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Advances in integrated disease management (IDM) for soil-borne plant pathogens: Innovative approaches and underlying action mechanism at molecular level, 2nd edition

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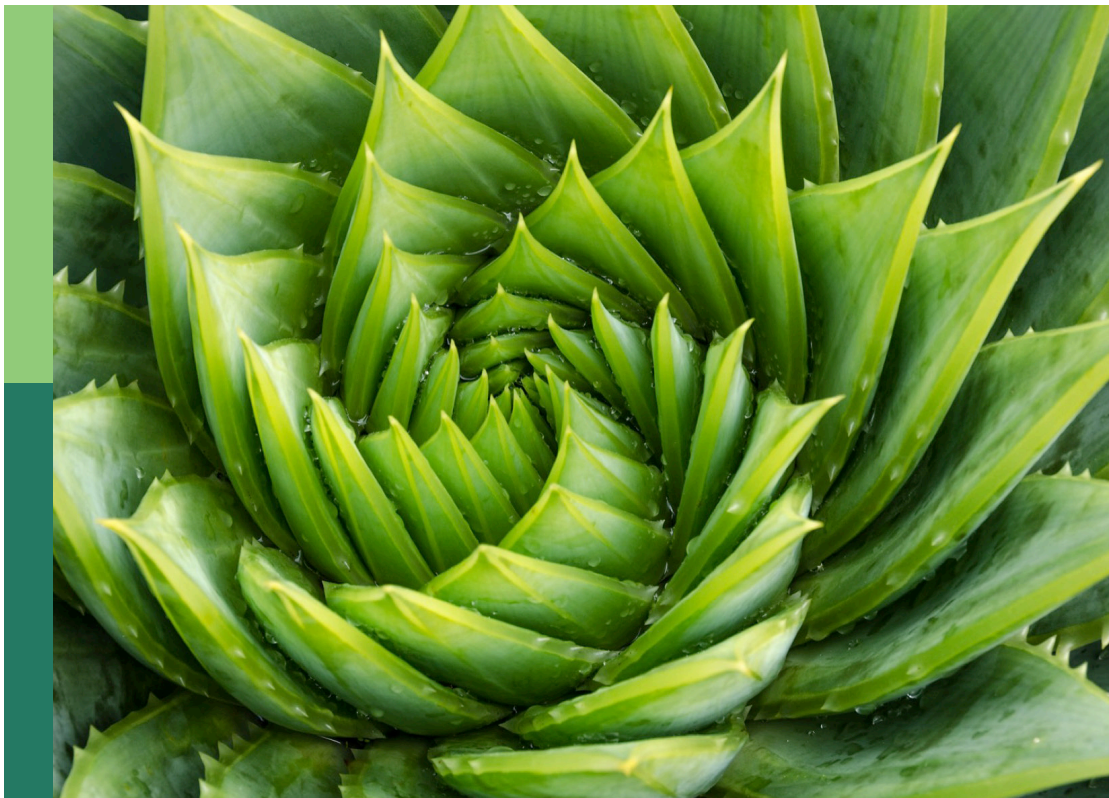

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Advances in integrated disease management (IDM) for soil-borne plant pathogens: Innovative approaches and underlying action mechanism at molecular level, 2nd edition

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Editorial: Advances in integrated disease management (IDM) for soil-borne plant pathogens: innovative approaches and underlying action mechanism at molecular level

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pests, pesticides, integrated disease management, diseases, soil-borne

Editorial on the Research Topic

Advances in integrated disease management (IDM) for soil-borne plant pathogens: innovative approaches and underlying action mechanism at molecular level

Soil-borne plant pathogens pose a significant threat to plants, and their management is comparatively more difficult than other plant pathogens because of their nature of the hidden enemy, long persistency in soil, vast genetic diversity and host range (Katan, 2017). However, researchers throughout the world are struggling to investigate effective management strategies to manage soil-borne plant pathogens (Panth et al., 2020). We proposed this Research Topic in order to make a useful collection of novel studies which involve the management of soil-borne plant pathogens and underlying action mechanisms. Among soil-borne plant bacterial pathogens, *Ralstonia solanacearum* is one of the most devastating agents affecting different plants in 45 families. Several management practices have been used effectively for the management of this bacterium in different plants, including the use of biological control agents (Wang et al.). However, because of difficulties in handling, culturing, and maintaining biocontrol agents or the issues related to their practical efficacy in soil, the use of their antimicrobial compounds is considered a good alternative. Ye et al. investigated an antibacterial furoic acid compound, 5-(hydroxymethyl)-2-furoic acid, produced by the fungus *Aspergillus niger*. The soil application of this compound effectively controlled the *R. solanacearum* population in soil by direct killing effect as well as enhancing host resistance in tomato plants. Wang et al. reviewed other studies for the management of this bacterium, including biological, organic, breeding, genetic engineering, physical, cultural, chemical and nano-technological approaches.

Nanotechnology emerged as one of the most rapidly advancing sciences of the twenty-first century, and plant scientists revealed the effective application of nanotechnology for the management of plant pathogens. Dutta et al., in their review, focused on several aspects of the use of nanotechnology in the management of soil-borne plant pathogens highlighting the

role of nanoparticles as protectants and inducers of plant defense against soil-borne plant pathogens. Uncovering the mechanism of host resistance and discoveries of host genes/proteins that regulate host resistance against soil-borne plant pathogens will help plant genetic engineers develop plants that can better fight against soil-borne plant pathogens. Tian et al. reported that small G-protein StRab5b positively regulates potato resistance against the soil-borne fungus *Phytophthora infestans*. Pazarlar et al. found that *Bacillus cereus* EC9 protects tomatoes against *Fusarium* wilt through JA/ET-activated immunity. In another study by Ling et al., it was shown that *WRKY* genes confer resistance to *Cucumis metuliferus* against root-knot nematodes (RKN). The RKN-resistant variety of *C. metuliferus* (cm3) was found to specifically recruit beneficial bacterial communities in soil upon infestation with RKN, as reported by Song et al. Similarly, Sikandar et al. reviewed overall management strategies, including several recent efforts to improve host resistance against *Meloidogyne enterolobii*.

Enhancing host resistance is also a prominent action mechanism of many biocontrol agents. In their review, Dutta et al. unveil the molecular mechanism by which *Trichoderma* fungus, one of the most used and studied biocontrol fungus, induce host resistance against pathogens including the role of exogenous elicitors in the induction of host resistance. Zhou et al. proved that exogenous application of elicitor isotianil could significantly alleviate the symptoms of *Fusarium* wilt on the banana. They further confirmed that isotianil application might contribute to disease control by inducing host plant defense against *Fusarium* infection. Investigation into how the pathogens achieve virulence at the molecular level is also essential for understanding the action mechanism of the pathogen. Xu et al. revealed the role of cAMP phosphodiesterase in the formation of sclerotia and achieving virulence in *Sclerotinia sclerotiorum*, an important phytopathogenic fungus that causes stem rot and white mold disease.

Most investigations conducted for the management of soil-borne pathogens are based on greenhouse or controlled laboratory conditions. Although the outcomes of these studies provide useful insights into the action mechanism and control efficacy, field investigations are vital for assessing the practical applications of these strategies. Field assessments consider the complex interplay between plants, pathogens and soil, and environmental factors that can affect disease management and development. This more accurately illustrates how these strategies perform in real agricultural scenarios. Moreover, field assessment can give an actual clue of management strategy on economic feasibility.

Author contributions

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Conflict of interest

The author declares 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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Managingtomato bacterial wilt by suppressing *Ralstonia solanacearum* population in soil and enhancing host resistance through fungus-derived furoic acid compound

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Synthetic chemical pesticides are primarily used to manage plant pests and diseases, but their widespread and unregulated use has resulted in major health and environmental hazards. Using biocontrol microbes and their bioactive compounds is a safe and sustainable approach in plant protection. In this study, a furoic acid (FA) compound having strong antibacterial activity against soil-borne phytopathogenic bacterium *Ralstonia solanacearum* [causal agent of bacterial wilt (BW) disease] was isolated from *Aspergillus niger* and identified as 5-(hydroxymethyl)-2-furoic acid compound through spectroscopic analyses (liquid chromatography–mass spectrometry (MS), electron ionization MS, and NMR). The SEM study of bacterial cells indicated the severe morphological destructions by the FA compound. The FA was further evaluated to check its potential in enhancing host resistance and managing tomato BW disease in a greenhouse experiment and field tests. The results showed that FA significantly enhanced the expression of resistance-related genes (*PAL*, *LOX*, *PR1*, and *PR2*) in tomato and caused a significant reduction (11.2 $\log_{10}$ colony-forming units/g) of the *R. solanacearum* population in soil, resulting in the reduction of bacterial wilt disease severity on tomato plants and increase in plant length (58 $\pm$ 2.7 cm), plant biomass (28 $\pm$ 1.7 g), and root length (13 $\pm$ 1.2 cm). The findings of this study suggested that the fungus-derived FA compound can be a potential natural compound of biological source for the soil-borne BW disease in tomato.

KEYWORDS

antibacterial, soil-borne, plant disease, bio-control, natural product

Introduction

Tomato is one of the most commonly consumed vegetables and dominant crops in the world. The cultivation and the production of tomato play a vital role in food security as well as socioeconomic development of people at the national and local levels (Asgarian et al., 2016; Moola et al., 2021). China plays an important part in tomato production, having the largest tomato cultivation area with the highest yield in the world. The annual tomato yield of China is 64.27 million tons, which is almost equal to one-third of the global tomato yield (Cen et al., 2022). Over 40 diseases have been reported to threaten the tomato crop in China, with 10 of them, including bacterial wilt, causing extensive damage across the country (Chiwaki et al., 2005). Bacterial wilt, which is of soil-borne nature, is a serious disease affecting the quality and the quantity of tomato especially in southern China. The yield losses in tomato caused by *Ralstonia solanacearum* vary from 0% to 90% depending on the cropping pattern, pathogen strain, cultivar, soil type, and climate (Nion and Toyota, 2015).

The bacterial wilt pathogen *R. solanacearum* is a soil-borne phytopathogen that affects both non-solanaceous and solanaceous plants (Chandrashekara et al., 2012; Khan et al., 2021). This is a motile, strictly aerobic, G^-, and rod-shaped bacterium that has been reported to cause bacterial wilt disease in more than 180 plants of 45 plant families (Tahat and Sijam, 2010). In soil, the bacteria start an infection through the roots and severally affect the water transport by colonizing and blocking the xylem vessels, resulting in the development of wilting symptoms and signs including leaf yellowing, reduced growth, and death of the plants (Ahing and Wid, 2016; Iraboneye et al., 2021). This specific process of infection caused by *R. solanacearum* is known as vascular wilt disease and categorized as one of the most important bacterial diseases worldwide, causing severe losses to several economically important agricultural crops including tomato (Nion and Toyota, 2015).

Several studies have reported the difficulties of managing *R. solanacearum* because of its endogenous growth in plants, soil persistency in deeper layers, and water dispersal and its link with weeds (Nion and Toyota, 2015). Teli et al. (2018) also reported that management strategies such as crop rotation, field sanitation, and the use of disease-resistant cultivars have mostly failed to control *R. solanacearum*. Although synthetic chemicals have shown some effective results, they are linked with soil, underground water and air pollution, biodiversity loss, and residual toxicity impacts (Hassan and Chang, 2017; Fan et al., 2019; Malerba and Cerana, 2019). The adoption of alternative management techniques for BW management, such as the use of potentially effective biocontrol agents, has therefore gained more attention (Chamedjeu, 2018). However, there are issues with storage duration that come with using these microbes (Algam et al., 2010). In order to manage plant diseases, it is therefore necessary to search for alternative control measures, such as the use of naturally produced antimicrobial compounds or secondary metabolites (SMs) by microorganisms.

Among microbes, the fungus group has been reported to secrete a diversity of SMs; therefore, they have been considered a useful source to discover new biologically active compounds (Boustie and Grube, 2005). *Aspergillus* spp. are widely known for the production of chemically diverse SMs including cyclopentapeptide, pyranone, polyketide, and alkaloids that showed antiviral, anticancer, antioxidant, and antibacterial activities (Swathi et al., 2013; Holm et al., 2014; Ding et al., 2019). Several compounds isolated from *Aspergillus* spp. showed promising antibacterial activities. Diphenyl ether, isolated from *A. sydowii*, exhibited antibacterial activity against a range of bacteria (Liu et al., 2017). *Aspergillus* sp. produced an active compound, emodin, that showed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* (An et al., 2019).

This study was aimed to investigate the antibacterial compound produced by *Aspergillus* spp. against BW pathogen and evaluate it for the control of tomato BW disease. The most active antibacterial compound, identified as furoic acid (FA) compound, was found to have a destructive effect on bacterial cell morphology and caused an enhanced expression of disease resistance genes. FA was also active in decreasing the soil pathogen population and disease severity, thus resulting in improved plant growth.

Materials and methods

Microbes and culturing media

Previously identified fungus *A. niger* (obtained from College of Chemistry and Life Sciences, Chengdu Normal University, Chengdu, China) was used for the extraction of antibacterial compound. Potato dextrose agar and potato dextrose broth were used for culturing and for the seed broth preparation of *A. niger*. Solid-state fermentation (SFM) was used for *A. niger* fermentation. Beef extract peptone broth (BEPB) and agar medium were used for culturing *R. solanacearum* and for antimicrobial tests. The composition of each medium is presented in Supplementary Table S1. Bacterial wilt pathogen *R. solanacearum* (biovar 3, race 1) was cultured for its logarithmic phase on beef extract peptone solid medium at 30°C and 150 rpm/min for 16 h (Liu et al., 2016). The activated strain was grown to its logarithmic phase in 35 ml of BEPB under shaking incubation at 150 rpm/min and 30°C. The culture of the bacteria was centrifuged at 6,000 rpm, and by using sterilized distilled water the concentration of 10^8 CFU/ml was maintained.

Extraction and identification of antibacterial compound

Shallow plate fermentation method was used for fungal fermentation. SFM was inoculated with 15% (v/m) spore suspension of *A. niger* with 1×10^6 spores/ml concentration and incubated for 1 week at 28°C. After the production of spores, the solid matrix was dried for 36 h and ground into particles. The spore powder of *A. niger* was collected, and the spore concentration was measured as 2×10^8 spores/g. Crude ethanolic extract of spore powder was prepared by extracting spore powder four times for 4 h at 80°C using 10 L ethanol each time. In 300 ml of distilled water, 120 g of the extract was dissolved and then successively extracted four times with 300 ml of ethyl acetate (EA), petroleum ether (PE), and water to produce EA, PE, and water fractions. Separation of crude fractions of PE was done under the regulation of antimicrobial activity. Using silica gel column chromatography, the PE extract was fractionated, and the gradient was eluted. Thin-layer chromatography (TLC) analysis was used to evaluate the polarity composition of the fractions (200 ml), and those that have a similar composition were then concentrated and combined. The TLC analysis of the PE extract showed that it comprises different metabolites having various levels of bioactivity. Thus, additional fractionation was required to obtain more metabolites. Lastly, this separation procedure produced eight fractions (F1–F8) (Figure 1). The antimicrobial potential-guided separation of the most active fraction F8 was performed using reversed-phase silica gel column chromatography (CC), eluted with water–acetone (1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:1) to yield five sub-fractions (F8-1, F8-2, F8-3, F8-4, and F8-5). F8-1 (4.634 g) was again subjected to CC using silica gel (300–400 mesh) and eluted through petroleum ether–acetone (10:4). The further purification of sub-fraction was performed through silica gel CC with petroleum ether–acetone (5:2, v/v) as eluent to yield a white crystal compound (360 mg). The compound was subjected to liquid chromatography–mass spectrometry (MS), electron ionization MS, and NMR (^{1}H-NMR and ^{13}C-NMR) spectroscopic analysis, and by matching the obtained data with already published data, the structure of the compound was determined.

Antibacterial activity

The crude extract and the fractions were tested for antibacterial activity against BW wilt pathogen *R. solanacearum*. At first, 100 mg/ml concentration of crude extract and active fractions were prepared separately, and then 10 µl of this concentration was poured to 1 ml of BEPB. Furthermore, this was inoculated with 100 µl *R. solanacearum* suspension [10^8 colony-forming units (cfu)/ml] and incubated under shaking incubation at 28°C and 160 rpm to logarithmic phase. Dimethyl sulfoxide was kept as solvent control, and streptomycin was used as the positive control. Using a UV–vis spectrophotometer (Shenyang Ebetter Optics Co., Ltd.), the bacterial cell density was monitored at $OD_{\lambda max}$ (420 nm). The bacterial inhibition rate I_R was calculated by using the following formula: $I_R = (OD_0 - OD_1)/OD_0 \times 100$. OD_0 and OD_1 represent the $OD_{\lambda max}$ of treatment and blank control, respectively.

