, and no. of fingers/hand of bio-immune treated plants were significantly higher compared to the control.

KEYWORDS

***Fusarium oxysporum* f.sp. *cubense* TR4, banana, wilt disease, tissue culture, bioimmune, LC-MS, tolerance mechanism, field trails**

1 Introduction

Musa spp. bananas are a common economic crop grown in tropical and subtropical areas (Wang et al., 2021), and one of the most popular exported fruits that serve as a staple diet for >400 million individuals globally (Sandborn, 2022). The banana crop economy has endured significant losses owing to an outbreak of *Fusarium* wilt since 1860, caused by *Fusarium oxysporum* f.sp. *cubense* race 1 (*Foc* R1). The replacement of *Gros Michel* by Cavendish cultivars, resistant to *Foc* R1, solved the problem of Fusarium wilt on banana to a great extent (Buddenhagen, 1990; Ploetz, 2015). Intensive cultivation of Cavendish cultivars in the sub-tropics of South Africa, Australia under low temperatures (10 to 35°C) resulted in a *Foc* variant as Race4 showing extensive wilting (Su et al., 1986; Bubici et al., 2019). *Fusarium* wilt induced by *Foc* TR4 is the most damaging banana disease, restricting production worldwide (Ploetz and Pegg, 2000; Damodaran et al., 2020). Of late, there has been a severe epidemic of *Foc* TR4 in the northern part of India across provinces of Bihar and Uttar Pradesh, reducing banana production in one of the biggest banana-producing belts of the country (Damodaran, 2018). The herbaceous perennial nature of banana and the polycyclic behavior of disease contribute for huge epidemics there by causing dramatic economic losses with period of time (Ismaila et al., 2023). The colonization tendency of *Foc*TR4 was proliferating more promptly and with increased pathogenicity.

Many initiatives have been performed to mitigate the impact of *Fusarium* wilt worldwide. Several scientists throughout the globe have explored a variety of strategies for the management of *Foc*TR4 including chemical and biological methods, altering cultural practices, and developing tolerant or resistant lines through breeding. The capacity of *Foc* TR4 to persist without its host is a crucial issue impeding the effective treatment of this disease. Chemical treatment methods including soil soaking with fungicide and corm injection failed to offer long-term disease control, while their recurrent usage has also posed environmental issues (Getha and Vikineswary, 2002). Soil fumigants, flood fallowing, and agricultural sanitation are used to moderately manage a vast region (Ploetz et al., 1990). Feasibility of biological suppression of *Foc* TR4 pathogen using beneficial microbes such as *Trichoderma reesei* (Damodaran et al., 2020) and *Streptomyces* sp. (Cao et al., 2022) are considered as a viable option to check the pathogen spread for a specific period of time. Strengthening the biological control measures for long-term sustainability in the management of the disease needs supplemental research approaches that can induce tolerance in the host (Kavino et al., 2008).

Bio-priming is a process where the fixed concentration of an effective microbe with proven growth promotion and disease control characteristics are primed (treated) to the rooted tissue culture plantlets that come out of the laboratory for primary and secondary hardening in the nursery stage to develop tolerant and disease-free material (Reddy, 2012; Jebakumar and Selvarajan, 2018). It is carried out at the late primary hardening stage in the poly house and continues in the secondary hardening phase before the plant goes for planting. Bio-priming of tissue culture plantlets to induce tolerance during primary and secondary hardening stages using bioagents was attempted earlier (Kavino et al., 2007a; Deshmukh et al., 2020). However, the response at the field level over the period of time was variable (Damodaran et al., 2021).

With the growing need for developing eco-friendly alternatives to disease management, in the recent past, the identification of antifungal chemicals and metabolites from the rhizosphere and endophytic biome of plants have been used for inducing adaptive innate immune responses in plant systems against specific pathogens (Fadiji and Babalola, 2020). Chemical metabolites- based induction of immune responses against specific pathogens and developing acquired or induced disease resistance in plants is a new alternative area of research in terms of environmental safety. Bio-immunization is a process where the bio-active antifungal metabolites of microbes with antagonistic or growth-promoting properties are obtained through selective extraction and integrated in the Murashige & Skoog (MS) rooting media in the laboratory to the shoot tips that are to be initiated for rooting at a known concentration to aid in inducing the plant innate immune response to biotic stress. The present study provides a unique one such bio-immuno formulation obtained from *Foc* TR4 antagonist *Trichoderma reesei* (CSR-T-3) attempted to study the efficacy in the development of immuno-booster antifungal compounds in the plants while they are in the shooting and rooting phase under aseptic laboratory conditions without contaminating the media and not creating somaclonal variation between the plantlets grown in MS shooting and rooting media. This could be a plausible novel approach for combating the frightening scenario brought on by the unexpected breakout of *Foc* TR4. In this context, a novel technique for bio-immunization of the multiple shoots generated during the *in-vitro* mass multiplication of banana plants cv. Grand Naine was attempted through a patent-protected protocol in the current study to impart field tolerance to the toxins generated during the pathogenesis of the host by the pathogen.

2 Materials and methods

2.1 Collections of *Fusarium oxysporum* f.sp. *cubense* TR4 and *Trichoderma reesei*

The biocontrol agent *Trichoderma reesei* was obtained from the Soil Microbiology Laboratory, ICAR-Central Soil Salinity Research Institute, Regional Research Station, Lucknow, India. It was previously isolated from the rhizospheric soil of banana cultivar G9 grown in salt-affected soil of Uttar Pradesh, India (Damodaran et al., 2020) and reported to control *Fusarium* wilt disease of Bananas (Damodaran et al., 2020; Yadav et al., 2021). Similar to this, the virulent *Fusarium oxysporum* f.sp. *cubense* tropical race 4 strain was obtained from the Soil Microbiology Laboratory, ICAR-CSSRI, RRS, Lucknow, India, which had made the disease's first report of an epidemic in India (Damodaran et al., 2018). Pure cultures of isolates were maintained for further studies.

2.2 Development of bio-engineered biomolecule

A biomolecule formulation from *Trichoderma reesei* "BIO-IMMUNE" was developed and patented (Patent File No. 202111003761) and the process of *in vitro* bio-immunization for immunizing the banana tissue culture plantlets during *in vitro* organogenesis was also patented. Both the biomolecule and the technique have been jointly patented by ICAR-CSSRI and ICAR-CISH, Lucknow, India. The culture was inoculated in potato dextrose

broth (PDB) and incubated at 35°C for 72 h and the cell free extract (CFE) was collected. CFE was precipitated with alcohol, purified, characterized using LC-MS analysis (as described in Section 2.8), and fractionated for isolating the biomolecule. A series of experiments were conducted to standardize the percentage of liquid formulation to be integrated into the MS rooting medium for *in vitro*-bioimmunization. Around 0.5% of the formulation was integrated into the MS rooting medium based on the factor that the plantlets did not produce any somaclonal variations.

2.3 Antagonistic potential of bio-immune formulation on *Fusarium oxysporum* f.sp. *cubense* TR4

The antifungal efficiency of bio-immune formulation against *Foc*TR4 was estimated using the poisoned food technique. Bio-immune formulation was added to PDA medium at 0, 0.5, 1, 1.5, 2.0, and 2.5% concentrations individually. The plates were inoculated with *Foc*TR4. The plate with 0% concentration was considered as control. All the plates were incubated at 28 ± 2°C for 9 days, and the growth was observed on the 5th, 7th, and 9th day after inoculation (DAI).

The estimated percent growth inhibition was calculated according to the following equation:

$$\%\ Inhibition = \frac{(A-B)}{A} X\ 100$$

where,

A is the radial growth of *Foc* TR4 in control plates, and.

B is the radial growth of *Foc* TR4 in treated plates.

Three replicates of this *in vitro* antagonistic potential experiment were performed and the data were statistically evaluated using SPSS software utilizing analysis of variance (ANOVA) and Tukey HSD.

2.4 Effect of bio-immunization on *in vitro* rhizogenesis, shoot proliferation, and hardening of G-9 banana variety

The explant of G9 cultivars were collected from banana plants grown in the *Foc*tolerant region of Bihar. New suckers were treated with bavistin for about 30 min before being cut off at a length of around 3–5 cm and thoroughly cleaned for 10 to 15 min under running water. All traces of bavistin were removed completely by repeated washing beneath a flowing tap water 4–5 times, then with distilled water. Shoot tips were prepared by trimming the corm and outer leaf sheath from the suckers. These shoot tips were exposed to the $HgCl_2$0.1% solution for five minutes and washed with sterile distilled water 4–5 times under aseptic conditions. All explants were grown on MS media (Murashige and Skoog, 1962) and bio-immunized with a bioengineered tukey HSD ($p \leq 0.05$) biomolecule. The unimmunized plants were considered as control. The pH was adjusted to 5.7 before autoclaving. All culture bottles were incubated at 25 ± 2°C with cool white fluorescent tubes for a 16 h photoperiod of light and dark. The materials were sub-cultured in the same medium at regular intervals of 25 days in order to produce several shoots. For measuring the growth parameters, the plantlets were carefully taken out of the culture flasks, and the roots were washed gently under running tap water to remove any agar that had become attached to the roots. The growth parameters root length, no. of roots and shoots, no. of leaves, average leaf length, and the weight of roots and shoots when they are fresh and dry were measured. The samples were dried at 65°C for 96 h in a hot air oven to determine the dry weight of root and shoot.

For banana hardening, coco peat and soil were mixed in a 1:1 ratio and filled in plastic pro-trays for primary, then polythene bags of size 7*4 for the secondary hardening phase. The details of treatments used were T1 = bio-immunized plants and T2 = unimmunized (control) plants. The experiment was replicated six times having a complete randomized design.

Tap water was filled into glass jars containing tissue-cultured plantlets that were being grown in a rooting media of the respective treatments. Plantlets were carefully removed from culture bottles and placed inside a plastic tub filled with water to avoid wilting and excessive transpiration. Plant roots were meticulously freed from the nutritional medium. Banana explant was transferred in plastic pro-trays for primary hardening and was sprinkled with water every hour on the first day after transplanting and then every three hours for the next week. Plants were then marked with tags, and the necessary observations were taken. After 30–35 days single banana explants were transferred in each plastic bag of corresponding treatments for secondary hardening. For the initial hardening trials, the Gothic arch-style greenhouse was built using a UV-stabilized poly-sheet, a thermal shade net, thermometers, hygrometers, and lux meters to measure temperature, humidity, and light intensity. They need to undergo many physiological and anatomical modifications to establish themselves under greenhouse conditions. The platelets are progressively exposed to conditions of decreasing humidity (70%) and rising light intensity after being exposed to the maximum humidity (90%) and diffused light, respectively. To accomplish this, they were transferred to shade houses for secondary hardening.