Analysis of bacterial cell morphology

Destruction in bacterial cell morphology caused by the most active antibacterial compound (FA) was investigated through scanning electron microscopy. Bacterial cells under the treatment of FA and control (without treatment) were fixed using 2.5% glutaraldehyde and phosphate buffer for 1.5 h at

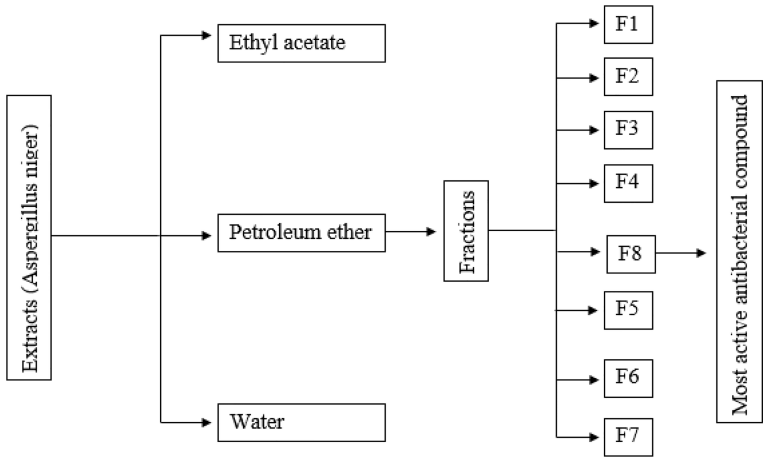

FIGURE 1
Schematic presentation of the isolation of the most active antibacterial compound.

45°C, followed by washing with phosphate buffer (0.1 M, pH 7.2) for 5 min and fixing in osmium tetroxide (OsO_4) for 1 h. Using phosphate buffer, the samples were washed, dehydrated in graded ethanol series (20%, 40%, 60%, 80%, and 90%) for 10 min each, and subjected to ethanol and CO_2 to reach the critical point. By using gold ions, the samples were coated and subjected to scanning electron microscopy (SEM) evaluation (Kamonwannasit et al., 2013).

Analysis of defense-related genes

Tomato plants (25 days old) were transplanted into sterilized culture medium pots (one plant per pot). Four treatments were maintained: T1, control plants without FA treatment and inoculation of pathogen (Ck); T2, plants inoculated with *R. solanacearum* suspension (10^8 cfu/ml) (Rs); T3, plants treated with 9 ml FA suspension (300 µg/ml) (FA); and T4, plants inoculated with 15 ml of pathogen suspension (10^8 cfu/ml) and treated with 9 ml FA suspension (300 µg/ml) (Rs+FA). According to the methods described by Kiefer et al. (2000), total RNA was isolated from the tomato plants. Briefly, 0.2 g of plant material was ground in liquid nitrogen to obtain tissue powder in which 1,000 µl TRIzol reagent was poured and mixed. The samples were incubated for 8 min on ice, followed by addition and mixing of 200 µl chloroform. The mixture was then centrifuged at 12,000 rpm for 12 min at 6°C. The obtained pellet was washed using ethanol (75%), mixed in 30 µl RNAse-free water, and stored at −80°C. The expression of defense-related genes (*PAL*, *LOX*, *PR1*, and *PR2*) was evaluated through real-time polymerase chain reaction (RT-PCR) using the isolated RNA from tomato plants under different treatments. The primers used for the analysis of defense-related genes are presented in Supplementary Table S2. For reference, Ubi3 was used. The RT-PCR reaction was conducted in 25 µl reaction mixture on DNA Engine Opticon2 Continuous Fluorescence Detection System. The composition of the reaction mixture and the operating conditions are given in Supplementary Table S3. RNAse-free water was used instead of cDNA for negative control. Following the extension stage, the fluorescence signal was evaluated immediately after 2-s incubation at 70°C, which prevents the possibility of primer dimer detection. The melting points of the PCR products were measured between 65°C and 95°C at the end of the cycles. Agar gel electrophoresis and melting curve analysis were used to confirm the specificity of the amplicons. The experiment was conducted in three replicates.

Pot experiment

A pot experiment was conducted in a greenhouse for evaluating the potential of FA to manage the bacterial wilt of tomato. Plastic pots (20 cm in diameter) were filled with 1 kg soil (sand/clay/silt = 25%:25%:50%) each, and 20-day-old tomato plants (cultivar Rio Grande) were transplanted (one plant/pot). In each pot, 15 ml of pathogen suspension (10^8 cfu) was added to initiate the bacterial wilt disease. After 3 days of inoculation with the pathogen, the rhizosphere of each plant was treated with 9 ml of sterilized water containing an increasing concentration of FA (control and 50, 100, 150, and 200 µg/ml). The treatments were applied through three holes surrounding the plant. Following horticultural recommendations, the plants were irrigated and fertilized for 50 days. Each treatment was applied in seven replicates using completely randomized design (CRD). The experiment was terminated after 50 days, and data were taken on the pathogen population in soil, disease severity, and plant growth parameters (plant length, root length, and plant biomass). Data on pathogen population and disease severity were converted to $\log_{10}$ and area under the disease progress curve (AUDPC) value, respectively, according to a previously described method (Khan et al., 2020). The experiment was repeated once, and the data of the two experiments were tested for significant difference and, in case of no significant difference, pooled for analysis.

Field experiment

Field evaluation of FA compound for controlling BW disease in tomato was tested in an experimental field at the College of Chemistry and Life Sciences, Chengdu Normal University, Chengdu 611130, China, in March 2021 and repeated in March 2022. The field area was divided into three sets of blocks, and every set contained three blocks, each with a size of 6 m^2. Tomato seedlings (20 days old) were transplanted in all blocks by maintaining two rows of tomato plants per block and eight plants in each row. The base of each plant in the two sets were treated with 100 ml of pathogen suspension (10^8 cfu/ml) mixed with 300 ml of water. The plants in one of these two sets were treated with 20 ml of FA suspension (400 µg/ml) at the rhizosphere (T1: Rs+FA), while the other set was kept as untreated inoculated control (T2: Rs). The plants in the third set of blocks were kept as untreated and un-inoculated control (T3: control). The experiment was terminated after 50 days, and data were taken on soil bacterial population, disease severity, and plant growth parameters (plant height, root length, and plant fresh biomass). Data on disease severity were converted to AUDPC value according to a previously described method. Soil bacterial population was calculated twice: once at the start after 24 h of pathogen inoculation (initial: Pi) and once at the end of the experiment (final: Pf). The difference between Pi and Pf was calculated and expressed as decrease in soil bacterial population. The experiment was repeated once, and the data of the two experiments were tested for significant difference and, in case of no significant difference pooled for analysis.

Statistical analysis

The experiments were conducted using CRD in the laboratory and greenhouse, while field tests were evaluated using randomized complete block design. The data were analyzed through Statistical Analysis System software version 8.0. Analysis was done using one-way analysis of variance. Tukey's multiple-range test ($P < 0.05$) was used to evaluate the significant differences among treatment means.

Results

Antibacterial evaluation and identification of antibacterial compound

Different solvent extracts of *A. niger* were tested for the inhibition of *R. solanacearum* growth. Among different extracts, the petroleum ether extract showed the highest antibacterial activity that was equal to the positive control streptomycin. The inhibition rate of petroleum ether extract and streptomycin was 89.4% and 91.3%, respectively, followed by ethyl acetate extract and aqueous extract that showed 39.5% and 23.6% inhibition rate, respectively (Figure 2A). Based on the maximum inhibition rate, petroleum ether extract was selected for further fractionation and analysis of antibacterial compound. The fractionation of petroleum ether extract resulted in eight fractions showing varying degrees of inhibition rate against *R. solanacearum*. Among these eight fractions, F4, F5, and F8 showed a clearly higher antibacterial potential compared with others, and among these three fractions, significantly highest inhibition rate of 90.2% was shown by F8, which was similar to that obtained by positive control which indicated the presence of antibacterial compounds in these three fractions and especially in F8 (Figure 2B). As the fractions F4 and F5 showed inhibition rates of less than 60% and F8 only exhibited more than 90% inhibition rate, F8 was further processed for separation and purification of the antibacterial compound. Finally, a compound of white crystal nature was obtained. Based on their spectroscopic data, it was identified as FA compound—5-(hydroxymethyl)-2-furoic acid—with molecular formula $C_6H_6O_4$ (Supplementary Figures S1, S2).

Analysis of bacterial cell morphology

Bacterial cell morphology under treatment of FA compound and without treatment was observed in SEM analysis. The micrographs clearly showed severe destructions in morphology of FA-treated bacterial cells. Compared with the control (Figure 3A), the FA-treated cells were swollen and the membranes were disrupted (Figure 3B). Leakage of cell content was obvious in the damaged cells, whereas in the untreated control the cells were in uniform morphology with a plain rod shape.

Analysis of resistance genes

The expression of host defense-related genes (*PAL*, *LOX*, *PR1*, and *PR2*) in tomato plants was analyzed through RT-PCR after 30, 60, 90, and 120 h of pathogen inoculation under FA treatment (Figure 4). The results showed that the FA-treated pathogen-inoculated plants showed an induced expression of resistance genes (*PAL*, *LOX*, *PR1*, and *PR2*) more than the basal level of expression in other groups where the plants were treated with pathogen alone, FA only, or un-inoculated and untreated control pants (Ck). The induction of *PAL* and *PR1* genes was highest at 5.7- and 20-fold, respectively, at 120 h, while genes *LOX* and *PR2* showed a maximum expression of 9.5- and 22-fold at 90 h, respectively, although plants with only pathogen inoculation or treated only with FA also showed an induced expression of these genes as compared with the control, but at a significantly lower rate and fold than the pathogen-inoculated plants that were treated with FA.

Greenhouse evaluation

Effect of FA compound on the growth of *R. solanacearum*-inoculated tomato plants

The potential of FA compound for controlling BW disease in tomato was investigated in a pot experiment. The results regarding plant growth parameters showed FA application to enhance plant growth (Table 1). Compared with untreated control plants, the plants treated with FA showed significantly higher plant length, root length, and plant biomass. The plant-growth-promoting effect of FA under bacterial wilt stress was dependent on its concentration. The lowest concentration of 50 µg/ml was not effective; however, at higher concentrations of 100, 150, and 200 µg/ml, FA application significantly enhanced the plant growth. The maximum plant length (64.4 ± 3.8 cm), biomass (74.4 ± 4.4 g), and root length (33.7 ± 2.3) were obtained by 200 µg/ml concentration, followed by statistically similar results obtained by 150 µg/ml concentration. Similar treatment effects were noticed in the repeated experiment (Table 1).

Effect of FA compound on AUDPC and soil suppression of *R. solanacearum* population

The application of FA compound also reduced the bacterial wilt disease severity and pathogen population in soil (Figure 5). Consistent with the results of the plant growth parameters, the lowest concentration of 50 µg/ml was not effective in reducing disease severity and pathogen population, while the highest two concentrations of 150 and 200 µg/ml gave similar results. The application of FA at 200 µg/ml concentration showed the highest

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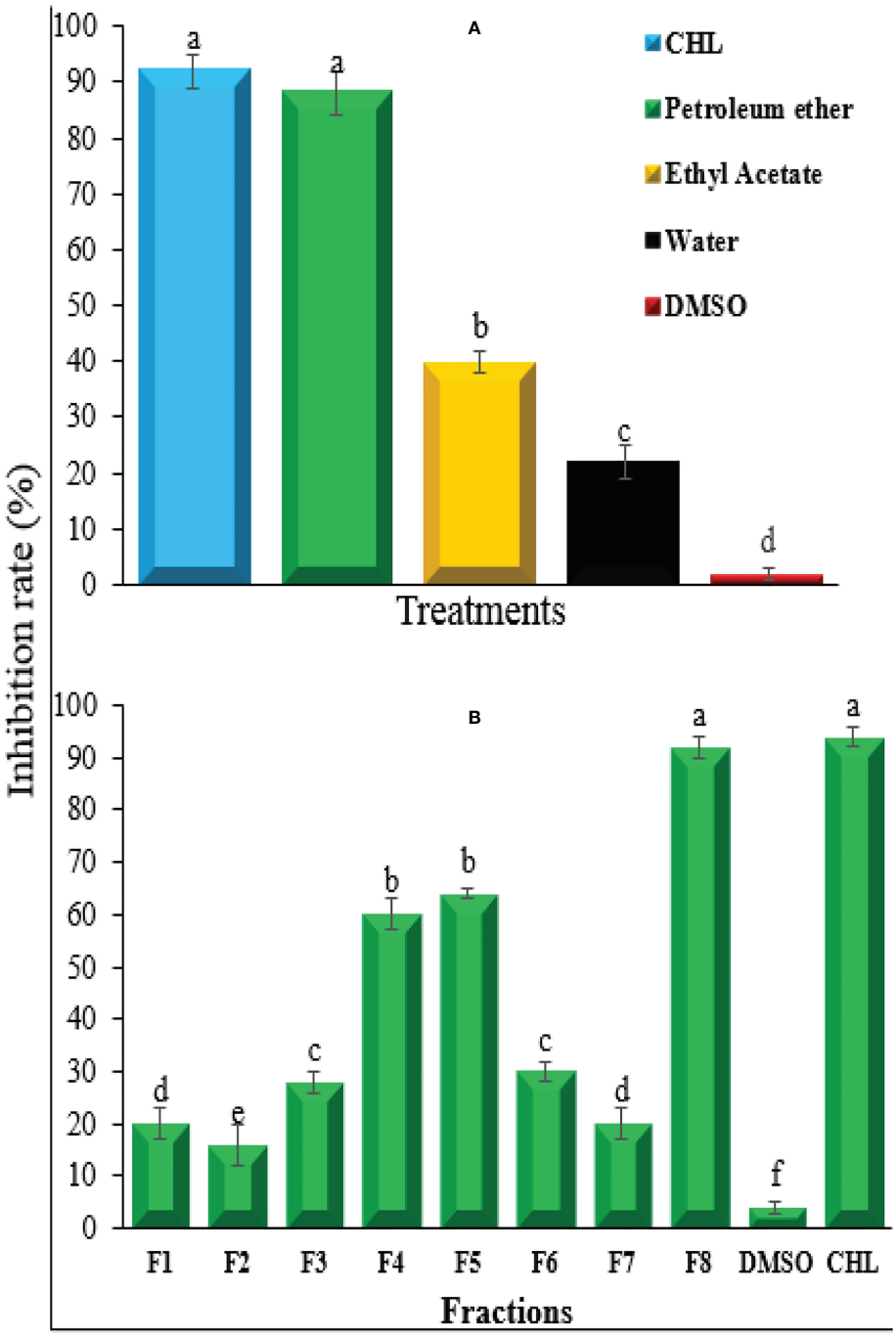

FIGURE 2
Growth inhibition rate of **(A)** niger extract using different solvents **(A)** and different fractions of petroleum ether extract **(B)** against bacterial wilt pathogen *Ralstonia solanacearum*. CHL, chloramphenicol; DMSO, dimethyl sulfoxide. Bars represent the standard error. Lower case lettering shows the significant difference among the treatments.

reduction in *R. solanacearum* population (3.1 $\log_{10}$ cfu/g) and the lowest AUDPC value (1850) followed by statistically similar results obtained by 150 µg/ml concentration. The treatments showed the same effects in the repeated experiment.

Field evaluation

Consistent with the greenhouse results, the application of FA also reduced the bacterial wilt disease severity and soil

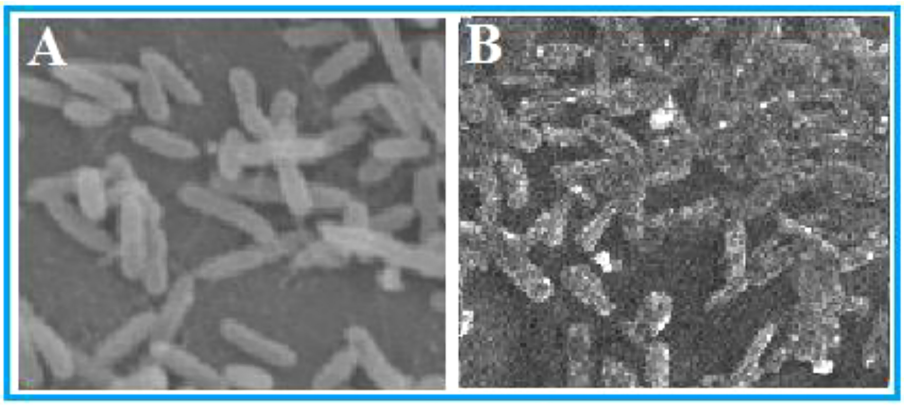

FIGURE 3
Morphological observations of *Ralstonia solanacearum* in SEM analysis. **(A)** Control: untreated bacterial cells and **(B)** cells treated with furoic acid compound.

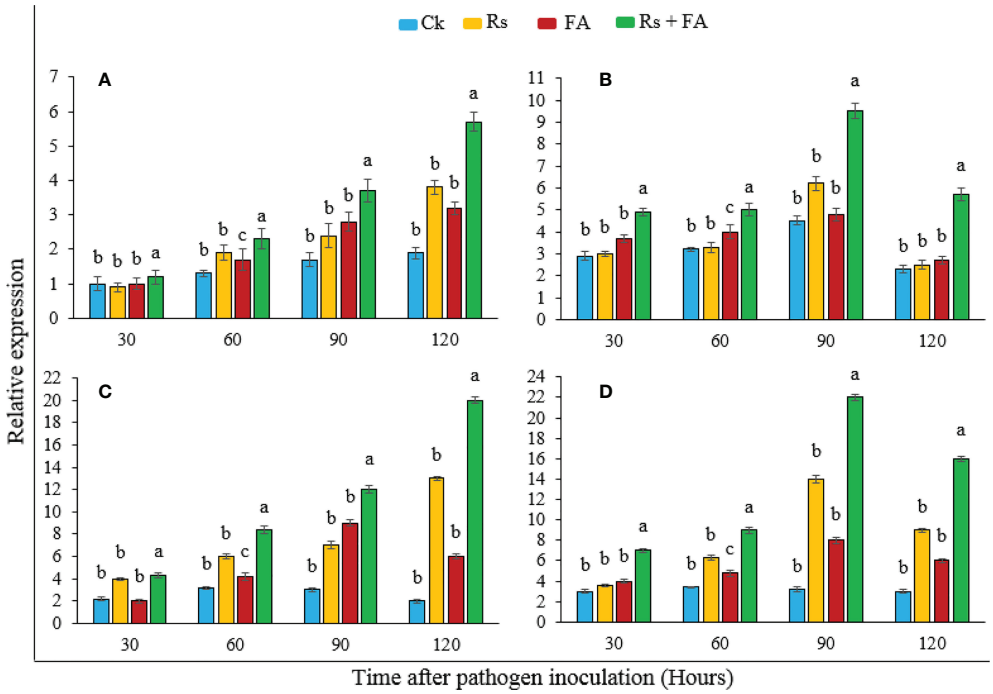

FIGURE 4
Relative expression of defense-related genes of tomato plants treated with furoic acid compound at different durations after pathogen inoculation. **(A)** *PAL*, **(B)** *LOX*, **(C)** *PR1*, and **(D)** *PR2*. Bars represent the standard error. Lower case lettering shows the significant difference among the treatments.

population of *R. solanacearum* in field evaluation (Figure 6). The plant growth was also enhanced by the application of FA (Table 2). Compared with untreated inoculated control plants, the plants treated with FA exhibited a significantly lower AUDPC value (1,670) and the highest decrease (11.2 $\log_{10}$ cfu/g) in soil population of *R. solanacearum*. The inoculated plants under the treatment of FA had improved plant length (58 ± 2.7 cm), plant biomass (28 ± 1.7 g), and root length (13 ± 1.2 cm) as compared with the untreated inoculated plants. The results of the FA treatment showed a similar plant growth as that recorded for untreated and un-inoculated healthy control plants.

TABLE 1 Effect of different concentrations of FA on tomato plant growth inoculated with bacterial wilt pathogen in pot experiment.

Treatments (FA concentration, µg/ml)	Plant length (cm)	Biomass (g)	Root length (cm)
March 2021			
Control	26.4 ± 2.3 c	30.6 ± 1.8 d	11.1 ± 1.1 c
50	28.2 ± 2.7 c	30.3 ± 2.6 d	12.2 ± 1.6 c
100	42.7 ± 3.2 b	39.8 ± 2.9 c	18.2 ± 2.1 b
150	59.3 ± 4.2 a	68.3 ± 4.2 b	30.1 ± 2.8 a
200	61.4 ± 3.8 a	74.4 ± 4.4 a	33.7 ± 2.3 a
March 2022			
Control	20.4 ± 2.2 c	25.4 ± 2.2 c	8.8 ± 0.8 c
50	23.3 ± 2.5 c	27.4 ± 1.8 c	10.2 ± 1.0 c
100	46.7 ± 3.5 b	34.2 ± 2.8 b	14.3 ± 1.6 b
150	57.2 ± 3.2 a	62.6 ± 3.2 a	26.5 ± 2.0 a
200	60.6 ± 4.3 a	65.3 ± 3.5 a	28.3 ± 2.5 a

Each value is a mean ± SE. Means with the same letters in a column are significantly different according to Tukey's multiple-range test ($P < 0.05$). The experiment was conducted twice: once in March 2021 and repeated in March 2022.
FA, furoic acid.

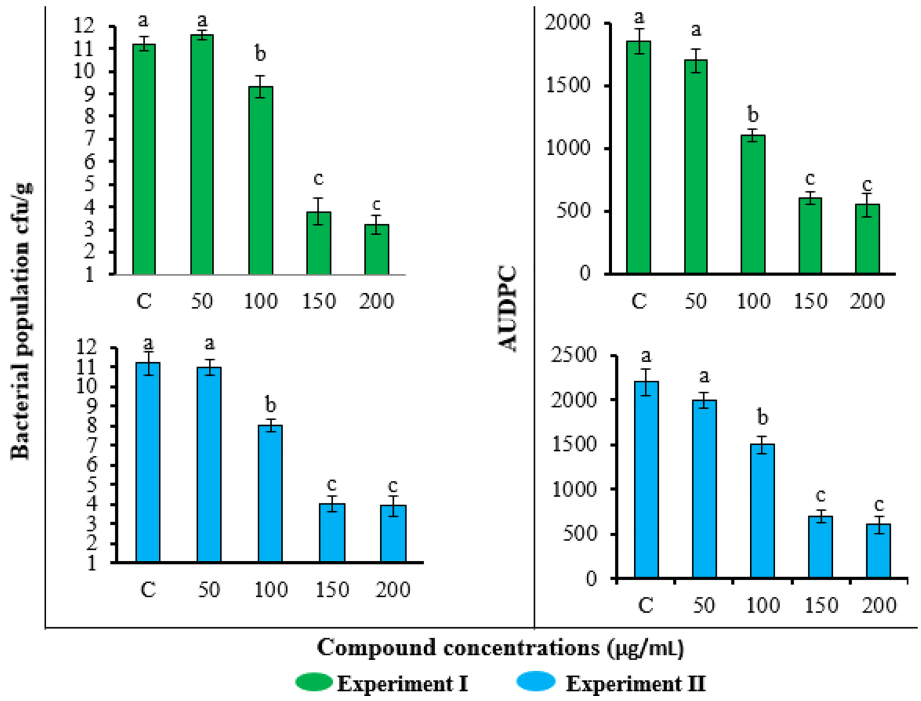

FIGURE 5
Effect of different concentrations of furoic acid on *Ralstonia solanacearum* population in soil and disease severity area under the disease progress curve value in pot experiment. C, control plants without any treatment. Bars represent the standard error. Lower case lettering shows the significant difference among the treatments.

Discussion

Alternative plant disease and pest control methods are being developed all over the world to reduce the use of synthetic agricultural pesticides. This will save humans and protect the environment from the harmful effects of synthetic chemicals. The investigation and utilization of naturally produced microbial products is one way to do this. Previous studies have successfully shown that, by employing natural products from plants or microbes, they were able to reduce

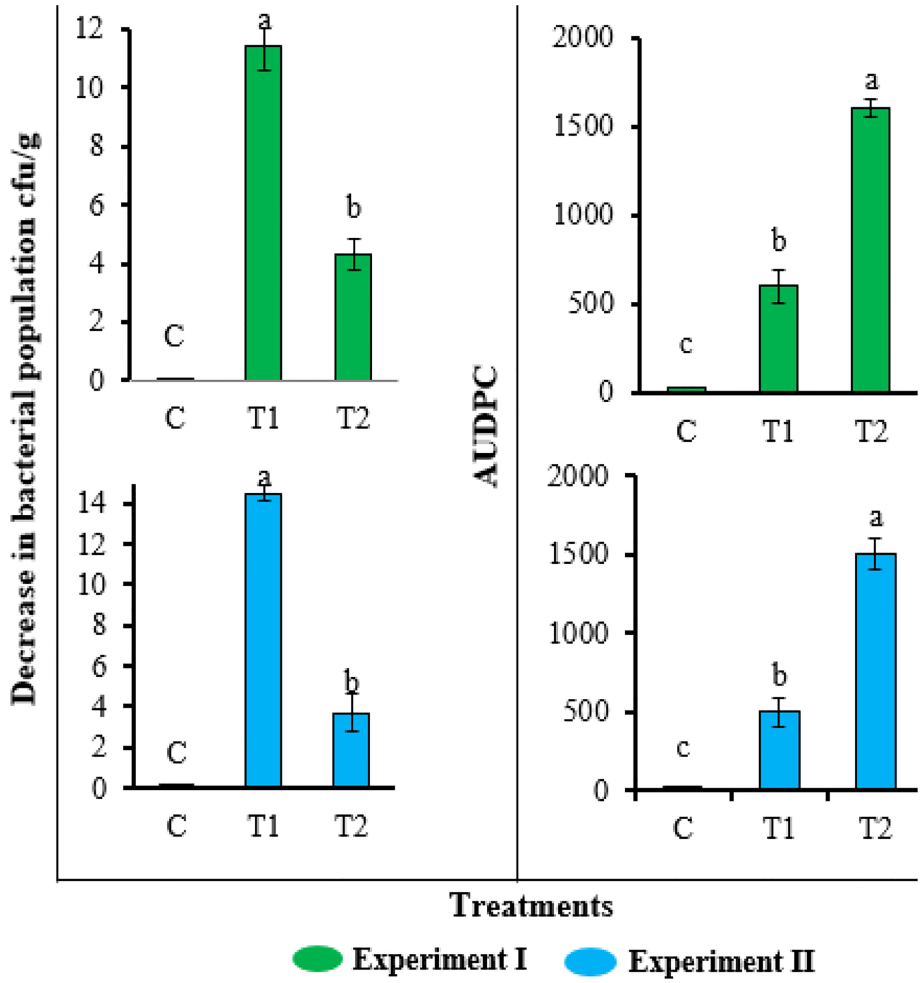

FIGURE 6
Effect of different concentrations of furoic acid (FA) on *Ralstonia solanacearum* population in soil and disease severity area under the disease progress curve value in field experiment. C, control plants; T1, plants inoculated with *R. solanacearum* and treated with FA (Rs + FA); T2, plants inoculated with *R. solanacearum* (Rs). Bars represent the standard error. Lower case lettering shows the significant difference among the treatments.

TABLE 2 Effect of different concentrations of FA on tomato plant growth inoculated with bacterial wilt pathogen in field experiment.

Treatments (compound concentration, µg/ml)	Plant length (cm)	Biomass (g)	Root length (cm)
March 2021			
Control	60 ± 3.6 a	69 ± 2.8 a	35 ± 2.3 a
Rs+FA	58 ± 2.7 a	70 ± 3.4 a	34 ± 1.8 a
Rs	26 ± 2.1 b	28 ± 1.7 b	13 ± 1.2 b
March 2022			
Control	52 ± 4.2 a	61 ± 3.2 a	28 ± 3.4 a
Rs+FA	54 ± 2.9 a	60 ± 2.6 a	30 ± 2.8 a
Rs	29 ± 1.8 b	32 ± 2.4 b	11 ± 1.4 b

Each value is a mean ± SE. Means with the same letters in a column are significantly different according to Tukey's multiple-range test ($P < 0.05$). The experiment was conducted twice: once in March 2021 and repeated in March 2022.
Rs, Ralstonia solanacearum; FA, furoic acid.

plant diseases. Among microbes, fungi have provided humanity with a variety of diverse bioactive compounds, thus becoming an effective group for research and development of new antimicrobial metabolites (Swathi et al., 2013). *Aspergillus* spp. has a variety of enzymes and produces several kinds of bioactive compounds. In this study, *A. niger* was investigated for the production of an antibacterial compound against *R. solanacearum*. Solvents of different polarities—water > ethyl acetate > petroleum ether—were used to extract the metabolites of *A. niger* spore powder. The metabolic extract obtained in petroleum ether demonstrated the highest antibacterial activity against *R. solanacearum*. Considering the presence of a main antibacterial compound in petroleum ether extract, it was further subjected to separation. Eight fractions, F1–F8, were obtained from petroleum ether extract, the most active fraction was analyzed, and FA compound was identified to have the highest antibacterial activity after spectroscopic analysis. The purified antibacterial FA compound was further evaluated for its effect on bacterial morphological destruction and the potential to manage the bacterial wilt of tomato in greenhouse and field conditions.

The results of the SEM analysis clearly demonstrated the membrane-damaging effect of FA compound against *R. solanacearum*. This compound is categorized as a furan ring derivative. Several studies reported the antimicrobial effects of furan ring derivatives against a range of pathogenic microbes such as *Bacillus megaterium*, *Alternaria alternate*, *Escherichia coli*, and *S. aureus* (Dai et al., 1989; Lewkowski, 2003; Zhang et al., 2019). The antibacterial effect could be ascribed to the enhanced permeability of the FA compound to the pathogen cell membranes, which causes membrane rupture and protein alterations. This eventually causes a disruption in cellular metabolism, which results in bacterial cell death (Premanathan et al., 2011; Vani et al., 2011; Akbar et al., 2020). The SEM results of our study also confirm this mechanism of antibacterial activity.

The results from plant experiments revealed that the application of FA caused a significantly enhanced expression of defense-related genes in tomato plants and suppressed the *R. solanacearum* population in the soil, resulting in the reduction of bacterial wilt disease severity and an increase in plant growth. Plant defiance can be improved through the use of exogenous elicitors in the form of plant- or microbe-based natural compounds. Fungal microbes have a large number of natural antimicrobial compounds that may act as natural elicitors, which act as host resistance inducer to enhance plant defense against pathogens. Several antimicrobial compounds such as aspyrone, γ-dehydrocurvularin, and penipratynolene were isolated previously from *Aspergillus* fungi against soil-borne pathogens (Kimura et al., 1996; Kusano et al., 2003). Kimura et al. (2007) isolated 5-hydroxymethyl-2-furoic acid from *Aspergillus* sp. and reported its antimicrobial activities against the root knot nematode. They also reported the improved plant growth of several crops when 5-hydroxymethyl-2-furoic acid was applied.

The large volume of the antimicrobial compound required for field broadcasting can be reduced by applying it selectively to the plant rhizosphere. In seed-bed soils for disease-free seedlings and for small-scale agricultural systems like those used in many developing countries, this selective treatment can be easily practiced. The use of this furoic acid compound derived from *Aspergillus* fungus has a high potential for consideration as an active integrated disease management component of BW management. As a natural product, it is environment-friendly, and it is difficult for pathogens to resist it. This compound can be used in combination with other IDM strategies to enhance the management of the bacterial population in the soil—for example, BW pathogen is killed by soil temperatures of 45°C or above for about 2 days (Kangkiattikajorn et al., 2007). The soil application of this compound with plastic mulching during scorching sunny days before tomato transplantation could effectively reduce the bacterial population in soil.

Conclusion

The findings of this study revealed the strong antibacterial potential of furoic acid compound derived from *A. niger* against soil-borne bacterium *R. solanacearum*, the causal agent of bacterial wilt disease. The compound caused severe morphological destructions to *R. solanacearum*. The application of this compound to the pathogen-infested soil transplanted with tomato plants enhanced the resistance of the tomato plants by increasing their expression of defense-related genes. This compound, when applied to the soil, effectively reduced the soil population of *R. solanacearum* and decreased the bacterial wilt disease severity, resulting in the improvement of plant growth. The current study suggests that the furoic acid compound has potential applications in the management of bacterial wilt disease in tomato and possibly in other crops.

Data availability statement

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

Author contributions

MY and SL: conceptualization, writing, review, editing, and supervision. MY and SL: writing—original draft and funding acquisition. JH and QY: methodology. MY, HF, and JH: investigation. QY: resources. 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/fpls.2022.1064797/full#supplementary-material

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Bacillus cereus EC9 protects tomato against Fusarium wilt through JA/ET-activated immunity

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The mechanisms of action and the limitations of effectiveness of natural biocontrol agents should be determined in order to convert them into end products that can be used in practice. Rhizosphere *Bacillus* spp. protect plants from various pathogens by displaying several modes of action. However, the ability of *Bacillus* spp. to control plant diseases depends on the interaction between the bacteria, host, and pathogen, and the environmental conditions. We found that soil drenching of tomato plants with the non-antifungal *Bacillus cereus* strain EC9 (EC9) enhances plant defense against *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). To study the involvement of plant defense-related phytohormones in the regulation of EC9-activated protection against *Fol*, we conducted plant bioassays in tomato genotypes impaired in salicylic acid (SA) accumulation, jasmonic acid (JA) biosynthesis, and ethylene (ET) production, and analyzed the transcript levels of pathways-related marker genes. Our results indicate that JA/ET-dependent signaling is required for EC9-mediated protection against *Fol* in tomato. We provide evidence that EC9 primes tomato plants for enhanced expression of *proteinase inhibitor I* (*PI-I*) and *ethylene receptor4* (*ETR4*). Moreover, we demonstrated that EC9 induces callose deposition in tomato roots. Understanding the involvement of defense-related phytohormones in EC9-mediated defense against Fusarium wilt has increased our knowledge of interactions between non-antifungal plant defense-inducing rhizobacteria and plants.

KEYWORDS

***Fusarium oxysporum* f. sp. *lycopersici*, rhizobacteria, biological control, defense priming, induced resistance, phytohormones**

1 Introduction

Tomato (*Solanum lycopersicum* L.), as a highly important crop for direct consumption and raw material for various products, has been cultivated extensively in many parts of the world for decades (Costa and Heuvelink, 2018). Fusarium wilt of tomato caused by the soil-borne ascomycete fungus, *Fusarium oxysporum* Schlecht f. sp. *lycopersici* (*Fol*), is one of the most destructive diseases of tomato (McGovern, 2015; Nirmaladevi et al., 2016). *Fol* is a hemibiotroph that penetrates through wounds at the root tip and lateral root formation zones. It colonizes the apoplast of the root cortex, and invades the xylem vessels, leading to wilting, yellowing, vascular discoloration, growth distortion, and eventually to the death of the plant (Di Pietro et al., 2003; Michielse and Rep, 2009; de Lamo and Takken, 2020). Chemical control of Fusarium wilt of tomato is not effective due to poor delivery of conventional fungicides to the xylem vessels, not to mention difficulties with soil treatments. Additionally, *Fol* chlamydospores are highly infectious and can survive on plant debris for long time, making disease control very challenging (De Cal et al., 1997; Hou et al., 2020). Meanwhile, one way of controlling *Fol* is through resistance breeding. Three Secreted In Xylem (SIX) effectors (Six1, Six3, and Six4), are required for virulence of *Fol* (Rep et al., 2005; Houterman et al., 2008; Houterman et al., 2009). These effectors, also referred to as Avr3, Avr2, and Avr1, are recognized by the intracellular resistance (R) proteins Immunity-3 (I-3), I-2, and I, respectively, in tomato (de Sain and Rep, 2015). Accordingly, *Fol* is differentiated into races 1, 2, and 3 based on its ability to overcome the corresponding *R* genes (Biju et al., 2017). However, when these *R* genes are combined in cultivars, novel *Fol* strains overcoming these may emerge (Takken and Rep, 2010).