For secondary hardening, a green shade net with a 50% light cutoff and micro-sprinklers were used for secondary hardening trials.

2.5 *In vivo* assay *Fusarium* wilt's suppression under pot conditions

Using a completely randomized design (CRD) with three replicates, a systematic pot experiment was conducted in June–July 2021 at the ICAR-CSSRI, RRS, Lucknow, India. For the pot experiment, clayey loam soil (pH 7.45, E.C. 0.32 dS/m, and organic carbon 3.0 g/kg) was taken from the Institute's research farm, sieved through a 20 mm sieve, and then sterilized in an autoclave at 121°C for 30 min for three consecutive days. The 5-day-old fungal culture was inoculated in potato dextrose broth (PDB), and then incubated for an additional 5 days at 28°C in an incubated shaker to create a fungal pathogen culture. The broth was filtered through two layers of cheesecloth to produce spore suspension. Using a hemocytometer, the filtrate spore count was changed from 10^5 to 10^6. About 100 mL of the diluted filtrate was poured into sterile soil. For the experiment, uniform-height secondary hardened banana plantlets of the G9 variety (susceptible and bio-immunized) were chosen. The plants were immersed for 30 min in pathogen spore suspension containing 10^6

conidia/ml before planting, as described by Pérez-Vicente et al. (2014). Four treatments *viz.*, T1 = bio-immunized plants with *Foc* TR4 (TFBI), T2 = unimmunized plants with *Foc* TR4 treated with ICAR-FUSICONT (TFTR), T3 = unimmunized plants with *Foc* TR4 (TF), and T4 = unimmunized plants without *Foc* TR4 (TC). The experiment was designed in a complete randomized form. At 0 and 90 days after planting, the phenological indicators banana plantlets, such as plant height (cm), plant girth (cm), and no. of leaves were observed. The disease scale-based scoring was done as described for *Foc* TR4 scoring by Damodaran et al. (2020). Analysis of variance (ANOVA) was used to statistically examine the data, and Duncan's multiple range tests ($p < 0.05$) were used to compare means.

2.6 Biochemical analysis of defense-related enzymes and phenol content

One gram of third leaf samples was homogenized in 2 mL of 0.1 M sodium citrate buffer (pH 5.0) and centrifuged for 20 min at 4°C at 10,000 rpm. The supernatant containing crude enzyme extract was used for testing the activity of chitinase (Boller and Mauch, 1988), and β- 1, 3-glucanase (Pan et al., 1991).

For the assessment of peroxidase (PO) method from Hammerschmidt et al. (1982), phenylalanine ammonia lyase (PAL) method from Ross and Sederoff (1992), and polyphenol oxidase (PPO) protocol by Mayer et al. (1965) were adopted. The enzymes were extracted in 0.1 M sodium phosphate buffer (pH 7.0). Using Zieslin and Ben-Zaken (1993) methodology, the total phenol content was estimated and expressed in terms of catechol equivalents g^{-1} of protein. The assays were carried out for the T1, T2, T3 and T4 treatments.

2.7 SEM analysis of bio-immunized and unimmunized control plants of banana

Scanning electron microscopy of the bio-immunized and unimmunized banana plants' root sections was done as per the protocol described by Kumar et al. (2018). Sections of the sucker area adjoining the roots were fixed in 3% glutaraldehyde dissolved in 0.1 M phosphate buffer (PH 7.0). The specimens were dehydrated using acetone at a series of concentrations (30 to 90% in increments of 10%) for 20 min, dried for 30 min, and mounted on a steel stub with double-sided carbon tape. The samples were finally coated with a film of gold–palladium alloy under vacuum and observed with a scanning electron microscope (SEM, Fei Quanta 200) at Babasaheb Bhimrao Ambedkar University, Lucknow, India.

2.8 Liquid chromatography coupled with mass spectrometric analysis of the leaf samples of bio-immunized and unimmunized control banana plants

Four different treatments *viz.*, T1 = bio-immunized plants with *Foc* TR4 (TFBI), T2 = unimmunized plants with *Foc* TR4 treated with ICAR-FUSICONT (TFTR), T3 = unimmunized plants with *Foc*TR4 (TF), and T4 = unimmunized plants without *Foc* TR4 (TC) plant samples underwent LC-MS analysis, to find key chemical components causing host tolerance in the pot trial. The freshly harvested leaves were chopped into little pieces, properly cleaned under running water, rinsed with sterile distilled water, and then dried in the shade for 15 to 20 days at room temperature. For LC-MS analysis 100 g of dry weight plant material was individually extracted with ethanol and ethyl acetate solvents (400 mL) overnight at room temperature. The Whatmann No. 1 filter paper was used to filter all the LC-MS extracts and was concentrated using a rotary evaporator. A small portion of the prepared plant extracts were collected in Eppendorf and sent to CSIR-Central Drug Research Institute, Lucknow, India for further LC-MS analysis.

The ESI-LC-MS of the TLC purified samples were analyzed using a Micromass Quattro II triple quadrupole mass spectrometer with a JASCO PU-980 HPLC Pump at CSIR-Central Drug Research Institute, Sophisticated Analytical Instrumentation Facility (SAIF), Lucknow, Uttar Pradesh, India for analysis. The water absorption (250_4.6mm_5I) column was used with acetonitrile: water +0.1% formic acid solvent system, Gradient elution was performed at 1.0 mL/min. The photodiode array was monitored at 200–650 nm and recorded at 220 nm. The mass spectra were scanned in the range 80–1,000 DA in 2.5 S. The ESO capillary was set at 3.5 kv and the cone voltage at 40 V. The m/z spectral chromatographs were analyzed, key metabolites were predicted based on already published reports, m/z database and presented for comparative analysis of the status of secondary metabolites in the banana leaf samples collected from bio-immunized and unimmunized grand nine plants both grown in similar sodic soil conditions. Key metabolites were identified based on previously published data and tabulated for comparison analysis between samples of all four treatments using the m/z spectral chromatographs.

2.9 Field study for evaluation of bio-immunized plants on disease-affected hotspots of Bihar

Field research was done as Inter-institutional research project of ICAR at two locations Nirpur, Purnia district (25° 43′ 38.626 N, 87° 08′ 18.455 E) and Dighari, Katihar district (Bihar, India) (25° 35′ 38.872 N, 87° 29′ 55.824 E) during 2020–21, 2021–22 to evaluate the field-level efficiency of bio-immunized banana plantlets in controlling *Fusarium* wilt. The selection of study area was based on the severity of the disease. Previously the technological intervention using ICAR-FUSICONT in one of the two locations i.e. Dighari of Katihar district was made through the formation of community groups of the cultivators from the affected areas. In the tissue culture facility of the ICAR-CSSRI, RRS, Lucknow, Uttar Pradesh, bio-immunized disease-free banana plantlets of cv. Grand Naine (G-9) were grown, and 50 plants per replication were utilized for each treatment. The four treatments were, T1- (TBI) treatment includes bio-immunized G-9 banana plantlets planted in a *Foc* infested field, T2- (TTR) includes un-immunized banana G-9 plantlets grown in *Foc* infected field and treated with the ICAR-FUSICONT (*Trichoderma reesei*

(CSR-T-3)), T3- (TBITR) includes bio-immunized G-9 banana plantlets grown in a *Foc* infected field treated with the ICAR-FUSICONT, and T4- (TC) included un-immunized G-9 banana plantlets grown in a *Foc* infected field. After five and nine months of planting, the plants under the treatment T2 and T3 were inoculated with 500 mL/plant with 3% ICAR-FUSICONT formulation. Using IBM SPSS statistical software, the recorded growth and yield data were subjected to an ANOVA test ($p < 0.05$) for each parameter. The disease incidence was calculated based on the symptoms that appeared on diseased plants. The disease incidence was calculated by: $DI = \frac{n}{N} X\,100$.

where, DI – Disease incidence; n = no. of plants diseased; N = total no. of plants observed.

3 Results

3.1 *In vitro* bioassay of bio-immune formulation against *Foc*TR4

Bio-immune formulation exhibited a significant antifungal potency ($p \leq 0.05$) against the *Foc* TR4 at different concentrations, compared with the control. The inhibition zone diameter ranged between 2.70 cm – 6.03 cm in different concentrations. On the 9th day after inoculation, the maximum radial growth (6.03 cm) was observed at a concentration of 0.5%, while minimum radial growth (0.47 cm) was recorded at 2.0% concentration (Table 1). Whereas no radial growth was observed in *Foc* TR4 at a 2.5% concentration of the bio-immune formulation, the data showed that with the increase in the concentration of the bio-immune formulation the percent of inhibition of *Foc*TR4significantly ($p \leq 0.05$) increases. The highest percent inhibition (100%) was observed at 2.5% concentration of bio-immune on the 5th, 7th, and 9th DAI followed by 100% on the 5th DAI, 96% on the 7th DAI, and 94% on the 9th DAI at 2.0% concentration, respectively, (Figure 1). It was observed that minimum concentration (0.5%) significantly ($p \leq 0.05$) reduced the growth of *Foc* TR4 by 44% (5th DAI), 42% (7th DAI), and 25% (9th DAI) respectively.

3.2 Effect of bio-immunization on *in-vitro* rhizogenesis, shoot proliferation, and hardening stages of tissue culture banana plants of G-9 banana variety

The result, which is represented in Table 1, makes it clearly evident that the biomolecule was highly effective in promoting the *in vitro roots* and shoot growth of banana plantlets. The highest significant ($p \leq 0.05$) values for root length (4.63 cm), number of leaves (4.37), roots (7.67), fresh root weight 59.67 mg, and dried root weight traits (12.67 mg), were observed in bio-engineered tissue culture banana plantlets compared to control for the same traits (3.73 cm, 3.70, 5.00, 39.60 mg, and 6.67 mg, respectively).

Similar to this, the results indicated that mean shoot proliferation rates of bio-engineered biomolecule were significantly ($p \leq 0.05$) higher after the basal cycle. The bio-engineered banana tissue culture plantlets showed significantly ($p \leq 0.05$) higher values (12.67, 3.43 cm, 2.03 mg, and 0.13 mg) for no. of shoots, average leaf length, fresh root weight, and shoot dry weight, respectively, compared to control 9.67, 5.43 cm, 3.70 mg, and 0.30 mg.

Before transplanting the banana tissue culture for the hardening process, the soil's pH and Ec were observed to be 7.6 and 0.13 ds/m, respectively. During the primary hardening, some growth metrics were assessed for banana transplants (Table 2). Overall, all observed vegetative growth metrics included, plant height (12.53 cm), root length (10.07 cm), plant girth (2.47 cm), no. of primary roots (6.47), no. of leaves (6.27), and leaf area (125.40 cm^2) were significantly ($p \leq 0.05$) higher in bio-immunized banana transplants compared to control by 28.17, 6.65, 21.86, 14.99, 14.35, and 6.56%, respectively. The survival rate (%) for the initially acclimated plantlets is the most crucial variable since it shows how well the bio-engineered biomolecule assists the primary acclimation of the banana

TABLE 1 Impact of *in-vitro* bio-immunization on rhizogenesis and shoot proliferation under laboratory.

Variables	Rhizogenesis		
	Bio-immunization	Control	*F value*
Root length (cm)	4.63 (±0.51)	3.73 (±0.21)	7.924
Number of leaves	4.37 (±0.42)	3.70 (±0.61)	2.689
Number of roots	7.67 (±1.21)	5.00 (±0.87)	9.595
Root fresh wt. (mg)	59.67 (±4.04)	39.60 (±6.22)	21.959
Root dry wt. (mg)	12.67 (±1.53)	6.67 (±1.06)	31.167

Variables	Shoot proliferation		
	Bio-immunization	Control	*F value*
Number of shoots	12.67 (±0.86)	9.67 (±1.70)	7.404
Avg. leaf length (cm)	3.43 (±0.40)	5.43 (±0.67)	19.780
Shoot fresh wt. (mg)	2.03 (±0.57)	3.70 (±0.36)	18.382
Shoot dry wt. (mg)	0.13 (±0.17)	0.33 (±0.03)	80.00

Each treatment was the mean of three replicates (bottles) per treatment with five roots/shoots per replicate. Values indicate the mean ± SD. There is significant difference between the treatment at $p < 0.05$.

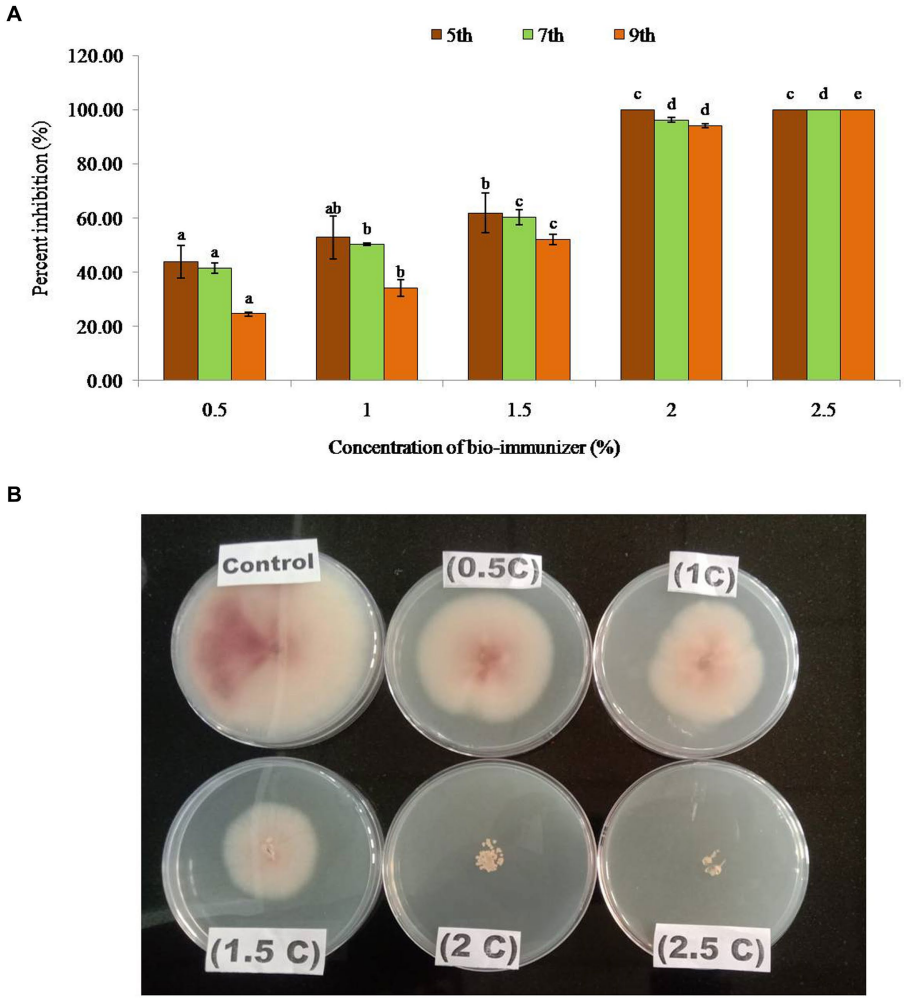

FIGURE 1
(A) Percent inhibition of bio-immunizer on the radial growth of *Fusarium oxysporum* f.sp. *cubense* TR4 under *in vitro* conditions. Values with standard deviation (mean ± SD) are displayed for $n = 3$. Bar graph with different lowercase letters is used to indicate significantly different values by Tukey HSD ($p \leq 0.05$). **(B)** Effect of bio-immunizer of the radial growth of *Fusarium oxysporum* f.sp. *cubense* TR4 under *in vitro* condition. This study evaluated the antifungal efficiency of a bio-immune formulation against *Fusarium oxysporum* f.sp. *cubense* TR4, utilizing the poisoned food technique. Different concentrations of the bio-immune formulation (0 to 2.5%) were individually incorporated into Potato Dextrose Agar (PDA) medium, and were subsequently point inoculated with *Foc* TR4, with the 0% as the control. The plates were incubated for 9 days at 28 ± 2°C, and the fungal growth was monitored on the 5th, 7th, and 9th day after inoculation (DAI). Results indicated a significant antifungal potency ($p \leq 0.05$) of the bio-immune formulation against *Foc* TR4 at various concentrations compared to the control. The observed inhibition zone diameter ranged from 2.70 cm to 6.03 cm. On the 9th DAI, the highest radial growth (6.03 cm) was observed at a concentration of 0.5%, while the minimum radial growth (0.47 cm) was recorded at the 2.0% concentration. Remarkably, no radial growth of *Foc* TR4 was observed at the 2.5% concentration of the bio-immune formulation. Moreover, the data exhibited a concentration dependent relationship, whereby an increase in the bio-immune formulation concentration resulted in a significant ($p \leq 0.05$) augmentation in the percent inhibition of *Foc* TR4. Notably, the highest percent inhibition (100%) was observed at the 2.5% concentration of the bio-immune formulation, followed by the 2.0% concentration, respectively. These findings showed that the bio-immune formulation possesses strong antifungal activity against *Foc* TR4, with potential implications for the management of Fusarium wilt disease in bananas.

transplants. Banana transplants that have been acclimated had a greater survival rate of up to 97.57% for the bio-immunized banana transplants compared to the control (94.53%) respectively.

Similarly, during secondary hardening, bio-engineered biomolecule significantly ($p \leq 0.05$) enhanced all the vegetative growth parameters (Table 3) *viz.* plant height (19.66%), root length (27.60%), plant girth (22.91%), no. of primary roots (7.28%), no. of leaves (4.22%), and leaf area (38.39%), respectively, compared to control. Likewise, the survival rate in unimmunized (control) plants (89.97%) was low compared to bio-immunized (93.23%) plants by 3.50%, respectively.

3.3 *In vivo* evaluation of bio-immunized plants under pot conditions against *fusarium* wilt

The use of bio-immunized plants considerably accelerates banana plant development, as seen by an increase in height and girth of the banana plants as compared to the control, in addition to suppressing *Fusarium* wilt (Table 2; Figure 2).

The ultimate height and girth immunized plants (TFBI) were measured to be 38.54 cm and 5.80 cm, respectively, were higher than those of the ICAR-FUSICONT-treated plants (TFTR) (34.78 cm

TABLE 2 Impact of *in-vitro* bio-immunization on growth of primary and secondary hardening banana tissue culture plantlets.

Variables	Primary hardening		
	Bio-immunization	Control	*F value*
Survival (%)	97.57 (±0.49)	94.53 (±1.17)	17.180
Plant height (cm)	12.53 (±3.02)	9.00 (±0.44)	4.030
Root length (cm)	10.07 (±0.31)	9.40 (±0.26)	8.163
Plant girth (cm)	2.47 (±0.21)	1.93 (±0.12)	15.059
Number of primary roots	6.47 (±0.38)	5.50 (±0.35)	10.646
Number of leaves	6.27 (±0.15)	5.37 (±0.40)	13.018
Leaf area (cm^2)	125.40 (±5.06)	117.17 (±3.79)	5.081

Variables	Secondary hardening		
	Bio-immunization	Control	*F value*
Survival (%)	93.23 (±1.07)	89.97 (±0.12)	27.560
Plant height (cm)	19.33 (±5.16)	15.53 (±1.27)	1.534
Root length (cm)	22.10 (±1.39)	16.00 (±0.60)	48.747
Plant girth (cm)	4.67 (±0.25)	3.60 (±0.20)	33.032
Number of primary roots	10.57 (±1.31)	9.80 (±0.10)	1.029
Number of leaves	6.40 (±0.17)	6.13 (±0.12)	4.923
Leaf area (cm^2)	265 0.93 (±2.63)	163.83 (±3.62)	1562.602

Values are the means of three replicates with sample size $n = 5$. Values indicates the mean ± SD. There is significant difference among the treatments at $p < 0.05$.

and 5.22 cm), *Foc*TR4 treated control (TF) plants (31.34 cm and 3.74 cm), and untreated control (TC) plants (26.90 cm and 3.06 cm) respectively. Remarkably ICAR-FUSICONT-treated plants showed a significant ($p \leq 0.05$) difference in percent increment in plant height and girth (TFTR) (20.43 and 28.46%) followed by bio-immunized plants (TFBI) (18.92 and 28.39%). The higher no. of leaves (7.00) was recorded in bio-immunized plants (TFBI) followed by untreated control plants (TC) 6.80, ICAR-FUSICONT treated plants (TFTR) 5.80 and *Foc* TR4 treated control (TF) plants 4.20.