In recent years, biological control of Fusarium wilt of tomato has attracted considerable attention (La Torre et al., 2016; Sallam et al., 2019; de Lamo and Takken, 2020; Doan et al., 2020; Vinchira-Villarraga et al., 2021). Several species of the bacterial genus *Bacillus* have proved to be promising biocontrol agents, since they protect through several modes of action, such as competition, parasitism, antibiosis, and induced resistance (Akram et al., 2016; Elanchezhiyan et al., 2018; Bhattacharya et al., 2019; Medeiros and Bettiol, 2021; Zhang et al., 2021). For the latter mechanism, some *Bacillus* species can activate immediate defense responses, predisposing the plant to react faster and stronger to subsequent pathogen attack, a mechanism known as defense priming (Tonelli and Fabra, 2014; Wang et al., 2014; Mauch-Mani et al., 2017).

Plants sense an attacking pathogen through recognition of microbe-associated molecular patterns (MAMPs), leading to pattern-triggered immunity (PTI). As a counterattack, effector proteins secreted by the pathogen hamper PTI to re-establish susceptibility. In turn, the effectors can be recognized by specific plant R receptors resulting in effector-triggered immunity (ETI), which is associated with local programmed cell death (Ngou et al., 2022). In addition, systemic defense pathways can be induced in the plant. Two main such interacting defense pathways have been described: "systemic acquired resistance" (SAR) and "induced systemic resistance" (ISR) (Conrath, 2006; Pieterse et al., 2014). Salicylic acid (SA), synthesized *via* isochorismate synthase or phenylalanine ammonium lyase, is a key phytohormone regulating the SAR reactions initiated by pathogens and various natural and synthetic elicitors (Ngou et al., 2022). On the other hand, jasmonic acid (JA) and ethylene (ET) mediate ISR, which is typically activated by nonpathogenic (and beneficial) rhizobacteria such as *Bacillus* spp. and *Pseudomonas* spp. (Bakker et al., 2007; Blake et al., 2021). NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) regulates SAR by recruiting TGACG-Binding (TGA) transcription factors to, for instance, activate *PR* genes that encode antimicrobial proteins and other genes that act as key elements in the crosstalk between SA and JA/ET-mediated reactions (Backer et al., 2019).

Bacillus spp. activate ISR in many plant species such as *Arabidopsis thaliana* (Nie et al., 2017), tobacco (Huang et al., 2012), tomato (Yoshida et al., 2019), maize (Xie et al., 2019), and rose (Chen et al., 2020). *Bacillus*-mediated defense responses depend not only on the transduction of JA/ET signaling, but also on SA-dependent signaling (Niu et al., 2011; Xie et al., 2021; Chaparro-Encinas et al., 2022). In addition, MAMPs and several metabolites produced by *Bacillus* spp., including well-known antimicrobial compounds such as surfactin and fengycins, have been shown to boost plant defense by activating ISR (Ongena et al., 2007; Jiang et al., 2016). Furthermore, volatile organic compounds from *Bacillus* spp. can also activate ISR (Rudrappa et al., 2010; Yi et al., 2016). Overall, the ability of *Bacillus* spp. to control plant diseases depends on the interaction between the bacteria, host, and pathogen, and the environmental conditions.

Recently, we reported that the antifungal *B. siamensis* strain DD6 and the non-antifungal *B. cereus* strain EC9 effectively suppressed root and stem rotting disease caused by *F. oxysporum* in the ornamental plant Kalanchoe (*Kalanchoe blosssfeldiana*) (Madriz-Ordeñana et al., 2022). Furthermore, expression analysis of the defense-related genes *PR1* and *LOX2* revealed activation of a primed state in Kalanchoe when the roots were colonized by EC9. These results prompted us to study the involvement of defense-related phytohormones in EC9-activated protection in the *Fol*/tomato pathosystem. In this study, we conducted plant bioassays in tomato genotypes impaired in SA accumulation, JA biosynthesis, and ET production, and analyzed the expression levels of pathway-related marker genes. We provide evidence indicating that JA/ET signaling is necessary for the biocontrol of Fusarium wilt of tomato activated by EC9. Furthermore, we show that EC9 primes the expression of JA/ET-related marker genes and induces callose deposition in tomato roots.

2 Materials and methods

2.1 Plant materials and growth conditions

The *NahG* transgenic tomato line (cv. Moneymaker), impaired in SA accumulation by expressing the *salicylate hydroxylase* gene (Brading et al., 2000), was kindly provided by Prof. Jonathan Jones (The Sainsbury Laboratory, Norwich, UK). The JA-deficient *spr2* (*suppressor of prosystemin-mediated responses2*) mutant (Howe and Ryan, 1999) and the corresponding wild-type cv. Castlemart were kindly provided by Dr. María Fernanda López Climent (Jaume I University, Castelló de la Plana, Spain). Transgenic *ACD* tomato, constitutively overexpressing (OE) the bacterial ACC deaminase gene resulting in compromised ET production (Klee et al., 1991), and the corresponding wild-type cv. UC82B were obtained from Dr. Birgit Jensen (University of Copenhagen, Denmark). The cv. Moneymaker was routinely used to analyze the expression pattern of marker genes for relevant phytohormones and to monitor callose deposition in roots. In all experiments, seeds were surface sterilized with 2.5% NaClO (v/v) and 0.05% Tween 20 for 15 min and 70% EtOH (v/v) for 1 min, followed by at least 3 times washing in distilled water. Sterilized seeds were planted in plastic pots containing peat moss substrate (Klasmann TS1, Germany). Plants were grown at 23°C on a 16/8 h day/night cycle with supplemental lightning of 160 µmol m^{-2} s^{-1} in the greenhouse.

2.2 Preparation of bacterial suspensions and fungal inoculum

B. cereus EC9 and *B. siamensis* DD6 strains were previously isolated from Kalanchoe-associated materials (Madriz-Ordeñana et al., 2022). The bacterial strains were grown on Luria-Bertani (LB) (Sigma-Aldrich, Germany) agar plates at 28°C for 24 h and a single colony was used to inoculate 50 ml of LB. The cultures were incubated at 28°C with a constant shaking at 125 rpm for 24 h. The cells were collected by centrifugation at 6000x g for 10 minutes and resuspended in 10 mM $MgCl_2$.

Fol 4287 (race 2) was kindly provided by Prof. Martijn Rep (University of Amsterdam, The Netherlands). The fungus was cultured on potato dextrose agar (PDA) (Scharlau Chemie, Spain) at 25°C for 7 days. Mycelial plugs (9 mm ø) were transferred to 100 ml of minimal medium (1% KNO_3, 3% sucrose and 0.17% Yeast Nitrogen Base without amino acids and ammonia) and incubated at 25°C with shaking at 150 rpm for 5 days. The spore suspension was filtered through four layers of sterile cheesecloth, centrifuged at 5000x g for 10 min and rinsed with sterile distilled water. The final concentration was adjusted to $1x10^7$ spores ml^{-1} (Di et al., 2017).

2.3 Antagonistic assay of *Bacillus* spp. against *Fol*

The agar disk diffusion method described by Shehata et al. (2016) was applied to test the *in vitro* antagonistic efficacy of DD6 and EC9. Briefly, the spore suspension of *Fol* prepared as described above was adjusted to a final concentration of $1x10^5$ spores ml^{-1} in freshly prepared and cooled PDA (~50°C) and poured into each sterile Petri dish. One hundred microliters of each bacterial LB culture at OD_{600} 0.25, 0.5, 0.75, and 1 were pipetted into 15 mm ø wells created in the PDA plates using a sterilized glass tube. Sterile LB medium was used as control. The antifungal activity of strains was determined by measuring the distance between opposed edges of the inhibition zone of each well (excluding the diameter of wells) after 5 days of incubation.

2.4 Protection efficacy *in planta* of strains DD6 and EC9 against *Fol*

Ten-days-old tomato plants were treated with 5 ml cell suspension of each strain at OD_{600} of 0.3 or 10 mM $MgCl_2$ (mock) by soil drenching. After one week, the plants were up-rooted and either inoculated with *Fol* spore suspension ($1x10^7$ spores ml^{-1}) or treated with water (for mock inoculation) by the root dipping method (Constantin et al., 2020). The inoculated plants were replanted on the same peat substrate. Disease severity (DS) was scored 3 weeks after inoculation according to the following scale (Constantin et al., 2019): DS0 = no symptoms; DS1 = brown vessel at the crown level; DS2 = one or two brown vessels at the cotyledon level and no outer symptoms; DS3 = three or more brown vessels with external wilting and growth distortion symptoms, DS4 = all vessels brown and severe wilting/stunning symptoms, DS5 = plant is dead.

For *Fol* recovery assay, approximately 3 mm thick tomato stems were sectioned using a microtome blade at the levels of the crown, cotyledon, 2nd and 4th nodes, and surface sterilized with 5% NaClO (v/v) for 15 min and 70% EtOH (v/v) for 1 min, then rinsed with sterile distilled water for 3 min at least three times. After air drying, the stem sections were placed on PDA medium under sterile conditions. The plates were incubated for 5 days at 25°C and the percentage of *Fol* colonization was expressed based on the outgrowth of fungal mycelium from stem sections (Di et al., 2017).

2.5 Quantification the biomass of *Fol* in tomato plants

To determine the relative amount of *Fol* biomass in the vascular tissue, sections of stems taken between the crown and the cotyledon of individual plants were used for genomic DNA

extraction using the Plant DNeasy kit (Qiagen GmbH, Germany). *Fol* biomass estimation was performed by qPCR on the extracted DNA using tomato and fungal specific primers (Supplementary Table S1) in the LightCycler96 System (Roche Diagnostics GmbH, Germany). PCR reactions were performed using the HOT FIREPol EvaGreen qPCR Mix Plus following the conditions recommended by the manufacturer (Solis BioDyne, Tartu, Estonia). Fungal biomass was estimated by calculating the ratio of fungal DNA to tomato DNA using serial dilutions from 0.002 to 20 ng of pure genomic DNA of each organism. Standard curves were fit by linear regression, and the amount of DNA was estimated by tracing the Ct-values against the corresponding known amounts of DNA.

2.6 Analysis of defense gene expression

Total RNA was extracted from the segment of the tomato stem between the cotyledons and the crown at 3, 7, 14 dpi using TRIzol reagent (TRI Reagent, R2050-1-200, Zymo Research) following the instructions provided by the manufacturer. Two stems randomly selected from different plants were pooled for RNA extraction. First-strand cDNA was synthesized from 1 µg RNA using a RevertAid First Strand cDNA Synthesis Kit (K1622, ThermoFisher Scientific, Walthamm MA, USA) with oligo(dT) primers, and the qRT-PCR assay was performed using EvaGreen 2× qPCR MasterMix (MasterMix-R, abm, Canada) in a PikoReal 96 real-time PCR system (Thermo Scientific, Burlington, Canada). Twenty microliter reaction mixtures consisted of 10 µl EvaGreen 2X qPCR MasterMix, volume of cDNA corresponding to 250 ng in final concentration, and 0.3 µM of reverse and forward primers. The conditions for PCR cycling were as follows: 95°C for 15 min and 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s. Relative transcript levels were calculated as described by Pfaffl (2001) and the values were normalized to the internal reference gene *α-tubulin*. The primer sequences for qRT-PCR used are listed in Supplementary Table S1.

2.7 Visualization of callose deposition in the roots

Sterilized tomato seeds were grown in vertically placed square Petri dishes, containing ½ x Murashige and Skoog medium (containing 1% sucrose) supplemented with 0.8% agar (pH: 5.7), in a growth chamber at 20°C and a 16/8 h day/night cycle (200 µmol m^{-2} s^{-1}). Seven-days-old tomato seedlings were lifted from the plates and the roots were dip treated with EC9 suspension or mock for 1 min. and returned to growth media. Twenty-four hours after treatment, the roots were dip-inoculated with *Fol* conidia suspension or sterilized water for 1 min. and replated. Roots were collected 24 h after inoculation and fixed in acetic acid:ethanol (1:3 v/v) for 4 h, followed by staining with aniline blue (0.1% w/v) in 150 mM K_2HPO_4 overnight in the dark (Schenk and Schikora, 2015). The roots were then mounted on a microscope slide and examined using a Leica DM 5000B fluorescence microscope using a DAPI filter. The number of callose spots was determined in the root segment between 1 and 2 cm from the primary tip.

2.8 Statistical analysis

All statistical analyzes were performed with Graph Pad Prism v.9 (San Diego, CA, USA) and SPSS v.25 (IBM, Armonk, NY, USA) software. Normality and homogeneity of variance were tested by the Shapiro-Wilk test. The effects of treatments on disease incidence, fresh weights, fungal recovery, *in vitro* antifungal effect, relative quantification of *Fol*, callose deposition were analyzed with the Student's *t*-test. If the data were not distributed normally, we applied the Mann-Whitney *U*-test. Comparison of the effects of the treatments on the gene expression profile of tomato plants was performed using one-way analysis of variance (ANOVA) and Duncan's *post hoc* multiple comparisons test. The data presented here were confirmed by at least two independent experiments.

3 Results

3.1 *B. cereus* EC9 protects tomato plants from *Fol* infection but does not show antifungal activity *in vitro*

We wanted to establish whether the antifungal and non-antifungal activity of DD6 and EC9, respectively, found in the *F. oxysporum*/Kalanchoe interaction (Madriz-Ordeñana et al., 2022), also applies to Fusarium wilt on tomato. Thus, we evaluated the fungal growth inhibition activity *in vitro*, as well as the development of disease symptoms in bacteria-treated plants 3 weeks after *Fol* inoculation. While DD6 strongly inhibited the mycelial growth of *Fol* at all the bacterial densities tested, the lack of inhibition of *Fol* growth by EC9 was not distinct from the control (Figure 1A). The evaluation *in planta* showed that for EC9, the development of symptoms, including vessel browning, stunting, growth distortion, and wilting, was greatly reduced in comparison with the mock-treated plants (Figures 1B, C). Similarly, treatment with EC9 resulted in higher fresh weight (Figure 1D) and a significantly lower amount of *Fol* biomass in tomato stems (Figure 1E). In contrast, treatment with the antifungal strain DD6 did not significantly reduce symptom development nor increased the fresh weight of the infected plants (Figures 1B–D). However, a lower level of fungal biomass was detected in DD6-treated plants in comparison to mock inoculated plants (Figure 1E).

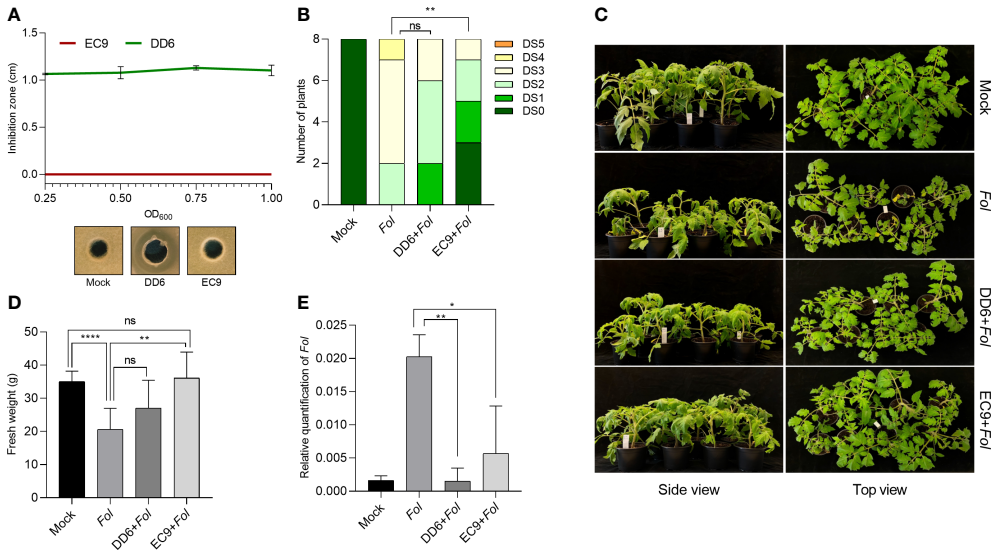

FIGURE 1
Treatment with *B cereus* EC9 enhances resistance to *Fusarium* wilt in tomato. **(A)** Inhibition zones caused by *Bacillus* spp. against *Fol* on PDA medium. The antifungal activity was determined after 5 days of incubation (n=5). **(B)** Disease severity score, **(C)** representative pictures of disease symptoms, and **(D)** fresh weight of tomato plants (cv. Moneymaker) at 21 days post-inoculation (dpi) (n=8). Ten days old tomato seedlings were treated with DD6, EC9 or 10 mM $MgCl_2$ (mock) by soil drenching and inoculated with *Fol* 1 week after treatment. **(E)** Estimation *in planta* (n=4) of *Fol* biomass in tomato stems. Quantification of *Fol* was carried out in plants at 21 dpi by calculating the ratio of fungal DNA to tomato DNA by qPCR. ns non-significant, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. The experiments were repeated at least two times with similar results. Bars represent the means of the indicated number of biological replicates ± standard error.