During one month of planting in the control treated with *Foc*TR4 plants, the disease progressed and the early symptoms of older leaf yellowing appeared in the infected plants. With time, unimmunized plants inoculated with *Foc* TR4 (TF) exhibited mortality between 60 to 90 days. At 90th day after planting, the treatment TF showed a significant ($p \leq 0.05$) high mean disease severity index (DSI) of 3.45 whereas, the bio-immunized plants (TFBI) and plants treated with ICAR-FUSICONT (TFTR) showed significant ($p \leq 0.05$) lower DSI (0.20 and 1.00) compared to *Foc* TR4 treated control (TF) (Table 2).

3.4 Analysis of defense-related enzymes and phenol content

Comparing bio-immunized plants to unimmunized plants, a significant increase in the production of all the analyzed enzymes, including phenylalanine ammonia lyase (PAL), β-1,3- glucanase, polyphenol oxidase (PPO), peroxidase (POD), phenol, and chitinase was observed (Figure 3). However, compared to the *Foc* infected hybrids, the control (uninoculated) plants usually had reduced enzyme activity. The enhanced antioxidant enzyme activities and PR proteins in Foc inoculated plants showed that the *Foc* concentration led to stress, which was detrimental to plant cells and increased antioxidant and reactive active scavenging systems indicating activation of host's defense mechanism.

The POD enzyme activity in banana leaves was significantly different among the four treatments ($F = 599.26$, df = 3, 8, $p = 0.00$). Both the treatments TFBI and TFTR led to a significant activity increase by 34.76 and 27.91% when compared with the *Foc* TR4 treated control (TF) similarly, increased by 39.98 and 33.67% compared to untreated control (TC) respectively. The activity of PPO varied significantly ($F = 10.27$, df = 3,8, $p = 0.004$) by 22.73% (TFBI), 10.53% (TFTR) compared to *Foc* TR4 inoculated unimmunized plants (TF), and by 40.91% (TFBI), 31.58% (TFTR), and 23.53% (TF) compared to *Foc* TR4 uninoculated control plants (TC). The PAL activity ranged from 1963.00 to 2472.25 nmol. Of trans-cinnamic acid g-1 fw min-1 in *Foc* TR4 treated plants (TFBI – TF). Whereas, 1286.05 nmol. Of trans-cinnamic acid g-1 fw min-1 was observed in *Foc* TR4 uninoculated control (TC) plants. Significantly higher ($F = 34787.98$, df = 3,8, $p = 0.00$) PAL activity was observed in bio-immunized*Foc*TR4 inoculated plants (TFBI) 2472.247 nmol. Of transcinnamic acid g-1 fw min-1 followed by ICAR-FUSICONT treated plants (TFTR) 2373.363 nmol. Of transcinnamic acid g-1 fw min-1, and *Foc*TR4 inoculated control plants (TF) 1963.003 nmol. Of transcinnamic acid g-1 fw min-1, respectively. The β-1,3-glucanase activates in banana leaves of the TFBI, TFTR and TF treatments were significantly ($F = 115509.2$, df = 3,8, $p = 0.00$) increased as compared with the control (TC) (Figure 3). However, the highest

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TABLE 3 Effect of bio-immunization and ICAR-FUSICONT [*Trichoderma reesei* (CSR-T-3)] treatment on *Fusarium* wilt management and growth of banana plantlets under field experiment in *Foc* TR4 infected fields.

Treat.	Plant height (cm)	Plant girth (cm)	No. of leaves/ Plant	3rd leaf Length (cm)	3rd leaf breath (cm)	Bunch wt. (kg)	No. of hands/ bunch	No. of fingers/ hand
Location 1: Nirpur, Purnia district, Bihar, India								
TBI	249.30c (±6.76)	68.70c (±3.18)	10.00b (±0.79)	222.40c (±5.71)	72.00c (±1.34)	28.25b (±1.59)	11.25b (±0.85)	17.50c (±1.05)
TTR	238.90b (±3.01)	57.95b (±5.17)	8.10a (±0.72)	200.40b (±6.12)	65.53b (±3.60)	28.70b (±1.13)	9.25a (±0.72)	15.20b (±0.83)
TBITR	257.30d (±5.41)	70.80d (±5.00)	11.15c (±0.88)	233.45d (±2.93)	73.65d (±1.35)	30.85c (±1.60)	12.30c (±0.66)	17.45c (±1.10)
TC	222.65a (±5.25)	53.65a (±1.50)	7.65a (±0.59)	186.65a (±4.76)	59.20a (±2.40)	16.19a (±1.11)	9.10a (±0.72)	14.60a (±1.10)
Location 2: Dighari, Katihar district, Bihar, India								
TBI	230.05b (±9.27)	66.45c (±3.84)	10.55b (±1.10)	196.10b (±5.21)	82.60d (±4.26)	25.65b (±2.13)	11.30b (±1.17)	18.95d (±3.14)
TTR	238.75c (±12.27)	62.60b (±2.16)	10.45b (±2.70)	207.75c (±11.76)	73.55b (±7.76)	27.10c (±2.05)	11.10b (±1.21)	15.00b (±1.30)
TBITR	249.40d (±4.12)	63.80b (±2.48)	12.35c (±1.04)	228.35d (±5.24)	80.05c (±5.80)	31.70d (±2.47)	12.40c (±0.75)	17.15c (±1.50)
TC	187.55a (±6.35)	55.15a (±2.30)	7.65a (±0.59)	147.45a (±6.26)	53.45a (±1.23)	14.13a (±2.47)	9.10a (±0.72)	12.90a (±0.79)

TFBI, Treatment Fusarium and bio-immunized; TTR, Treatment Trichoderma; TBITR, Treatment bio-immunized and Trichoderma; TC, Treatment control (No inoculations). Values are the means of ten replicates with the sample size $n = 5$. Means in the columns followed by the distinct letters are significantly different according to Duncan's multiple range test at $p = 0.05$. The standard deviation of the mean is indicated by values in parentheses.

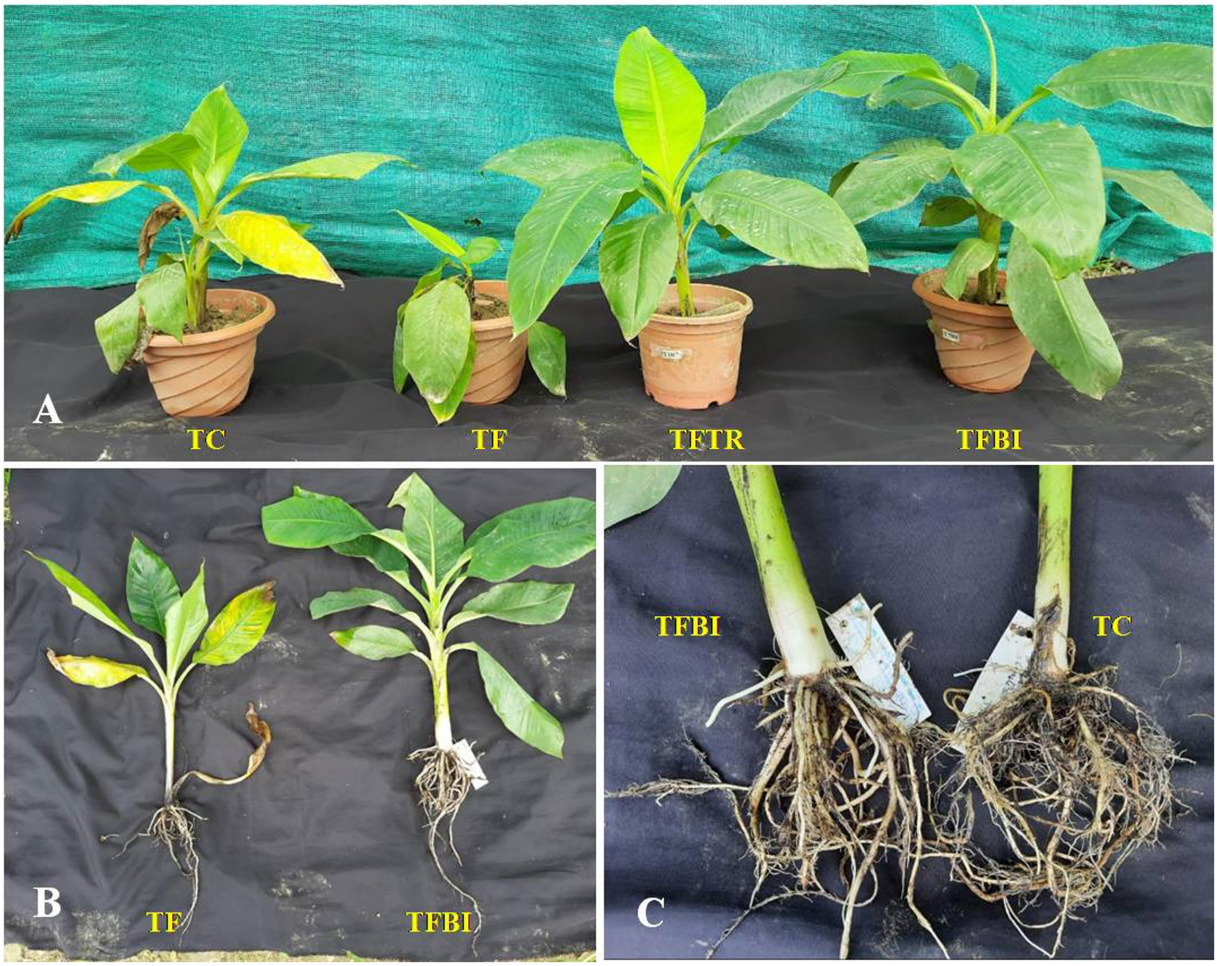

FIGURE 2
Superiority of *in vitro* bioimmunized plants over other treatments in terms of plant growth and root characters. **(A)** Plant growth enhancement of tissue culture raised banana plantlets under various treatments. TFBI, treatment *Fusarium* and bio-immunized; TFTR, treatment *Fusarium* and *Trichoderma*; TF, treatment *Fusarium* alone; TC, control; **(B)** complete TC plants; TF: Effect of *Foc* TR4 is visible in the plant growth showing leaf yellowing and poor root growth. TFBI- healthy plant with higher visible biomass along with sound root system; **(C)** magnified view of root system: TFBI – with dense and multiple adventitious roots; TC – shallow root system with lower no of adventitious roots.