3.2 *B. cereus* EC9-mediated protection is independent of SA signaling

We further intended to determine the involvement of plant defense hormone signaling pathways in EC9-mediated protection against *F. oxysporum* in tomato. Thus, we first investigated whether EC9-mediated protection involves SA-signaling. Here we made use of an SA-compromised *NahG* transgenic tomato line (Brading et al., 2000). As expected from Di et al. (2017), untreated *NahG* plants showed increased susceptibility to *Fol* compared to the wild-type cv. Moneymaker (Figures 2A–C). At the same time, disease severity in cv. Moneymaker was significantly reduced by treatment with EC9. However, this was also the case in EC9-treated *NahG* plants exhibiting reduced Fusarium wilt and increased fresh weight compared to the mock (Figures 2A–C). In addition, the fungal recovery assay revealed that EC9 treatment caused *Fol* to reach the upper parts of the stem only in a reduced number of plants in both cv. Moneymaker and *NahG* line (Figure 2D). To gain further insight into the role of SA signaling in EC9-mediated protection, the expression level of the SA biosynthesis-associated genes *ICS* and *PAL* was examined in EC9-treated and untreated cv. Moneymaker plants subsequently challenged with *Fol*. The level of the *ICS* and *PAL* transcripts are commonly used to indicate SA-responses in tomato (Di et al., 2017; Constantin et al., 2019). None of the treatments induced a significant change in the level of the *ICS* transcript at any of the tested time points (Figure 2E). For *PAL*, however, the transcript level in plants treated with EC9 alone or in combination with *Fol* moderately increased at 3 dpi. At 7 and 14 dpi, an increased PAL transcript level was observed only in *Fol*-inoculated plants, whether or not treated with EC9 (Figure 2F).

3.3 JA pathway is required for *B. cereus* EC9-mediated protection against *Fol*

Next, we turned to study the involvement of JA in the enhanced protection mediated by EC9 using the JA-deficient tomato line *spr2* (Howe and Ryan, 1999). Inoculation with *Fol* resulted in clear symptoms at 21 dpi in untreated *spr2* and the respective wild-type cultivar Castlemart, suggesting that none of these genotypes exhibited resistance to *Fol* (Figure 3A). While most of the wild-type plants showed severe disease symptoms, such as growth distortion and wilting at 21 dpi, disease development was significantly reduced in plants treated with EC9 (Figures 3A, B). In addition, **cv.** Castlemart plants treated with EC9 and inoculated with *Fol* showed higher fresh weight than mock-treated, *Fol*-inoculated plants (Figure 3C). In

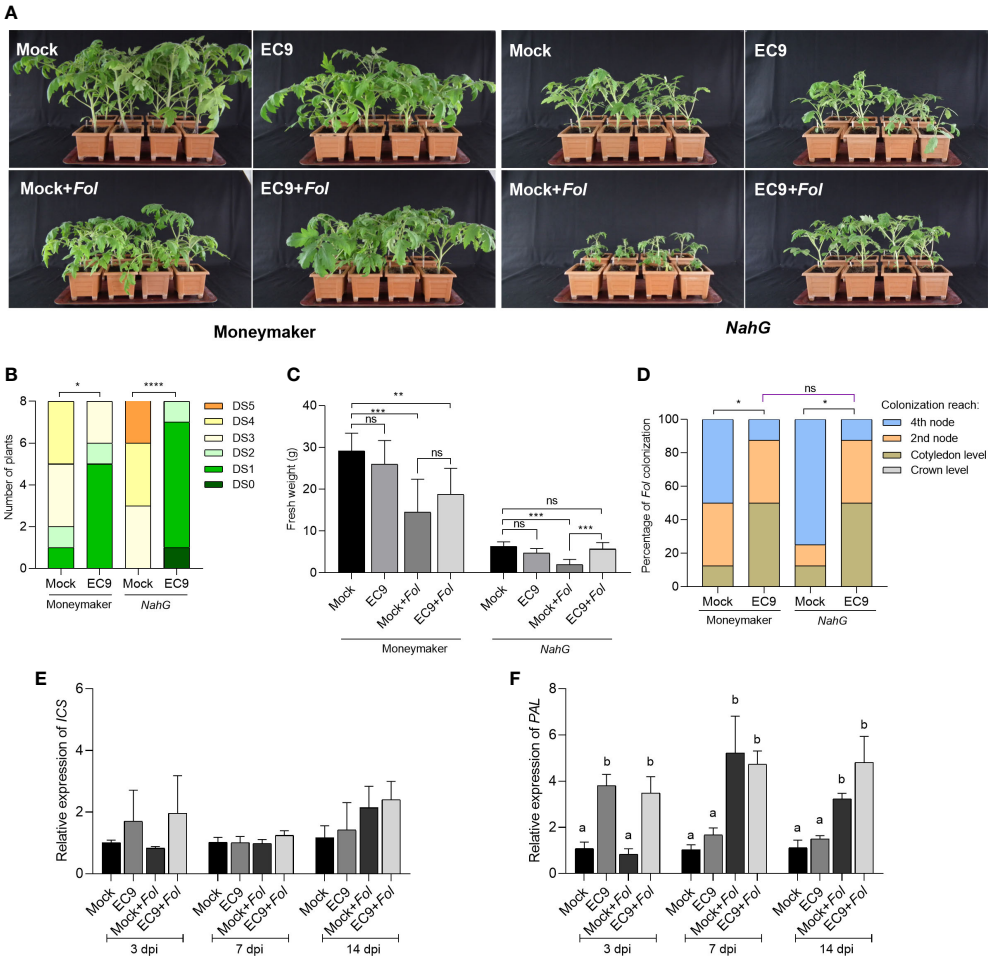

FIGURE 2
The SA signaling pathway is not involved in EC9-enhanced defense in tomato. **(A)** Disease symptoms, **(B)** disease severity score and **(C)** fresh weight of transgenic *NahG* plants and the corresponding wild type cv. Moneymaker at 21 days post-inoculation (dpi) (n=8). Ten-days old tomato seedlings were treated with EC9 or 10 mM $MgCl_2$ (mock) by soil drenching and inoculated with *Fol* 1 week after treatment. **(D)** Percentage of *Fol* infected stem sections at 21 dpi (n=8). ns non-significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P<0.0001$. The accumulation of transcripts of **(E)** *isochorismate synthase* (*ICS*) and **(F)** *phenylalanine ammonia-lyase* (*PAL*) in tomato plants (cv. Moneymaker) treated with EC9 or mock and inoculated with *Fol* was revealed by qRT-PCR (n=3). For gene expression analysis, stem pieces from the region between cotyledons and crown were harvested. The values were normalized to the internal reference transcript *α-tubulin*. Bars represent the means of the indicated number of biological replicates ± standard error. The experiments were repeated at least two times with similar results. Different letters indicate statistically significant differences between treatments.

contrast, EC9 treatment of *spr2* plants neither changed the level of Fusarium wilt significantly (Figures 3A, B) nor affected their fresh weight (Figure 3C). In addition, *Fol* colonization of the main stem was not affected by EC9 treatment in cv. Castlemart and *spr2* line (Figure 3D). The transcript level of JA signaling marker gene *PI-I* (Di et al., 2017) was monitored in stems of cv. Moneymaker plants where the roots were EC9-treated and mock-treated, 3, 7, and 14 days after *Fol* challenge. EC9 treatment alone did not affect the *PI-I* transcript level at the time points tested. Following *Fol* inoculation, however, EC9-treated plants exhibited a higher transcript level of *PI-I* at 3 and 14 dpi (Figure 3E).

3.4 *B. cereus* EC9 protects tomato plants against *Fol* in an ET signaling-dependent manner

JA signaling is partly linked to ET signaling (Robert-Seilaniantz et al., 2011). Therefore, we wanted to determine whether the ET signaling also is involved in the protection mediated by EC9. This was studied using the ET compromised tomato transgenic line overexpressing *ACD* (Klee et al., 1991). *Fol* inoculation of the *ACD* OE line and the corresponding wild-type cultivar UC82B plants showed clear disease symptoms at 21 dpi (Figure 4A). In addition, a significant reduction in disease severity was observed in EC9-

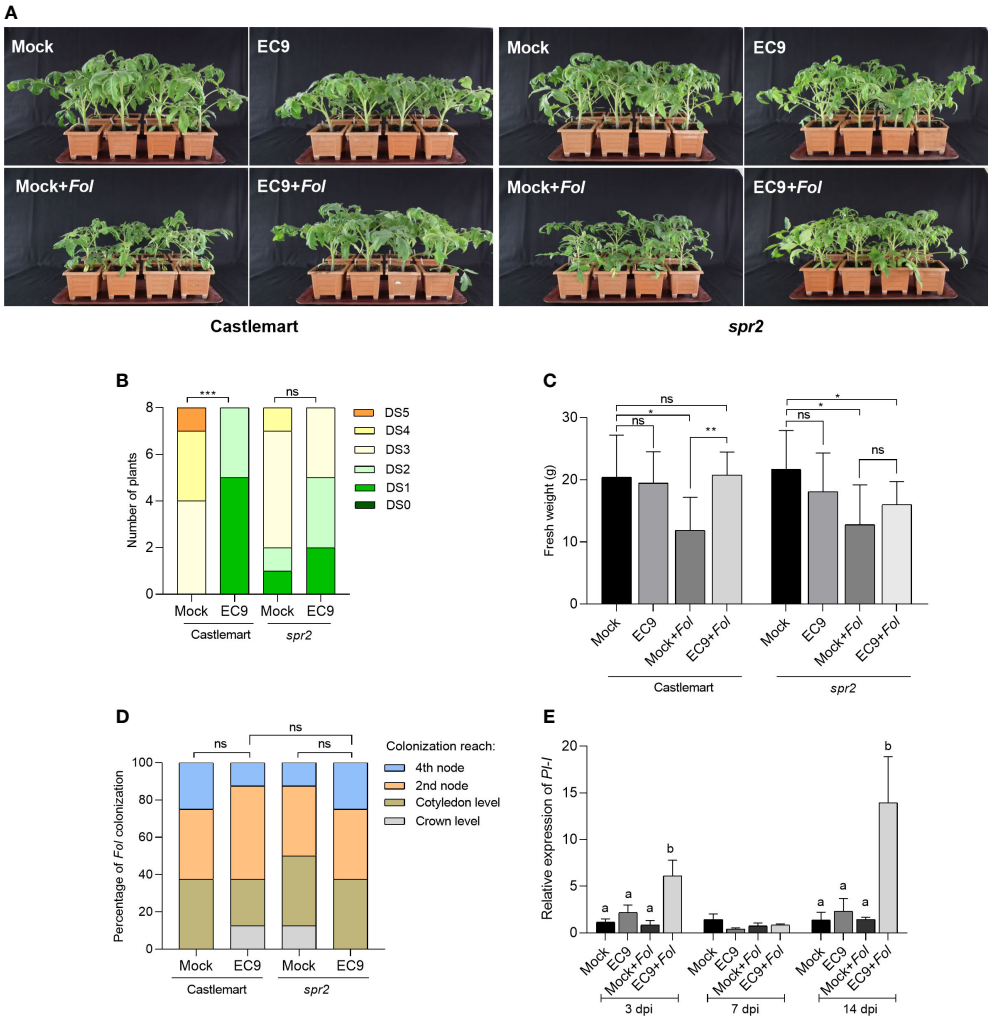

FIGURE 3
EC9-mediated enhanced defense against *Fol* involves JA signaling pathway. **(A)** Disease symptoms, **(B)** disease severity score, and **(C)** fresh weight of *Spr2* mutant and the corresponding wild type cv. Castlemart at 21 days post-inoculation (dpi) (n=8). Ten-days old tomato seedlings were treated with EC9 or 10 mM $MgCl_2$ (mock) by soil drenching and inoculated with *Fol* 1 week after treatment. **(D)** Percentage of *Fol* infected stem sections at 21 dpi (n=8). ns non-significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$,. The accumulation of transcripts of **(E)** *proteinase inhibitor I* (*PI-I*) in tomato plants (cv. Moneymaker) treated with EC9 or mock and inoculated with *Fol* was revealed by qRT-PCR (n=3). For gene expression analysis, stem pieces from the region between cotyledons and crown were harvested. The values were normalized to the internal reference transcript *α-tubulin*. Bars represent the means of the indicated number of biological replicates ± standard error. The experiments were repeated at least two times with similar results. Different letters indicate statistically significant differences between treatments.

treated UC82B plants, confirming that this line is useful for studying EC9 function. In contrast, no significant enhanced protection was observed in the *ACD* OE line (Figures 4A, B). The fresh weight and *Fol* colonization of the main stem were not significantly affected by EC9-treatment in cv. UC82B and *ACD* OE line (Figures 4C, D). We performed qRT-PCR to measure the transcript level of the ET signaling marker gene *ETR4* (Di et al., 2017) in stems 3, 7, and 14 days after *Fol* challenge of cv. Moneymaker plants, root-treated with EC9 and mock-treated. Treatment with EC9 alone did not trigger any changes in the *ETR4* transcript level at the tested time points, whereas *Fol* inoculation alone resulted in higher *ETR4* transcript accumulation at 7 and 14 dpi. However, when also treated with EC9, *Fol*-inoculated plants showed increased *ETR4* transcript levels at 7 and 14 dpi, in comparison to *Fol* inoculation alone (Figure 4E).

3.5 *B. cereus* EC9 induces callose deposition in tomato roots

To analyze whether EC9 activates immune responses in roots, which is the site of *Fol*'s primary attack, we studied the level of

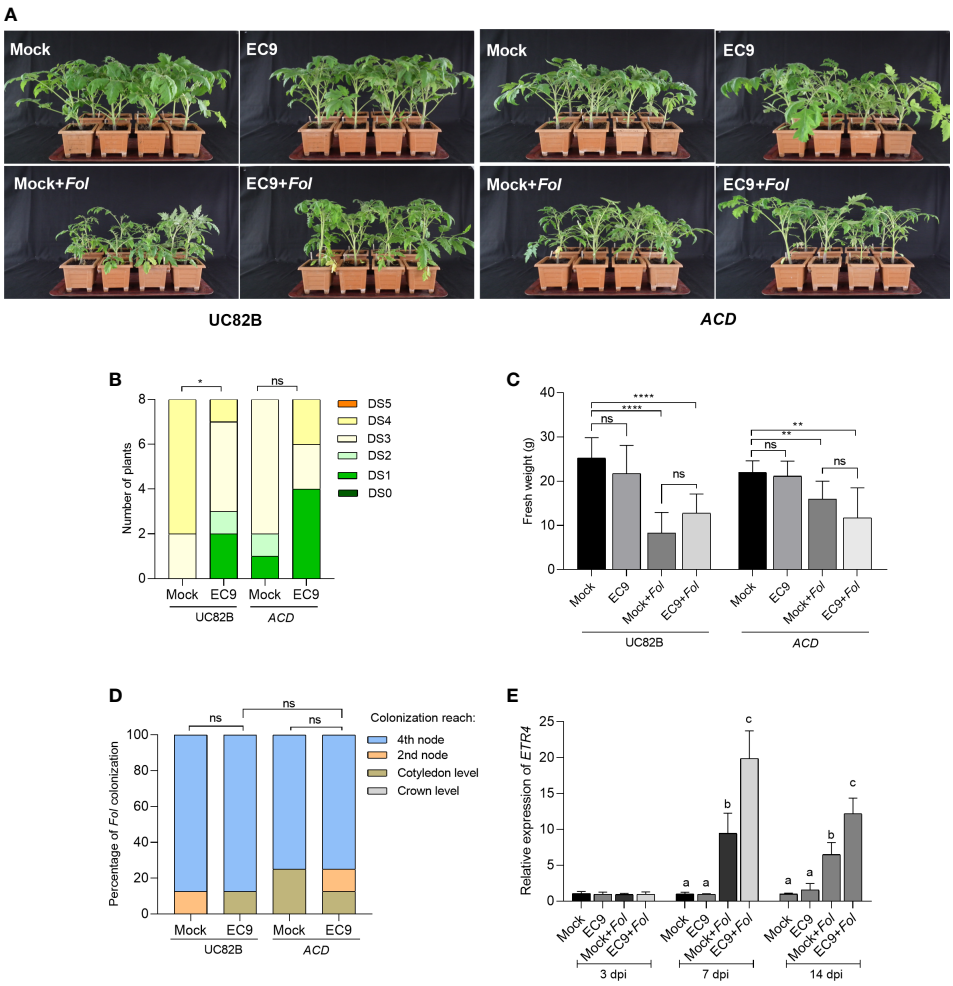

FIGURE 4
ET is involved in EC9-mediated enhanced defense against *Fol*. **(A)** Disease symptoms, **(B)** disease severity score, and **(C)** fresh weight of *ACD* transgenic line and the corresponding wild type cv. UC82B at 21 days post-inoculation (dpi) (n=8). Ten-days old tomato seedlings were treated with EC9 or 10 mM $MgCl_2$ (mock) by soil drenching and inoculated with *Fol* 1 week after treatment. **(D)** Percentage of *Fol* infected stem sections at 21 dpi (n=8). ns non-significant, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. **(E)** The accumulation of transcripts of *ethylene receptor 4* (*ETR4*) in tomato plants (cv. Moneymaker) treated with EC9 or mock and inoculated with *Fol* was revealed by qRT-PCR (n=3). For gene expression analysis, stem pieces from the region between cotyledons and crown were harvested. The values were normalized to the internal reference transcript *α-tubulin*. Bars represent the means of the indicated number of biological replicates ± standard error. The experiments were repeated at least two times with similar results. Different letters indicate statistically significant differences between treatments.

callose in this tissue. Callose deposition was determined in the elongation zone of EC9-treated and untreated cv. Moneymaker tomato roots 24 hours after *Fol* inoculation. Treatment with EC9 alone resulted in significantly increased callose deposition. Roots inoculated with *Fol* had an even higher number of callose spots, whether or not pre-treated with EC9 (Figures 5A, B).

4 Discussion

Bacillus-mediated plant disease protection can rely on direct effects of bacteria-derived antimicrobial metabolites on pathogens, widely known as antibiosis, or indirect effects by activating defense in the host plant (Pérez-García et al., 2011; Blake et al., 2021). We have previously identified antifungal (*B. siamensis* DD6) and non-antifungal (*B. cereus* EC9) strains and have shown that both strains provide enhanced protection against Fusarium disease in Kalanchoe (Madriz-Ordeñana et al., 2022). In the present study, we firstly demonstrate that treatment of tomato plants with the antifungal strain DD6 did not significantly reduce *Fol* symptom development despite showing strong antifungal effect *in vitro*. However, we could confirm our previous findings on the role of the non-antifungal strain EC9 as a biocontrol agent (Figure 1). To explore whether

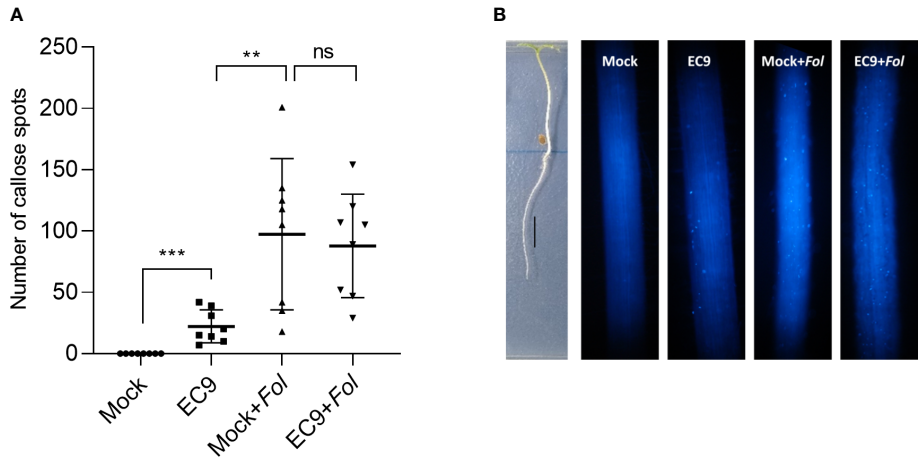

FIGURE 5
B cereus EC9 induces callose deposition in tomato roots. **(A)** Number of the callose spots in roots (cv. Moneymaker) (n=8-10) 24 hours post-inoculation. **(B)** Representative images of tomato roots of 7 days old tomato plants, grown on ½ x MS medium positioned vertically, treated with EC9 or mock and inoculated with *Fol* at 24 hours post-treatment. Presence of callose spots were visualized by aniline blue staining and fluorescence microscopy. ns non-significant, $^{**}P < 0.01$, $^{***}P < 0.001$. Data points correspond to the mean ± standard error. Experiments were repeated two times with similar results.

the mechanisms behind the EC9-mediated protection against Fusarium wilt of tomato involves immunity and whether it involves one or more of the major plant hormone signaling pathways, we conducted plant assays in different phytohormone impaired genotypes and analyzed the expression of related marker genes. Our results suggest that EC9 enhances defense against *Fol* in tomato, possibly by priming of the JA/ET signaling pathways rather than the SA pathway.

The increasing demand for commercial products that are based on biocontrol agents such as *Bacillus* spp. requires that the mechanisms underlying *Bacillus*-mediated protection are properly investigated for various *Bacillus*-plant-pathogen interactions. Successful commercialization of *Bacillus* spp. has so far relied mostly on their production of antimicrobial metabolites capable of directly inhibiting the pathogen. The ability of members of the *B. subtilis*-like group to biosynthesize specialized compounds with antimicrobial activity have made this species attractive for use as biocontrol agents (Caulier et al., 2019; Penha et al., 2020). Thus, treatment of tomato seeds with antifungal *B. velezensis* AP3 (Medeiros and Bettiol, 2021), *B. subtilis* MMS9 (Patel and Saraf, 2017), and *B. amyloliquefaciens* FZB24 (Elanchezhiyan et al., 2018) suppressed Fusarium wilt of the plant. In our study, the antifungal DD6, which contains biosynthetic gene clusters (BGC) associated with well-known antimicrobial secondary compounds, was unable to significantly decrease the development of symptoms of Fusarium wilt disease in tomato (Figure 1). This result contradicts our previous findings (Madriz-Ordeñana et al., 2022) showing that DD6 treatment by soil drenching effectively controls *F. oxysporum* in Kalanchoe. This inconsistency for DD6 may be attributed to differences in bacteria-plant species adaptation, root bacterial colonization patterns and environmental conditions. Interestingly, although DD6 treatment did not result in a reduction in Fusarium wilt disease symptoms, this strain caused a significant reduction in pathogen biomass in tomato stems. That could have arisen from the suppressed fungal mycelium by DD6 that clogs the xylem vessels resulting in wilting symptoms in plants (Di Pietro et al., 2003).

Many *Bacillus* strains isolated from various ecological niches have been identified as potential biocontrol agents. However, only a limited number have been reported to be inducers of plant immunity as their main mode of action (Köhl et al., 2019). In this study, we found that pretreatment with EC9 mediate protection against Fusarium wilt of tomato (Figure 1). Remarkably, EC9 does not exhibit fungi-toxic activity against *Fol in vitro* and does not possess BGCs, associated with antimicrobial compounds, as predicted using antiSMASH analysis (Madriz-Ordeñana et al., 2022). This suggests that EC9-mediated protection is based on enhanced defense rather than direct antagonistic effects. Furthermore, we did not observe significant growth promoting activity in EC9-treated tomato plants (Figure 1). This is in contrast to other *Bacillus cereus* strains, such as AR156 and YN917, that have been shown to promote plant growth in different species (Niu et al., 2011; Zhou et al., 2021).

The phytohormone SA and JA/ET signaling pathways have fundamental roles in the regulation of plant immunity (Glazebrook, 2005; Bari and Jones, 2009; Pieterse et al., 2014). Although the general concept that JA/ET-mediated activation of ISR is triggered by root-associated beneficial bacteria, it has been

shown that, depending on host-bacteria-pathogen interactions and experimental conditions, *Bacillus* species can also trigger SA-mediated defense pathways. It is in addition known that these pathways are interconnected in signaling networks, but the relationship between beneficial microorganisms, harmful pathogens and these pathways is not strictly defined (Spoel and Dong, 2008; Thaler et al., 2012). Treatment with *B. cereus* AR156 by soil drenching increased *PR1* transcript level, ROS accumulation, and callose deposition in *A. thaliana*, and it improved the level of defense against the necrotrophic pathogen *Botrytis cinerea* independently of JA/ET and NPR1-mediated signaling (Nie et al., 2017). Additionally, SA-dependent signaling is also required for AR156-mediated protection against the hemi-biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Niu et al., 2011). Extracellular polysaccharides of this bacterium were suggested to confer MAMP-mediated activation of ISR (Jiang et al., 2016). Phthalic acid methyl ester secreted by *B. subtilis* IAGS174 (Akram et al., 2015) and phenylacetic acid secreted by *B. fortis* IAGS162 (Akram et al., 2016) have been proposed as potential ISR activators alleviating the symptoms caused by *Fol*. To determine the involvement of the SA pathway in EC9-mediated protection against *Fol*, we examined the *NahG* transgenic line that is compromised in the accumulation of SA. As shown previously (Di et al., 2017; Constantin et al., 2019), we found that *NahG* makes the plants more susceptible to *Fol* (Figure 2). However, EC9-mediated protection was not abolished in *NahG* plants, suggesting that it is SA-independent (Figures 2A–C). We also tested the transcript levels of the SA biosynthesis-related *ICS* and *PAL* genes in cv. Moneymaker. The unaffected *ICS* expression and the increased transcript level of *PAL* in *Fol*-inoculated plants at 14 dpi (Figures 2E, F) are consistent with the findings of Constantin et al. (2019). The transcript profile for *ICS* agrees with our argument that EC9-mediated defense does not occur through the SA pathway (Figure 2E). However, because PAL transcript level is increased by EC9 treatment alone and following pathogen infection at 3 dpi, it can be speculated that SA signaling may still function in an EC9-mediated defense against *Fol* in tomato (Figure 2F).

To test the involvement of the phytohormones JA and ET in EC9-mediated protection against *Fol*, we used the *spr2* mutant and the *ACD* OE line, which are deficient in JA biosynthesis and ET production, respectively. We found that pretreatment with EC9 provided protection against the Fusarium wilt of tomato in the corresponding wildtype cultivars. Since protection by EC9 was ineffective in both *spr2* and *ACD* OE lines, we suggest that EC9 activates a defense that is dependent on the JA and ET pathways (Figures 3A, B; Figures 4A, B). Similar findings have been reported for different *Bacillus* spp. in *Arabidopsis* (Nie et al., 2017) and tomato (Yan et al., 2002). Interestingly, Wang et al. (2018) demonstrated in *Arabidopsis* that *B. cereus* AR156, which is genetically clustered within the *B. cereus sensu lato* group together with EC9 (Madriz-Ordeñana et al., 2022), activates pattern-triggered immunity in a SA, JA, and ET-independent manner. The *PI-I* and *ETR4* genes are fundamental for JA- and ET-dependent signaling, respectively, and they have been reported as marker genes for related pathways in tomato-*Fol* interactions (Di et al., 2017; Constantin et al., 2020). We tested the transcript levels of *PI-I* and *ETR4* in cv. Moneymaker plants, either treated with EC9 or mock, and subsequently challenged with *Fol*. Our results agree with those of Di et al. (2017) who reported that *PI-I* expression was not affected by *Fol* inoculation alone, whereas *ETR4* expression was up-regulated. Noteworthy, EC9 alone did also not induce accumulation of the *PI-I* and *ETR4* transcript prior to *Fol* inoculation. However, the *PI-I* transcript level was augmented following the *Fol* challenge in EC9-treated plants. Similarly, although *ETR4* expression was upregulated in *Fol*-inoculated plants, its expression level was significantly higher when EC9-treatment was combined with *Fol* inoculation (Figures 3E, 4E). While the transcripts are quantified in the stem tissue of plants root-treated with EC9, these results suggest that EC9 systemically primes tomato plants for enhanced expression of *PI-I* and *ETR4*. This is in line with the defense gene priming effects of *B. cereus* AR156 (Niu et al., 2011; Wang et al., 2013; Wang et al., 2014). Madriz-Ordeñana et al., (2022) also proposed that EC9-mediated enhanced defense in Kalanchoe against *Fusarium oxysporum* is associated with defense priming.

Deposition of callose at the site of pathogen attack is one of the early defense responses and has been well documented to play an important role in effective inhibition of pathogen invasion (Luna et al., 2011; Ellinger et al., 2013). MAMP-elicited callose deposition is considered a marker of PTI not only in leaves but also in roots (Luna et al., 2011; Li et al., 2016; Tran et al., 2017). In addition to PAMPs, such as purified flg22 or chitin from pathogenic bacteria or fungi, elicitors like peptidoglycans from root-associated beneficial rhizobacteria *Bacillus subtilis* were shown to induce callose deposition in roots (Millet et al., 2010; Newman et al., 2013). In the present study, we demonstrated that EC9 treatment induces callose deposition in roots. However, we did not find significant differences in the number of callose spots between mock-treated and EC9-treated plants when inoculated with *Fol* (Figure 5), suggesting that EC9 treatment does not predispose the host plant for increased callose deposition following *Fol* inoculation. Nevertheless, while the increased callose deposition in EC9-treated roots is an indication of PTI, we speculate that other PTI-related defense responses rather than callose deposition might play a role in the suppression of the *Fol*. We suggest that the systemic priming effect that we observe on the *PI-I* and *ETR4* marker transcripts in stem tissue is linked to a role JA and ET signaling in the protection against *Fol*. Interestingly, colonization of *Arabidopsis* roots by the related strain AR156 does not systemically induce callose deposition in the leaves. Yet, it primes plants for enhanced callose deposition after pathogen attack (Niu et al., 2011; Nie et al., 2017). Furthermore, this strain is capable of inducing callose

deposition when infiltrated into *Arabidopsis* leaves (Wang et al., 2018).

5 Conclusion

This study provides evidence that the non-antifungal EC9 has strong potential for priming plant immunity against *Fol* in tomato in a JA/ET signaling-dependent manner. These findings provide new insights into the molecular mechanism of EC9-activated protection towards Fusarium. Furthermore, our results indicate that activation of induced resistance through priming of defense mediated by beneficial microorganisms is an interesting approach for identifying new biological control agents with alternative modes of action.

Data availability statement

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

Author contributions

SP, KM-O, and HT-C: conceptualization. SP and KM-O: methodology, investigation and data curation. SP: formal analysis. SP and HT-C: funding acquisition. HT-C and KM-O: supervision. SP: writing-original draft: SP, KM-O, HT-C: writing-review and editing. 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1090947/full#supplementary-material

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Small G Protein StRab5b positively regulates potato resistance to *Phytophthora infestans*

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Rabproteins are the largest members of the small G protein family and are widely distributed in eukaryotes. It comprises eight subfamilies and is responsible for regulating vesicle transport, plant growth and development, and biotic and abiotic stress responses. In this study, the small G protein gene *StRab5b* was cloned from potato, and its biological information, expression profile and induced expression level, overexpression and gene silencing were examined on regulating potato resistance to *Phytophthora infestans* using PCR, qPCR and Virus-induced gene silencing (VIGS). Our results indicate that the amino acid of StRab5b shows the highest and lowest homology with NbRab5b in *N. benthamiana* and StRab in potato respectively. *StRab5b* expression varied among different potato tissues and varieties, and was induced by *P. infestans* infection. Transiently ectopic expression of *StRab5b* in *N. benthamiana* enhanced its resistance to *P. infestans*, whereas, silencing of *StRab5b* and its homologous gene facilitated pathogen infection in potato and *N. benthamiana* respectively. Furthermore, stable expression of the *StRab5b* gene in potatoes enhanced its redox-stress response capacity, as manifested by the accumulation of H_2O_2 in infected leaves and subsequent increase in the activity and expression of ROS scavenging enzymes, thereby attenuating the development of *P. infestans* and ultimately reducing the lesions on infected potato leaves. In addition, the *LOX* gene transcripts and JA level were upregulated rapidly after inoculation with *P. infestans*. Collectively, our results suggest that *StRab5b* positively regulates the resistance against potato late blight (PLB) *via* JA-mediated defense signaling pathway.

KEYWORDS

Small G proteins, potato late blight, antioxidant enzyme activities, plant disease resistance, gene expression, hormone signaling pathways

1 Introduction

The potato (*Solanum tuberosum* L.) is the most important non-gramineous crop worldwide. However, its tuber yield and quality are devastatingly affected by PLB caused by *P. infestans*. In China, in the early 1950s and the 1960s, PLB caused yield losses amounting to approximately 1 billion dollars (Li et al., 1997). Furthermore, the frequent variation in dominant races of *P. infestans* in potatoes in recent years has been considerable, resulting in a loss of disease resistance (Saville et al., 2016). At present, it is extremely difficult to control PLB, and an effective way is urgently required to excavate novel functional genes for investigating their resistance functions.

Plants have developed sophisticated pathogen-surveillance systems and can mount defense responses against microbial attack. Microbial parasites in the environment constantly challenge plants, which in turn, defend themselves through the innate immune system. Specifically, plants detect potential invaders, including bacteria, fungi, and oomycetes, mainly through a two-layered immunity system. First, when the host plants defend pathogen infection, the pattern recognition receptors (PRRs) located on the cell membrane recognize the pathogen associated molecular patterns (PAMPs) of *Phytophthora* pathogens, and elicit the first layer of immune system (PAMP-triggered immunity, PTI) (Naveed et al., 2020). Microbe-associated molecular patterns (MAMPs) are motifs of microbes which can be recognized by plants in response to infection (Robinson and Bostock, 2015). Then, MAMPs triggered PTI defense responses that can effectively counteract potential microbes. Second, pathogens secrete and deliver effectors into the host cells, that neutralize the functions of immune regulators (Saville et al., 2016). Resistance proteins (R) recognize pathogen effectors, activating a rapid immune response known as effector-triggered immunity (ETI), which results in host cell death, a phenomenon known as the hypersensitive response (HR). These pathogen effectors can evolve new functions through mutations to suppress immunity and promote disease progression (Wang et al., 2019).

During the establishment of HR, Salicylic acid (SA), JA, and ethylene (ET) biosynthesis are activated through PTI, ETI, and systemic acquired resistance (SAR). The accumulation of SA, JA and ET leads to enhanced resistance to pathogens in plants (Li et al., 2004). Particularly, recent studies have reported that the transcription factor *StbZIP61* is involved in the SA signaling pathway that regulates the defense response of potato against *P. infestans* (Zhou et al., 2018) and enhances potato resistance to salt stress (Efimova et al., 2019). Similarly, activating JA and ET signaling, instead of SA signaling by eliciting β-cryptogein, triggers resistance to *P. infestans* (Staŕy et al., 2019).