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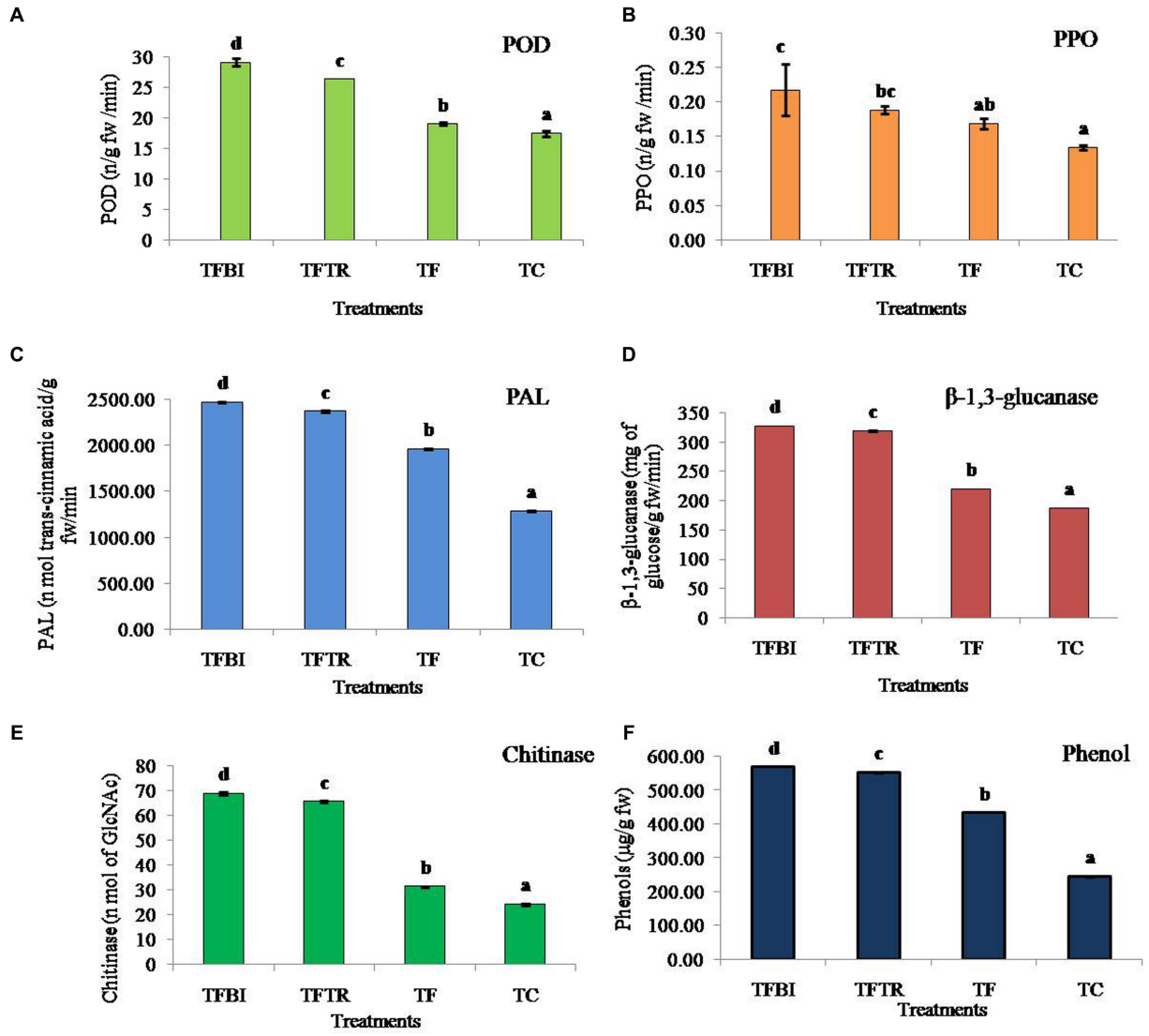

FIGURE 3
Effect of treatments of on different antioxidant and defense enzymes **(A)** Peroxidase (POD) (ng^{-1} fresh wt.), **(B)** Polyphenol oxidase (PPO) (ng^{-1} fresh wt.), **(C)** Phenylalanine ammonia lyase (PAL) (nmol of trans-cinnamic acid g^{-1} fresh wt. min^{-1}), **(D)** β-1,3-glucanase (mg of glucose g^{-1} fresh wt. min^{-1}), **(E)** Chitinase (nmol of GlcNAc g^{-1} fresh wt. min^{-1}), and **(F)** Phenol (µg g^{-1} fresh wt.) of banana plants of G9 variety. Where, T1 = bio-immunized plants with *Foc* TR4 (TFBI), T2 = unimmunized plants with *Foc* TR4 treated with *Trichoderma* (TFTR), T3 = unimmunized plants with *Foc* TR4 (TF), and T4 = unimmunized plants without *Foc* TR4. Values are mean of three replicates with ± standard deviation. Means denoted by different letters indicate significant difference between the treatments ($p \leq 0.05$) (DMRT).

β-1,3-glucanase activity was recorded in TFBI, showing 32.92% increase followed by TFTR (31.04%) relative to the *Foc*TR4 inoculated control (TF). The least β-1,3-glucanase activity 187.66 mg of glucose g-1 fw min-1was observed in *Foc*TR4 uninoculated control (TC) plants. The same pattern of antioxidants was seen for chitinase enzyme and phenol production. The chitinase activity varied significantly ($F = 115509.2$, df = 3,8, $p = 0.00$) by 54.42% (T 1), 49.60% (TFTR) compared to *Foc*TR4 inoculated control (TF), and by 64.70% (TFBI), 62.92% (TFTR), and 22.57% (TF) relative to the *Foc*TR4 uninoculated control (TC) plants. The phenol quantity varied significantly ($F = 172980.7$, df = 3,8, $p = 0.00$) by 23.89% (TFBI), 21.16% (TFTR) compared to inoculated control (TF).

3.5 Scanning electron microscopy analysis of different treatments *FOC* TR4 challenge inoculation and bio-immunization

SEM analysis of four treatments indicated that the lignifications of the cells have been identified in the treatment TFTR and TFBI. Quite contrarily, the tissues were found to be damaged in TF, and lesser lignified cell membranes were observed in the control TC. The close-up view of the cells in TFTR and TFBI indicates the metabolite induced lignin biosynthesis which could be one of the adaptive mechanisms conferring tolerance against the pathogen *Foc* invasion (Figure 4).

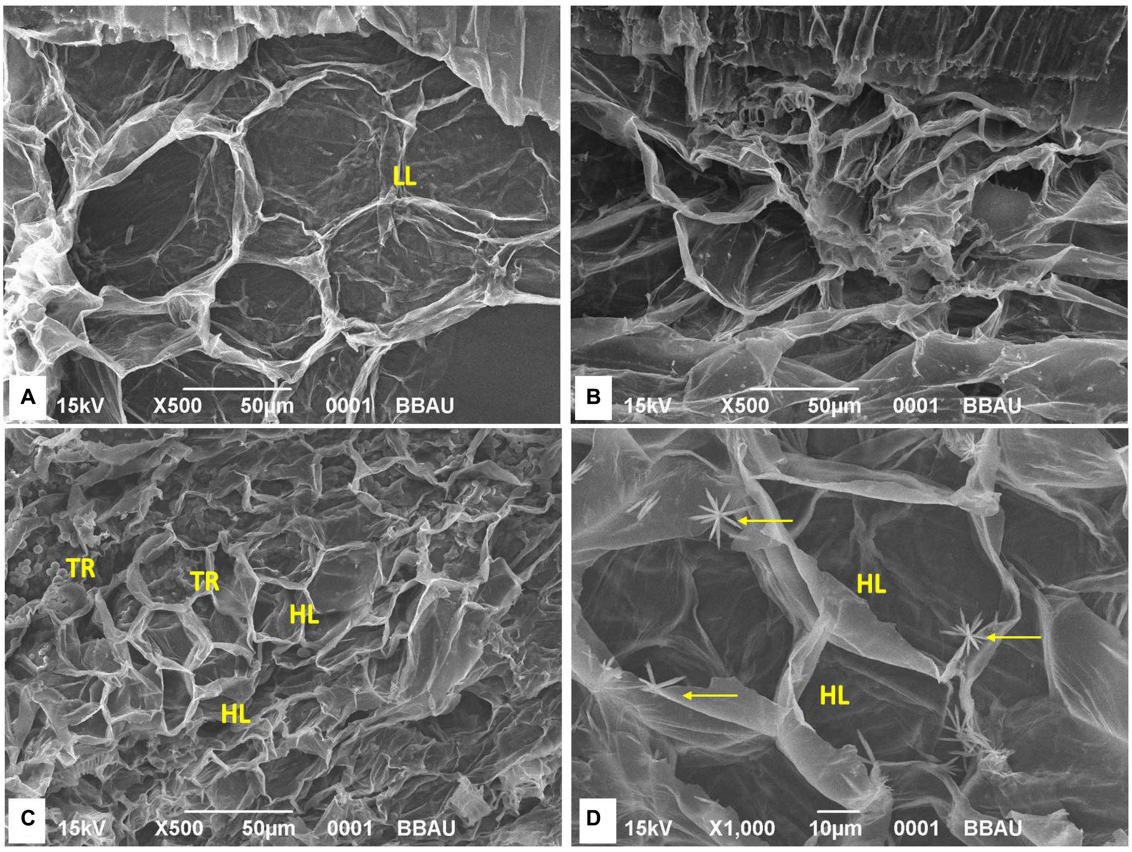

FIGURE 4
Scanning electron microscopy (SEM) analysis of different treatments. Lignification of the cells has been identified in the treatment TFTR and TFBI while tissues were found to be damaged in TF and lesser lignifications of cells has been noticed in TC. SEM images indicates that lignifications of cells has been induced both in TFTR and TFBI speculating that the metabolite induced lignin biosynthesis could be operating in conferring tolerance against the pathogen Foc invasion. **(A)** TC, Treatment Control; **(B)** TF, Treatment *Fusarium* alone; **(C)** TFTR, Treatment Fusarium+*Trichoderma reesei*; **(D)** TFBI, Treatment Fusarium+Bio-Immunizer. Where markings in yellow indicate intensity of lignifications. LL, Low lignifications; HL, High lignifications; TR, *Trichoderma reesei*.