In addition, when plants are subjected to biotic or abiotic stress, rapid accumulation of reactive oxygen species (ROS) occurs, leading to programmed cell death (PCD) and HR. However, cells have evolved a set of ROS-scavenging enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) to balance cellular ROS levels (Beers and Sizer, 1952). Recent evidence suggests that the higher activities of POD and SOD play key roles in scavenging excessive ROS in late plant-pathogen interaction. Higher levels of H_2O_2 were also represented in potato transgenic plants after inoculation with *P. infestans* (Zhang et al., 2014). Zhang Y et al. (2020) found that ROS can be scavenged by SOD, POD, and CAT activities. In sweet potato, SOD and APX activities of transgenic plants were increased under salt stress (Yan et al., 2016). In conclusion, ROS and antioxidant enzymes are involved in the signal pathway of plant-pathogen interaction and stress response.

The RAS superfamily of small G proteins are divided into five subfamilies: RHO, RAB, RAS, RAN and ARF. RAB proteins belong to the largest subfamily of RAS superfamily. RAB GTPase generally regulates its activity through two regulatory proteins: Guanine nucleotide exchange factors (GEFs) are used to dissociate the GDP to form an active GTP-RAB-effector, and GTPase activating proteins (GAPs) are used to decompose GTP into GDP. GDP-RAB combines with GDI to form inactive GDP-RAB-GDI, thus entering the next regulation cycle (Ebine and Ueda, 2009). RAB proteins are widely distributed in plants, animals, and microorganisms. To date, 52 *Rab* genes have been cloned from rice (*Oryza sativa* L.) and 57 from *Arabidopsis thaliana* (Rutherford and Moore, 2002; Du et al., 2013). In potato, the *Rab* gene *StRab5b* is located on the 11th chromosome of the genome (https://solgenomics.net/locus/82544/view). Previous studies have shown that Rab proteins regulate both abiotic and biotic stress responses in plants, plant hormone-mediated signal transduction, plant growth and development, and vesicular trafficking (Yamaguchi-Shinozaki et al., 1990; Moshkov and Novikova, 2008; Szumlanski and Nielsen, 2009; Falchi et al., 2010; Gao et al., 2012). Research of plant bacterial diseases showed that the overexpression of *OsRab11* in rice resulted in elevated resistance to the pathogen *Pseudomonas syringae* through induced JA response genes (Hong et al., 2013). Small G protein, *VmRab7*, regulates the vegetative growth and pathogenicity of *Valsa mali*, and knockout of *VmRab7* blocks autophagy (Zhang et al., 2021). In previous studies on plant fungal diseases, *Rab2* was significantly expressed in the early stage of infection by wheat leaf rust (*Puccinia triticina*). The active *OsRac1* caused HR responses and substantially reduced disease lesions against rice blast fungus (Ono et al., 2001). In Arabidopsis, *RabG3bOX*-transgenic plants displayed unrestricted hypersensitive PCD against a fungal toxin and a fungal pathogen *Alternaria brassicicola* (Kwon et al., 2009). Additionally, higher expression of *TaRab7* implied that it was related with defense to wheat-stripe rust fungus (Liu et al., 2012). Moreover, the overexpression of the small G protein *StRab* caused small lesions on potato leaves after inoculation with *P. infestans* (Gao et al., 2012), indicating that the *Rab* genes and their associated proteins show potential to influence disease progression. However, limited research has been done on the resistance of small G protein to PLB.

In this study, we aimed to elucidate the role of the small G protein *StRab5b* in the regulation of potato resistance against late blight. For this purpose, the *StRab5b* gene was cloned, gene sequences and expression profile were analyzed; transient expression and gene silencing were performed in *N. benthamiana* and potato. Stable expression of *StRab5b* in potatoes was obtained and used to confirm its role in regulating potato resistance against *P. infestans*. These results lay a stone for resistant breeding against potato late blight.

2 Materials and methods

2.1 Plant material, *P. infestans* strain, and primers

Potato cultivar 'Desiree' was used for gene amplification and genetic transformation. Plants were grown *in vitro* on Murashige and Skoog (MS) medium, under a luminous flux intensity of 3,000–4,000 Lx, 80% relative humidity, and a photoperiod of 16 h/8 h light/dark, at 25°C. The *P. infestans* strain (14-3) was kindly provided by Prof. Francine Gover, Wageningen University and Research (WUR), Netherlands. Tubers of six potato varieties 'Zihuabai' (ZHB), 'Desiree' (DXR), 'Longshu No.7' (L7), 'Zhongshu No.4' (ZS4), 'Atlantic' (DXY) and 'Shepody' (XPD), were planted in a nutrient pot and cultivated in a chamber under a luminous flux density of 18,000 Lx, 75% relative humidity, and a photoperiod of 16 h/8 h light/dark, at 25°C. These plants were used for expression profile analysis. Desiree seedlings cultivated in pots for 20 d were selected for induced expression.

Primers were designed using Primer 5.0 software and synthesized by the Beijing Hooseen Biotechnology Company, China. The primer sequences used in this study are listed in Supplementary Table 1.

2.2 *P. infestans* culture and zoospore suspension preparation

Luria-Bertani liquid medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl) was prepared, and H_2O was added to bring the volume to 1,000 mL. YEB liquid medium (5 g beef extracts, 5 g tryptone, 5 g sucrose, 1 g yeast extract, $MgSO_4{\cdot}H_2O$ 0.5 g) with pH 7.0 was prepared, and H_2O was added to bring the volume to 1,000 mL. Agar powder (15 g) was added to each mixture media to prepare the corresponding solid medium. *P. infestans* was induced to produce zoospores at 4°C for 4 h. Zoospore suspensions were adjusted to a concentration of 5×10^5 mL^{-1} sporangia using a hemocytometer (Neubauer-improved, Germany). Approximately 10–15 µL of spore suspension was inoculated on plant leaves.

2.3 RT-PCR and RT-qPCR analysis

Relative expression quantitation was performed using Quantity one software for semi-quantitative RT-PCR (*St Rab5b* and *PDS*), and *GAPDH* was used as a reference gene. Q-PCR was performed on a LightCycler (96, Roche, Basel, Switzerland) using the SYBR Premix Ex Taq II Kit (TaKaRa Bio, Shiga, Japan). We analyzed the expression levels of *ACS*, *LOX*, *NPR1*, *SOD*, *APX1*, *POD*, *CAT2*, and *PIO* and used *Actin* as a reference gene.

2.4 Cloning of *StRab5b* and overexpression vector construction

Total RNA was extracted from potato plantlets grown for 30 d using the RNAiso reagent (TaKaRa Bio), and genomic DNA was extracted using the DNAiso reagent (TaKaRa). The quality of RNA and DNA was determined by performing gel electrophoresis on 1% agarose gels. The first cDNA chain was synthesized according to manufacturer instructions of a HiScript II reverse transcription Kit (Nanjing Vazyme Biotechnology Co., Ltd).

The PCR-amplification mixture (50 µL) included 10 µL of 5X Prime Star Buffer (Mg^{2+} plus), 4 µL of dNTP Mix (2.5 mM), 1 µL of Primer *Rab5bF/Rab5bR*, 1 µL of cDNA template, 0.5 µL of Prime STAR HS DNA Polymerase, and 32.5 µL of ddH_2O. Target fragments were purified using the EasyPure Quick Gel Extraction Kit (Beijing TransGen Biotech Company, China) according to manufacturer's instructions. Plasmid extraction was performed according to the manufacturer's instructions of the TIANprep Mini Plasmid Kit II (Beijing TIANGEN Biotech Company). PLG-Rop and pBIA1300-221 were provided by Prof. Yan Zhao, from the Institute of Genetics and Development, Chinese Academy of Sciences, Beijing, China. WUR kindly provided the *P. infestans* strains, pTRV1 and pTRV2 vectors. Fastdigest endonucleases *Kpn*I, *Bgl*II, *Xba*I, and *Sac*I were purchased from Thermo Fisher Scientific, (Waltham, MA, USA).

Phylogenetic analyses were performed with DNAMAN software. The *Rab* or *Rab5b* sequences of *Oryza sativa* (AF323991), *Nicotiana benthamiana* (DQ335217), *Solanum tuberosum* (ABK96799 and AK323357), *Hevea brasiliensis* (KC577146), *Physcomitrella patens* (AB379973), *Phoenix dactylifera* (AK287492), *Nicotiana sylvestris* (X63875), *Mangifera indica* (KF768563), *Athaliana* (D89824), *Selaginella moellendorffii* (KF516567), *Medicago sativa* (X79278), *Triticum aestivum* (X59133), *Lotus japonicus* (Z73939), and *Medicago truncatula* (BT147767) were retrieved from Genbank separately.

The *StRab5b* gene was linked to the pEASY-Blunt Simple cloning vector and transferred into DH5α cells (TransGen Biotech Company, Beijing, China). Positive DH5α colonies were screened and sequenced. Target gene purification was performed using the EasyPure Quick Gel Extraction Kit (TransGen Biotech Company). Recombinant vectors PLG-Rop

and pEASY-Blunt Simple-*StRab5b* were digested with *Bgl*II and *Kpn*I to obtain the PLG-*StRab5b* vector. Recombinant plasmid PLG-*StRab5b* and vector pBIA1300-221 were digested with *Xba*I and *Sac*I to obtain the recombinant plasmid pBIA1300-*StRab5b*. Plasmid pBIA1300-*StRab5b* was transferred into *Agrobacterium tumefaciens* GV3101 for preservation.

2.5 Measurement of lesions on inoculated potato and *N. benthamiana* leaves

The length and width of the leaf lesions formed on the plant leaves were measured using an electronic caliper (DL91150, China). Each lesion was measured three times (Vleeshouwers et al., 1999). The lesion area (A1) was calculated using the formula: $A1 = 1/4\pi \times length \times width$.

2.6 Expression profile and induced expression of *StRab5b*

Inoculation method of *P. infestans* for analysis of the expression profile of *StRab5b* was described as follows. The seedlings of different potato varieties were cultured in a pot (10 cm height * 10 diameter) for 50 d. The abaxial surface of potato leaves was scratched with sterile toothpicks and inoculated with 10 uL zoospore suspension (5×10^5 mL^{-1}) of *P. infestans* on one side; the other side was inoculated with water as control. The inoculated leaves were placed in a plastic box with wet filter paper on the bottom and kept in an incubator (day and night 16 h/8 h, 25/18°C).

Induction of *StRab5b* after inoculation with *P. infestans*: Potato seedlings ('Desiree') were planted in a pot (10 cm height * 10 diameter) for 20 d, then inoculated with 10 µL of sporulation of *P. infestans* (5×10^5 mL^{-1}). Then, inoculated leaves were collected at 0, 12, 24, 48, 72, 96, and 108 h post-inoculation (hpi). Using potato *GAPDH* as a reference gene, RT-PCR was performed to detect the relative expression levels of *StRab5b*, and the results were analyzed using Quantity one software.

2.7 Transient expression of *N. benthamiana* and VIGS-mediated gene silencing in *N. benthamiana* and potato

A. tumefaciens containing pBIA1300-*StRab5b* and pBIA1300 were inoculated in 10 mL YEB liquid medium (100 µg/mL rifampicin and 50 µg/mL kanamycin). The oscillating culture was incubated overnight at 180 rpm, followed by centrifugation for 2 min at 7,104×*g* to collect the bacteria; then, the culture was resuspended in a liquid mixture containing 150 µM acetyleugenone, 10 mM $MgCl_2$, and 10 mM MES. The bacterial solution was suitable for use when the OD_{600} value reached 0.5–0.6. The bacterial liquid (15–20 µL) was drawn using a needle-free syringe, injected into the right side of the abaxial surface of the *N. benthamiana* leaf, and incubated for 24 h (night and day alternate 16 h/8 h) at 21°C. The *N. benthamiana* leaves were inoculated with *P. infestans* spores and incubated for 4 h at 4°C before being transferred to a 21-23°C incubator with 80% humidity. The lesion area was measured at different inoculation time points.

Recombinant vectors pTRV2 and pEASY-Blunt Simple-*StRab5b* were digested with *Xba*I and *Sac*I to construct the silence-expression vector pTRV2-*Rab5b* (98% amino acid identity with *Rab5b* of *N. benthamiana*). The *Agrobacterium tumefaciens* vector pTRV1 was provided by WUR. Plasmid pTRV2-*Rab5b* was then transferred to *A. tumefaciens* GV3101. *A. tumefaciens* plasmids were resuspended with VIGS mixture containing 20 mM $MgCl_2$, 100 mM MES, and 20 mM acetosyringone. The photobleaching phenotype was obtained by inhibiting the expression of endogenous phytoene desaturase gene (*PDS*). A 536 bp fragment of *StPDS* (95% amino acid identity with *PDS* of *N. benthamiana*) was linked with pTRV2 and transformed into *A. tumefaciens* GV3101 (pTRV2-*PDS*). Thereafter, *A. tumefaciens* containing pTRV1 was mixed with pTRV2-*PDS* or pTRV2-*StRab5b* at a 1:1 ratio after the OD_{600} value reached 0.5–0.6. A needle-free syringe was used to draw 20 µL of the bacterial suspension to inject into *N. benthamiana* and potato leaves. Three to four leaves of each plant were inoculated. After photobleaching, leaf samples were collected to identify the relative expression levels of *PDS*, *StRab5b* and *StRab5b* homologous gene using RT-PCR, after 25 d (*N. benthamiana*) and 60 d (potato). *GAPDH* was used as a reference.

2.8 Generation of transgenic potato lines

Potato stems (2 cm long) were placed in the suspended *Agrobacterium* solution, soaked for 10 min, and dried with sterile filter paper. Stems were then transferred to the co-culture medium (MS + 1.0 mg/L IAA + 0.2 mg/L GA3 + 2.0 mg/L ZT + 0.5 mg/L 6-BA, pH 5.8) and cultured in darkness for 2–3 d. Thereafter, they were transferred to the bud inductive-differentiation medium (MS + 1.0 mg/L IAA + 0.2 mg/L GA3 + 2.0 mg/L ZT + 0.5 mg/L 6-BA + 50 mg/L Kan + 300 mg/L TMT, pH 5.8), and incubated at 25°C under a light intensity of 4,000 µmol/m^2·s, and a photoperiod of 16 h/8 h. The medium was replaced at 2-week intervals. When adventitious buds grew to 2.0 cm, they were transferred to the rooting medium (MS + 50 mg/L Kan, pH 5.8). When plantlets were formed, total leaf RNA was extracted, and positive transgenic plants were identified using PCR and q-PCR separately.

2.9 Trypan blue staining to monitor dead cells

Leaves infected with *P. infestans* were decolorized in anhydrous ethanol by boiling for 2 to 2.5 h. Decolorized leaves were placed in 0.5% trypan blue solution (0.5 g trypan blue dissolved in 100 mL distilled H_2O), stained for 10–15 min, washed twice with distilled water, and then treated with lactophenol water (lactic acid, phenol, and dd H_2O mixed in a ratio of 1:1:1).

2.10 DAB staining to quantify the accumulation of H_2O_2

Leaves infected with *P. infestans* were soaked overnight in 1 mg/mL 3,3′-Diaminobenzidine (DAB) dyeing solution (0.1 g DAB dissolved in 100 mL distilled water, pH 3.8). The leaves were decolorized by boiling for 2 h in absolute ethanol, then treated with lactophenol water (lactic acid, phenol, and water were mixed in a ratio of 1:1:1), and photographed. The H_2O_2 content of *P. infestans*-inoculated transgenic plants was determined as described by Zhang et al. (2014).

2.11 ROS scavenger enzyme activity measurement

Activities of APX CAT, POD, and SOD enzymes were determined according to manufacturer's instructions using the corresponding kits (APX-1-W, CAT-1-W, POD-1-Y and SOD-1-W), purchased from Suzhou Keming Biotechnology Co., Ltd., (Suzhou, China). The infected leaf samples were collected from all treated plants. The data were analyzed using SPSS 16.0.

2.12 Determination of JA content

0.1 g smples collected from potato leaves were cut into pieces, and grinded into powders in liquid nitrogen. The content of JA in potato was determined by plant JA ELISA Kit (Jiangsu Jingmei Biological Technology Co., Ltd) (Yi et al., 2020).

3 Results

3.1 Amplification of *StRab5b*

The potato *StRab5b* gene was amplified using Prime STAR HS DNA Polymerase (Takara). The fragment identified *via* agarose gel electrophoresis and the amplicon size was approximately 607 bp. To analyze the conserved domains of StRab5b, Rab 5b proteins isolated from rice, *N. benthamiana*, lotus, and *Physcomitrella patens* were retrieved from Genbank and used for multi-sequence analysis. The results suggested that StRab5b contained the basic general characteristics of Rab proteins, including RabF2 (83–87), RabF4 (YYRGA, 102–106) and RabF5 (131–135) domains, which are the conserved domains of the Rab proteins. Additionally, it also contained the GTP/GDP binding domain, phosphate binding loop G1 (39–46), G2 (79–80), and the coordination of GTP β and γ phosphate motifs G3 (88–91) and G5 (176–178), binding motifs NKAD (146–149) and ETSA (174–177) (Figure 1A). Therefore, the potato StRab5b protein belongs to Rab protein family.