3.6 Metabolic profiling of bioactive metabolites in whole plant samples of banana plantlets under different treatments of *FOC* TR4 challenge inoculation and bio-immunization by using LC-MS analysis

Metabolic profiling of whole plant extracts of banana plantlets under different treatments by LCMS showed a significant increase in the synthesis of bioactive compounds classified mainly as flavonoids, quinones, phenolic acids, and derivatives of lipids and amino acids in bio-immunized treatment than the others. A total of 27 bioactive compounds with repeated peaks of high intensity were characterized in the treatment TFBI involving bio-immunization and *Foc*TR4 challenge inoculation, 28 with the treatment TFTR involving *Trichoderma reesei* isolate CSR T-3 and challenge inoculation with *Foc* TR4and 17 under treatment TF comprising of *Foc*TR4 challenge inoculated plantlets (Table 1). In the untreated control TC involving plantlets that were neither bio-immunized nor challenge inoculated with *Foc*TR4 only nine bioactive compounds comprising carbohydrate, phenol derivatives with high-intensity peaks were detected. The heatmap with linkages (Figure 5A) distinctly separates the TFBI and TFTR treatments with TF and TC with dissimilarity of 74.39%. The PCA analysis of the detected metabolites showed that the differentially accumulated metabolites (DAM) grouped distinctly with the respective treatments. The first and second principal components depicted 47.04 and 27.36% variation, respectively (Figure 5B). The divergence of the vectors of the TFTR treatment involving *Trichoderma reesei* CSR T-3 isolate and TFBI involving the bio-immunization from the PCA signifies the higher contribution of the PC (principal components) while the convergence of the vectors of TF treatment with *Foc* TR4 alone and TC signifies the least contribution of the PC (Figure 5B). We observed five differentially accumulated metabolites (SF Sinefungin, AA Arachidonic acid, QRR Quercetin glucoside rhamnoside-rhamnoside, RM Rhamnose, and TZ Trans zeatin) in treatment TBI involving bio-immunization and *Foc* TR4 challenge inoculation whereas the presence of these compounds were not observed in other treatments.

3.7 Multi-location trial and assessment of the performance of bio-immunized plants

Field trials were conducted for two consecutive years (2020–21 and 2021–22) in the field of susceptible soil at two locations Nirpur,

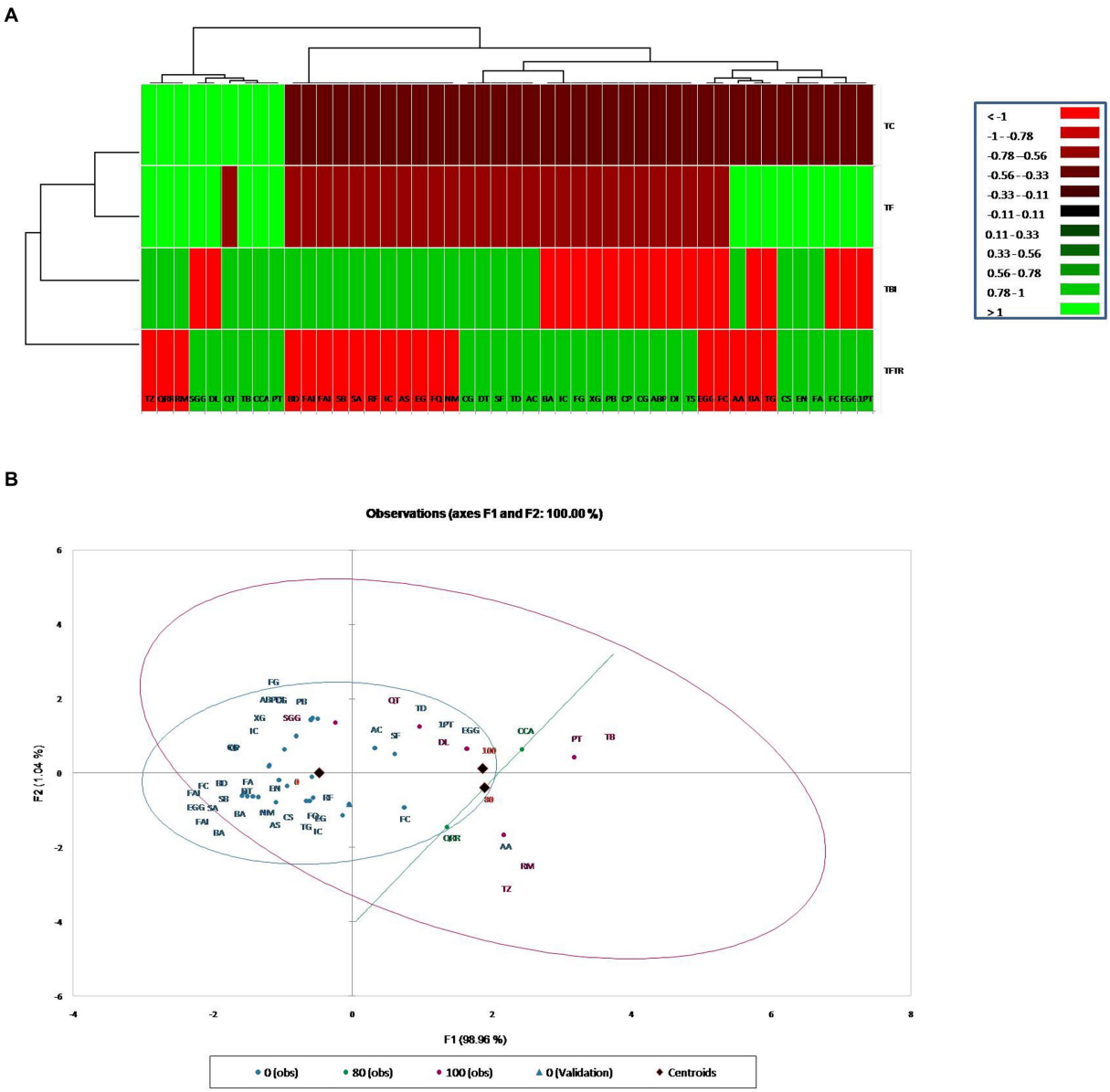

FIGURE 5
Cluster analysis for classification and categorization of metabolites obtained in the LC-MS data pooled from the four treatments. **(A)** Heat map showing profile of different metabolites expressed in banana plants under different treatments along with vertical and horizontal clustering marked on the top and left panels. *X*-Axis denotes metabolites and *Y*-axis represents treatments. **(B)** Principal component analysis showing clustering of metabolites into different groups generated through XLSTAT software. Treatments: TC, Treatment Control; TF, Treatment *Fusarium* alone; TFBI, Treatment Fusarium+Bio-Immunizer; TFTR, Treatment Fusarium+*Trichoderma reesei*. Metabolite: TD, Tomatidin; FQ, Flavonoid quercetin; NM, Neodiosmin; EG, Epigallocatechin gallate; CCA, Cryptochlorogenic acid; QRR, Quercetin-glucoside-rhamnoside-rhamnoside; AS, Acteoside; AC, Anthocyanin; IC, Isocytric acid; RM, Rhamnose; RF, Rushflavanone; PT, Phloretin; SF, Sinefungin; DT, Dehydrotomatine; TZ, Trans Zeatin; QT, Quercetin; TB, Thebaine; EN, Enniatin A; CG, Corilagin; FA, Fusaristatin A; AA, Arachidonic acid; CS, Chlamydosporal; Sa, Surfactin isomar A; SB, Surfactin isomar B; FAI, Fengycin A isomar; BD, Bacillomycin D isomar; SGG, Soyasapagenol E-3-O-rhamnosyl glucosyl glucuronide; DI, Diglucoside isomer; DL, Demethyloleuropein; TS, Tabersonine; ABP, A-D-Glucose-1,6-bisphosphate; CG, Catechin-0-gallate; CP, β caryophyllene; PB, Peptaibols; XG, Xyloglucan; FG, Fenigycin; IC, Iturin-C19; EGG, Epi gallocatechin-O-gallate; 1PT, 1-Palmitoyl-2-10-hydroxy-5,8,11-tridecatrienoic acid; BA, Beauveric acid; FC, Fusarin C; EGG, Epi gallocatechin-O-gallate; BA, Beauveric acid; FC, Fusarin C; TG, Trigalloyl glucose.

Purnia district, and Dighari, Katihar district (Bihar, India) (Figure 1). It was observed that bio-immunized plants significantly ($p \leq 0.05$) enhanced the growth and yield compared to unimmunized plants. At location 1, the maximum plant height and plant girth were recorded in TBITR (257.30 cm, 70.80 cm) followed by TBI (249.30 cm, 68.70 cm) and TTR (238.90 cm, 57.95 cm) compared to untreated control plants (TC) (222.65 cm, 53.65 cm), respectively. Similarly, each treatment caused a significant ($p \leq 0.05$) increase in no. of leaves/plant, 3rd leaf length, and breadth compared to control as indicated by Duncan's multiple range tests. The highest increase in no. of leaves/plant (31.39%), 3rd leaf length (20.04%), and 3rd leaf breadth (19.62%) in TBITR were significantly higher than that found in TBI (23.5, 16.07, and 17.78%), TTR (5.56, 6.86, and 9.66%) compared to control (TC). Based on DMRT analysis bunch weight, no. of hand/bunch, and no.

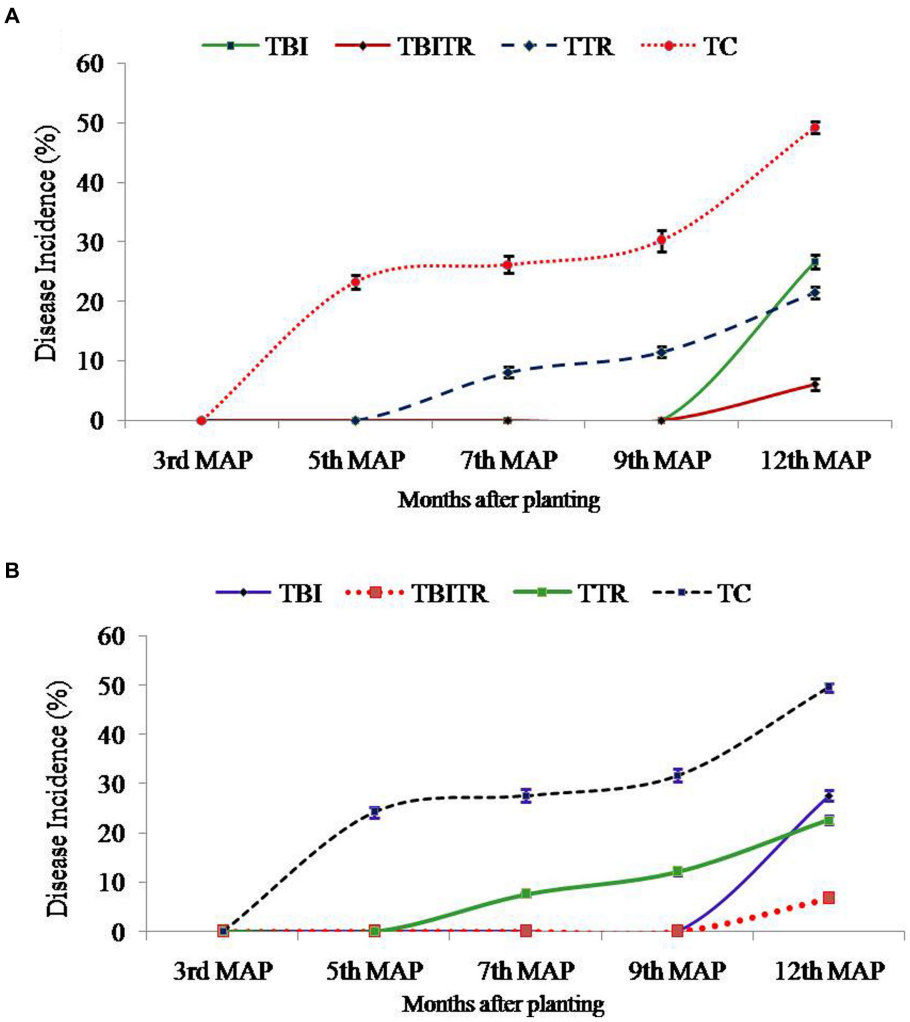

FIGURE 6
Disease incidence analysis of *Foc* TR4 in Grand Naine (G9) banana plants throughout the two year field trial at two different locations **(A)** Nirpur, Purnia district, Bihar, India and **(B)** Dighari, Katihar district, Bihar, India under infected fields.

of finger/hand were statistically ($p \leq 0.05$) higher for the treated plants compared to control (Table 3). At location 1, the highest bunch weight (30.85 kg), and no. of hand/bunch (12.30) was observed in treatment TBITR whereas, a maximum no. of fingers/ hand was found in TBI (17.50) significantly similar to TBITR (17.45) followed by TTR and TBI, respectively.

At location 2, the highest plant height was observed in TBITR (249.40 cm) and plant girth in TBI (66.45 cm), which were significantly ($p \leq 0.05$) higher than control (TC). The maximum no. of leaves/plant, 3rd leaf length (12.35 and 228.35 cm) was found in TBITR, while the highest 3rd leaf breath (82.60 cm) was recorded in TBI significantly ($p \leq 0.05$) higher than other treatments and control as indicated by DMRT. Similarly, the highest bunch weight (31.70 kg), no. of hand/bunch (12.40), was observed in TBITR inoculated plants, while the maximum no. of finger/hand (18.95) was observed in TBI followed by TBITR, TTR, and control (TC), respectively. Percentage disease incidence was calculated for all the treatments planted at two locations in Nirpur, Purnia district, and Dighari, Katihar district (Bihar, India) over the years 2020–21 and 2021–22 (Figure 6). At location 1, in the case of control (TC) the appearance of the disease started after 3rd month of planting, at the final growth stage (12th MAP) the mean incidence recorded was 49.50%. In the case of CSR-T-3 (TTR) treated plants the incidence was found (21.50%) on the 12th MAP and the appearance of the disease started after the 5th month after planting. However, in the case of bio-immunized plants (TBI) disease appearance started after the 9th month after planting with a disease incidence was 26.70% whereas, when we combine bio-immunized plants along with rotational application of CSR-T-3 (TBITR) appearance of disease started at 9th month after planting like TBI but, disease incidence lower down at (6.10%). A similar kind of trend was observed at location 2. The highest disease incidence (49.50%) was observed in control (TC) after the 12th MAP followed by bio-immunized plants (TBI) (27.60%), CSR-T-3 treated (TTR) (22.60%), and bio-immunized plants with application of CSR-T-3 (TBITR) (6.70%) respectively. In the case of TC, the disease incidence started at the 3rd MAP while, in TTR the disease incidence started at the 5th MAP. In the case of TBI and TBITR, the disease incidence started at the 9th MAP.

4 Discussion

Banana is one of the major important commercial fruit crops of India, known for being cultivated in Maharashtra, Karnataka, Tamil Nadu, Andhra Pradesh, Odisha, Gujarat, and parts of the North East. Recently, banana cultivation has been expanding significantly in Uttar Pradesh and Bihar covering about 69,380 ha and 31,070 ha, respectively (Damodaran et al., 2021). In the recent past, the cultivation of commercial cultivar G9 of banana has been threatened by the outbreak of a virulent strain of *Fusarium* wilt tropical race 4. The pathogen is known for its rapid proliferation and devastation of the plantation within a short period causing a huge economic loss to growers. Control of the disease has been a great challenge to researchers across the globe to date. In an attempt toward successful management, the complex polycyclic nature of the pathogen and its ability to mutate into different Vertical Compatibility Groups (VCG's) warranted the need for supportive technologies to build resistance in the host. According to Chow et al. (2008) engineered peptide-based biopolymers have recently attracted much attention as a new class of materials. One such effort resulted in the development of a lipo-polypeptide-based biomolecule from *Trichoderma reesei* and *in vitro* bio-immunization technology. To the best of our knowledge, no single report is presently describing lipo-polypeptide-based biomolecule used in the process of bio-engineering into the banana plantlets (bio-immunization) in tissue culture root regeneration media. *Trichoderma* spp. is known as a potential biocontrol agent (El Komy et al., 2015). The metabolites secreted by *Trichoderma* spp. suppresses the plant pathogenic microorganisms and enhances plant growth (Kubicek et al., 2001; Contreras-Cornejo et al., 2015). *Trichoderma* spp., considered a potent biocontrol agent, that can survive under stressed conditions, antagonistic against phyto-pathogenic microorganisms, induces a defense mechanism in plants, and helps in plant growth promotion by secreting several secondary metabolites (Adnani et al., 2017; Poveda et al., 2020), PR proteins (Yadav et al., 2021), enzymes (Tariq et al., 2020), siderophores, phytohormones and phosphate solubilizing enzymes (Doni et al., 2014). In our findings, we observed that bio-immune formulation significantly inhibited the growth of the pathogen *Foc*TR4 under *in vitro* conditions.

We observed that bio-immunization enhanced the *in vitro* rooting and plant growth of banana plantlets. *In vitro* bio-immunization of the host plant is an advanced technology in tissue culture to develop disease-tolerant, disease-free planting material through bio-fortification of plantlets with bio-immunizing formulation. According to Chatenet et al. (2001) tissue culture allows the production and propagation of disease-free genetically homogeneous plant material. The process can also be used to develop plants resistant to various types of stresses (Bouquet and Torregrosa, 2003). According to Pati et al. (2013), the maximum mortality of micro-propagated plants occurs during the acclimatization process as plantlets undergo several morphological, physiological, and biochemical changes. In our study, a 97.57% survival rate was observed at the primary hardening stage and 93.23% at the secondary hardening stage. Uzaribara et al. (2015) recorded a 95% survival rate during the primary hardening of red banana (*Musa acuminate*) whereas, Rai et al. (2014) reported a 96% survival rate. However, mortality of plantlets may be due to injuries to the root system during transferring and sudden exposure to the harsh environment. Successful acclimatization and survival rate in banana plantlets (80–100%) under *in vitro* greenhouse conditions has been reported by several scientists (Rahman et al., 2013; Ahmed et al., 2014; Hossain et al., 2016). In the current study, the technology of *in-vitro* bio-immuniztion for producing disease-free, *Fusarium* wilt-tolerant banana plantlets were tested for its bioefficacy through a pot culture experiment where the bio-immune treated plantlets exhibited significantly low disease index (0.67) compared with the *Foc* TR-4 challenge inoculated control that showed 3.78 disease index signifying the host induced tolerance due to bio-immunization. It also significantly enhanced the plant growth compared to the control due to the presence of PGPR properties of *Trichoderma reesei* in the bio-immunized plantlets. According to Cai et al. (2015) *Trichoderma* spp. have been reported to increase plant growth by releasing several hormones that enhance root development and plant growth, which in turn increases the secretion of root exudates resulting in the availability of nutrients for microbial growth (Elad, 2000; Carvalhais et al., 2015). In the same line, Yedidia et al. (2001) reported that *Trichoderma* spp. increases the length of primary and lateral roots which results in enhanced nutrient uptake by the plants. They exert biocontrol action against fungal phytopathogens by inducing resistance in host plants and promoting plant growth (Fiorentino et al., 2018; Zhao et al., 2020). *Trichoderma* sp. was found to enhance the concentration of essential nutrients in the shoots and roots of cucumber and tomato seedlings (Azarmi et al., 2011).

The results obtained in this study revealed that *in vitro* bio-immunization of banana plantlets during organogenesis through the bio-immune formulation of *T. reesei* showed better activity that increased defense-related enzymes PAL, PPO, POD, β-1,3-glucanase, chitinase, and phenolics by a greater amount. Our findings are in line with Damodaran et al. (2020), who reported that *T. reesei* enhanced β-1,3-glucanase, POD, chitinase, PPO, and PAL with higher phenolic contents. Singh et al. (2021) reported that *Trichoderma* spp. showed significantly higher defense-related enzymes such as PAL, POD, and PPO. Joseph et al. (1998) reported that POD inhibited the mycelia growth and spore germination of *Pseudocercospora abelmoschi* and *P. cruenta*. Kavino et al. (2007b) showed enhanced POD and PAL activity in bacterial strain-treated banana plantlets against *Banana bunchy top virus*. Another enzyme PPO is known to catalyze the oxidation of phenolic substances into highly reactive quinines (Araji et al., 2014). Karthikeyan et al. (2006) reported that a consortium of *Trichoderma viride* with *Pseudomonas fluorescens* enhanced the PPO activity in coconut plants against *Ganoderma*. Consortia of *Trichoderma* spp. and with other bacterial isolates have been reported to increase the defense enzyme activity of PAL, POD, and PPO during pathogen invasion (Jain et al., 2012; Singh et al., 2013; Singh and Singh, 2014). Peroxidase (POD) is one of the most important enzymes that is responsible for lignin synthesis and developing plant resistance against phytopathogen (Bruce and West, 1989) and restricts fungal growth (Macko et al., 1968). Several scientists reported that POD activity was elicited during the pathogen infection in cucumber (Yedidia et al., 1999; Chen et al., 2000), tomato (Nandakumar et al., 2001; Ramamoorthy et al., 2002), banana (Damodaran et al., 2020), and brinjal (Singh et al., 2021). Similarly, a significantly higher amount of PAL activity was observed in brinjal (Singh et al., 2021), chickpea (Singh et al., 2013), pea (Jain et al., 2012), tomato (Singh and Singh, 2014) and banana (Damodaran et al., 2020). Phenolic compounds present in plants have antimicrobial properties and serve as signaling molecules (Hammerschmidt, 2005).

Our findings from secondary metabolite profiling using LCMS have furthered the host tolerance attributed by bio-immunization

technology by confirming the role of antifungal secondary metabolites present in the *Trichoderma reesei* isolates CSR-T-3contributing to the suppression of *Foc*. The versatile nature of antifungal secondary metabolites from endophytic *Brachybacterium paraconglomeratum* isolated from the resistant cultivar YKM5 was proved to be responsible for the suppression of *Foc* under *in vitro* conditions (Ravi et al., 2022). In the current study, we observed high expression of antioxidants like Quercetin and Quercetin rhamnosyl-rutinoside to the level of intensity 100 and 80%, respectively, in the bio-immunized TBI treatment challenge inoculated with *FOC* TR4. Likewise, co-culturing of *B. velezensis* YRBBR6 along with *Foc* KP (Pisang Awak strain) induced the secretion of secondary metabolites, *viz.*, dihydroacridine, nonanol from the antagonistic bacteria inhibit plant pathogens and promote plant growth (Khalid and Keller, 2021). Quercetin isoflavonoids known to have antibacterial and antiviral activity are induced in plant systems under stress conditions to enable immune response (Johari et al., 2012; Lachowicz et al., 2020). The challenge inoculation of *Foc* TR4 significantly shows the presence of fungal toxins of *Fusarium* sp. like Fusaristatin A, Fusarin C, and Beauveric acid signifying the intense activity of pathogen in the plant system due to *Foc* TR4. However, the presence of fungal toxins was found to be at a lower level running from (4 to 22%) intently signifying the role of bio-immunization in TBI treatment in the suppression of toxins produced by *Foc*TR-4 in the presence of the antifungal metabolites. Besides this, the intensity of lignifications in the cell walls of bio-immune (TFBI) and *Trichoderma reesei* treatment (TFTR) indicates the mechanism of induction of host defense through signal transduction and regulation of lignin biosynthesis pathway. The mechanism of host defense conferred by the bio-immune molecule is explained in Figure 7 wherein it is speculated that the bio-immunized plants exhibited activation of the host defense system in terms of enhanced secondary metabolite production pathway, pathogenesis-related proteins (PR proteins) and reactive oxygen species scavenging system (ROS scavengers) by the

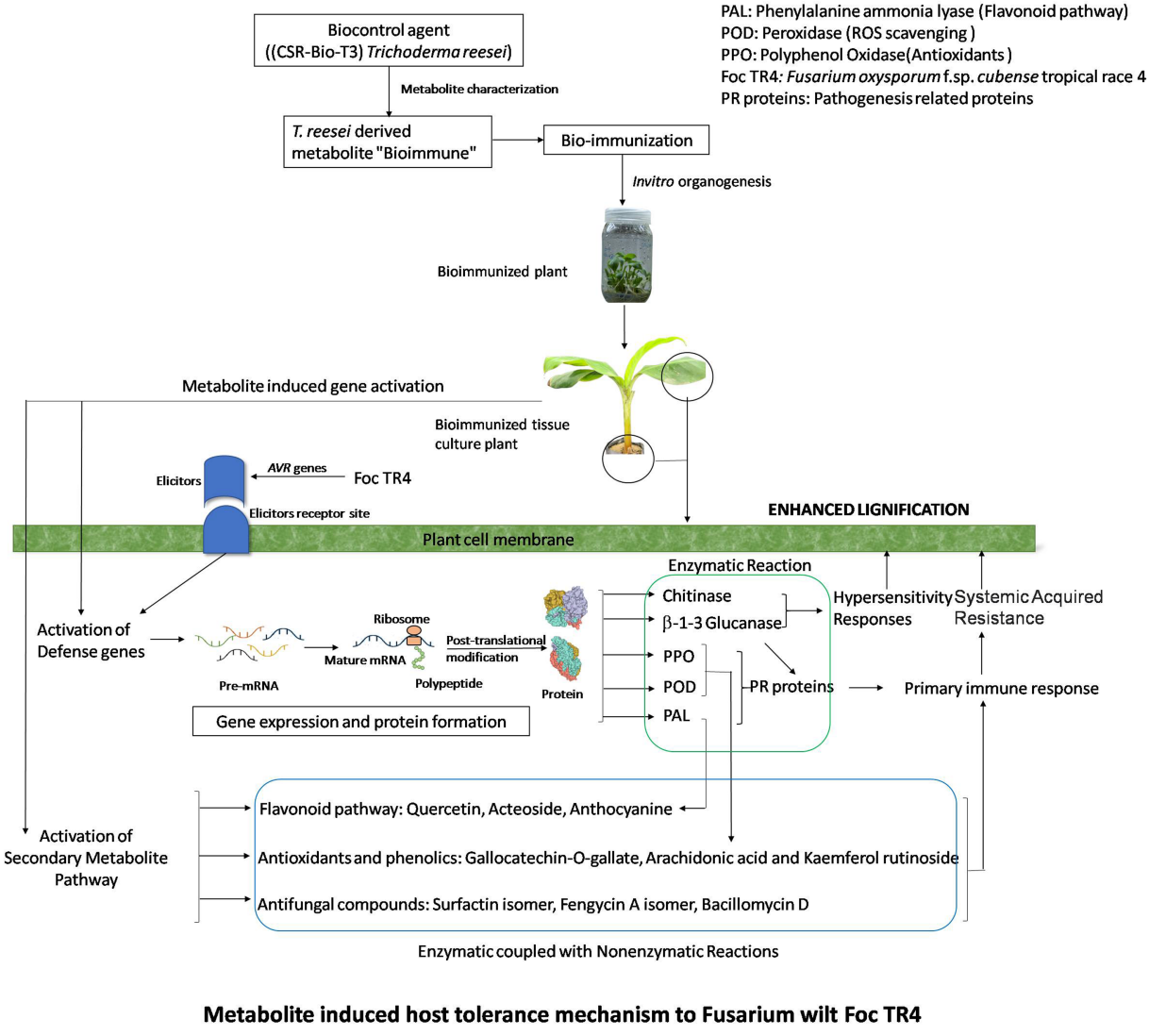

FIGURE 7
Metabolite induced host tolerance mechanism to Fusarium wilt *Foc* TR4. Bioimmunized plants showed improved host defense mechanism (HDM). HDM is activated by enhanced secondary metabolite pathway, ROS scavenging enzymes, PR proteins which lead to lignifications of cells.

production of antifungal compounds, antioxidants, and phenols, besides enhanced lignification in the cells (evident from SEM images, Figure 4).

Field performance of the different treatments of banana plants also indicated that TFBI outperformed the other treatments in terms of yield, bunch weight, and plant growth characters. The expression of disease symptoms is also very negligible in these two treatments thus proving that this strategy is a rationalized field translational success report of *in vitro* Bio-immunized plants for the management of Fusarium wilt disease of banana. Earlier, Damodaran (2018) demonstrated successful control of *Foc* TR4 using ICAR-FUSICONT during the late stages of plant growth whose principal microorganism is *Trichoderma reesei*. This report is an advancement over the previous report, in which earlier days of plant growth as well as infections during the initial 4-5 months are also taken care of; as ICAR-FUSICONT works well in the later stages.

5 Conclusion

Over the past decade, Fusarium disease has become a serious problem for banana growers not only in terms of economic loss but also because of the disposal of diseased plants. The major setbacks that affect the management of the disease include a lack of information on the resistant gene pools, no effective chemical control, and a lack of virulent biological control agents. However, in our previous study, we had identified *Trichoderma reesei* as a potential biological agent for the management of the pathogen strain. We report here another novel approach of metabolite-induced host immune system for combating the Fusarium wilt disease of banana. This study is a holistic approach to a technological intervention that has been validated in the field for the management of Fusarium disease in banana (*Foc* TR4).

Data availability statement

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

Author contributions

TD conceived, planned, and designed the experiments. MMi conducted the *in vitro* lab experiments and contributed to manuscript preparation with TD. MMu designed, analyzed the LC-MS, enzyme analysis, field data, and interpreted the results for elucidation of mechanism. SR designed the field experiments, data collection, performed the statistical analysis of the data. KY, SKi, and AK did *in vitro* Bioimmunization work. PD, PB, and RG performed the experiments of dual culture, pot, and field experiments. SK performed LC-MS, enzyme analysis, data interpretation, and prepared conceptual mechanism figure. 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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