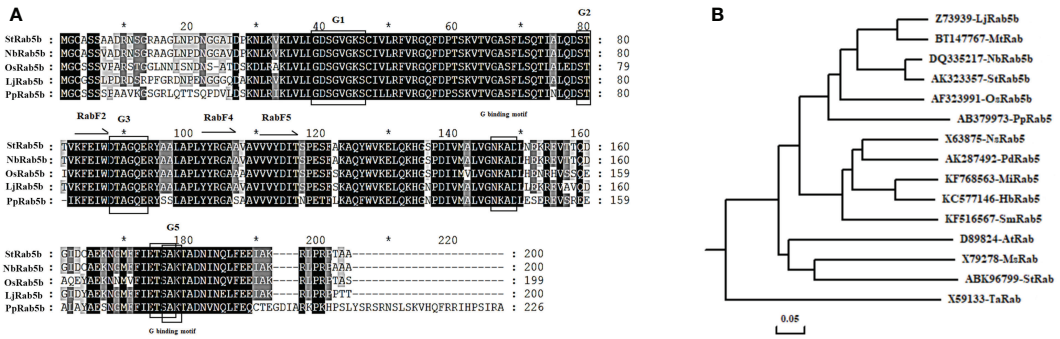

FIGURE 1
Conservative domain and phylogenetic tree analysis of StRab5b **(A)** The conserved domains of *StRab5b*. **(B)** Phylogenetic tree of *StRab5b*. The phylogenetic tree was constructed with DNAMAN v. 6.0. The accession number of each protein is as follows: *Oryza sativa* (AF323991), *Nicotiana benthamiana* (DQ335217), *Solanum tuberosum* (ABK96799 and AK323357), *Hevea brasiliensis* (KC577146), *Physcomitrella patens* (AB379973), *Phoenix dactylifera* (AK287492), *Nicotiana sylvestris* (X63875), *Mangifera indica* (KF768563), *Arabidopsis thaliana* (D89824), *Selaginella moellendorffii* (KF516567), *Medicago sativa* (X79278), *Triticum aestivum* (X59133), *Lotus japonicus* (Z73939), and *Medicago truncatula* (BT147767). The symbol * represents the middle value between two sequence numbers.

Furthermore, the phylogenetic tree was constructed with StRab5b and the other 15 Rab proteins, derived from Oryza sativa, Nicotiana benthamiana, Solanum tuberosum, Hevea brasiliensis, Physcomitrella patens, Phoenix dactylifera, Nicotiana sylvestris, Mangifera indica, Arabidopsis thaliana, Selaginella moellendorffii, Medicago sativa, Triticum aestivum, Lotus japonicus, and Medicago truncatula, showed that StRab5b exhibited the highest similarity (95%) with NbRab5b, whereas, it showed the lowest similarity (44%) with TaRab (Figure 1B).

3.2 Expression profile analysis of *StRab5b* gene and its induced expression in potato

The relative expression levels of *StRab5b* in 'ZHB', 'XPD', 'L7', 'DXR', 'ZS4', and 'DXY' showed a decreasing tendency (Figure 2A). The expression profiles indicated that the expression levels of *StRab5b* differed substantially among different organs; the highest expression was in young leaves, followed by expression in stems, old leaves, and roots (Figure 2B). The tendency of symptoms in 'Desiree' leaves at different time points increased firstly at 12 hpi, followed by reaching the highest point 72 hpi, then dropping to basal level at 108 hpi, indicating the induction of *StRab5b* by *P. infestans* (Figures 2C, D).

3.3 Transient expression enhanced resistance and gene silencing reduced resistance to *P. infestans* in *N. benthamiana* and potato

Transient expression of *StRab5b* in *N. benthamiana* leaves was performed *via* infiltration, followed by inoculation with *P. infestans*. The lesions on the control leaves gradually increased after inoculation, and reached the largest size (102.1 mm^2) at 108 hpi; it is much bigger than the lesion size on leave which transiently expressed *StRab5b*, indicating that transiently ectopic expression of *StRab5b* could enhance *N. benthamiana* resistance to *P. infestans* (Figure 3A).

To verify the function of *StRab5b* on regulating the resistance to *P. infestans*, VIGS was used to silence *StRab5b* and *NbRab5b* in potato and *N. benthamiana* respectively. *PDS* gene was taken alone as an indicator. When photobleaching was observed on *N. benthamiana* and potato leaves at 25 and 60 dpi,

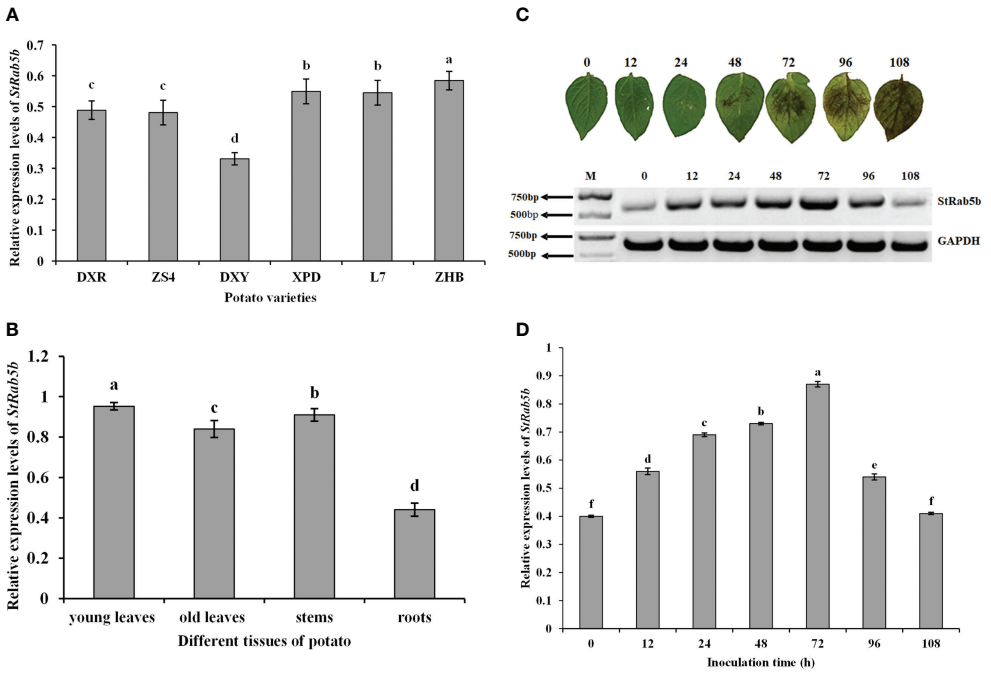

FIGURE 2
Expression pattern and inducible expression of the *StRab5b* gene in potato **(A)** Relative expression levels of *StRab5b* in six different potato varieties. **(B)** Relative expression levels of *StRab5b* in different tissues in cultivar 'Desiree'. **(C)** Phenotype of potato leaves inoculated with *Phytophthora infestans* and the relative expression levels of *StRab5b* in cultivar 'Desiree' at 0–108 hpi. Results were obtained from three biological replicates. **(D)** Quantification of relative expression of *StRab5b* in cultivar 'Desiree' at 0–108 hpi. Different lower case letters show significant differences at $p < 0.05$. Error bars represent the standard deviation (SD) of three biological replicates.

respectively, the expression of *StRab5b*, *NbRab5b* and *PDS* genes was quantified using qRT-PCR and the average silencing efficiencies of *StRab5b*, *NbRab5b* and *PDS* were 59%, 65%, and 100%, respectively (Figures 3B, D). The leaf lesion was recorded upon inoculation with *P. infestans*. The lesion area on the infiltrated leaves was 259.9, 253.4, 284, and 152.8 mm^2 at different time points, which were significantly larger than that on control leaves at the same time point. The average increase in the rate of the lesion area was 3.1 mm^2/h (Figure 3C). However, the leaf lesion areas of potato were 67.4, 83.7, 63.6, and 40.1 mm^2 at 96 hpi, respectively (Figure 3E).

3.4 Overexpression of *StRab5b* enhanced potato resistance to *P. infestans*

Positive transgenic lines were obtained *via* Agrobacteria-mediated transformation of *StRab5b* in potato cultivar 'Desiree' (Figure 4A). The relative expression levels of *StRab5b* among the nine transgenic lines were variable, but significantly higher than its expression levels in control plants. After 96 hpi, the leaf lesions among three transgenic lines (2, 3, and 8) did not differ extensively (5.1, 5.4, and 5.6 mm^2, respectively) (Figure 4C). Meanwhile, the lesions on control leaves increased gradually and reached 96.9 mm^2 at the same inoculation point (Figure 4B). The dead cells on potato leaves were monitored with trypan blue staining; much less blue color was observed on transgenic leaves, indicating a lower number of dead cells on transgenic leaves compared with those in control leaves (Figure 4D). However, Quantitative analyze the amount of colonization of *P. infestans* showed that there were significant differences among the three transgenic lines and EV. In addition, the phenotypic characteristics of transgenic plants showed that the stems, leaves and tubers of transgenic plants presented as red, while the control remained green (Supplementary Figures 1A, B). The anthocyanin accumulation in leaves and tubers of transgenic

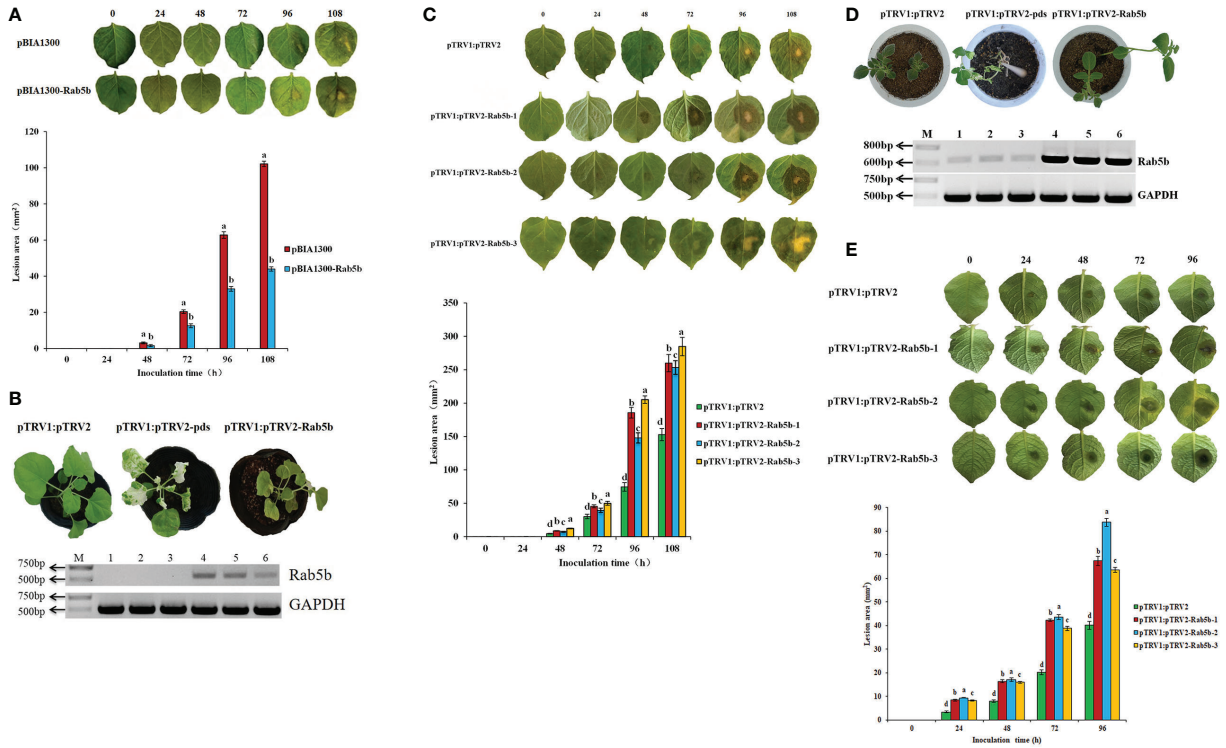

FIGURE 3
The function of the *StRab5b* gene was verified by both transient expression and VIGS in *Nicotiana benthamiana* and potato **(A)** The differences of lesion in *Nicotiana benthamiana* inoculated after transient expression of *StRab5b* homologous gene at 0–108 hpi. **(B)** The Establishment of VIGS system and identification of *StRab5b* homologous gene silencing efficiency in *N. benthamiana*. Results were obtained from three biological replicates. Photobleaching occurs after 25 dpi. Lanes 1-3 represents the plants injected with pTRV1:pTRV2-*Rab5b*, and lanes 4-6 represents the plants injected with pTRV1:pTRV2 **(C)** The leaf symptoms and the lesion area size of silenced *StRab5b* homologous gene in *Nicotiana benthamiana* at different times. **(D)** The formation of VIGS system in potato and determination of *StRab5b* gene silencing efficiency. Results were obtained from three biological replicates. Photobleaching occurs after 60 dpi. Lanes 1-3 represents the plants injected with pTRV1:pTRV2-*Rab5b*, and lanes 4-6 represents the plants injected with pTRV1:pTRV2. **(E)** The leaf symptoms and the lesion area size of silenced *StRab5b* gene in potato at different times. Error bars represent the standard deviation (SD) of three biological replicates. Different lower case letters indicate significant differences at $p < 0.05$.

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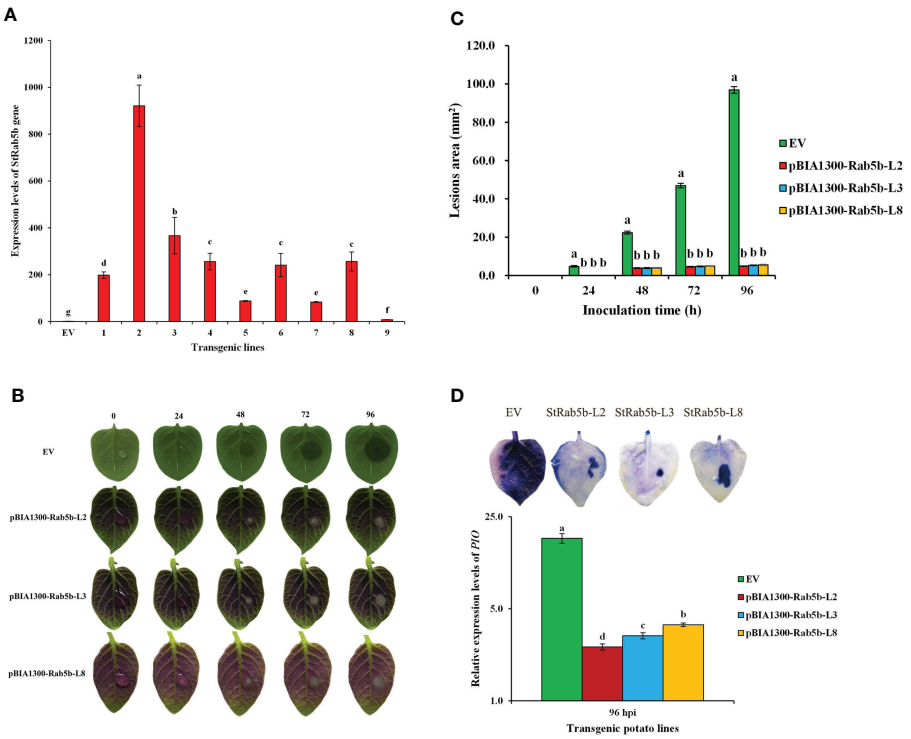

FIGURE 4
Identification of resistance to *Phytophthora infestans* in transgenic potato plants **(A)** The relative expression levels of *StRab5b* gene in 9 transgenic lines. Different lower case letters indicate significant differences at $p< 0.05$. Error bars represent the standard deviation (SD) of three biological replicates. **(B)** The differences of lesion area on transgenic plants inoculated with *P. infestans* at 0–96 h Results were obtained from five biological replicates. **(C)** Quantification of the lesion area on transgenic plants inoculated with *P. infestans* at 0–96 h Different lower case letters indicate significant differences at $p< 0.05$. Error bars represent the standard deviation (SD). **(D)** Trypan blue staining and the amount of colonization after inoculated with *P. infestans* in transgenic leaves. Results were obtained from three biological replicates. *PIO* gene is an indicator of *P. infestans* and *Actin* is a reference gene.

lines were greatly higher than that in the EV control (Supplementary Figure 1C).

3.5 H_2O_2 is involved in StRab5b-mediated resistance to *P. infestans*

DAB staining was performed to detect whether H_2O_2 was involved in the resistance of *StRab5b* to late blight. A brownish color was observed in the *StRab5b* transgenic leaves at 24 hpi and the color deepened at 48 and 96 hpi. However, no signal was observed in control leaves at 24 hpi, and brownish color on control leaves was lighter than that on the transgenic leaves (Figure 5A). The quantification of H_2O_2 accumulation is shown in Figure 5B. The accumulation of H_2O_2 increased after inoculation, peaked at 72 hpi, then decreased in three transgenic and control plants. Compared with the H_2O_2 accumulation level in control (0.097 μmol/g), the highest H_2O_2 level of 0.156 μmol/g was detected in transgenic line 2 at 72 hpi, followed by other two transgenic lines 3 and 8 with values of 0.136 and 0.132 μmol/g, respectively. The value of H_2O_2 was significantly higher than that in control plants after inoculation. These results suggested that *StRab5b* positively regulates potato resistance through H_2O_2 accumulation.

3.6 The activities and related-genes transcripts of antioxidant enzymes increased after inoculation

Four antioxidant enzyme activities (APX, CAT, POD, and SOD) were monitored after inoculation. The results suggested that APX, CAT, POD, and SOD activities in *StRab5b*-transgenic leaves were substantially higher than those in control plants at different inoculation time points (Figure 6). Particularly, SOD, POD, and APX showed a decreasing to- increasing pattern after inoculation, whereas, CAT showed an increasing to- decreasing pattern. Maximum activities of APX and SOD appeared at 72 hpi (54408.5 nmol/min·g and 2180.2 U/g), maximum activities of POD were 566.8 U/g at 96 hpi, except CAT, which had 2 peaks

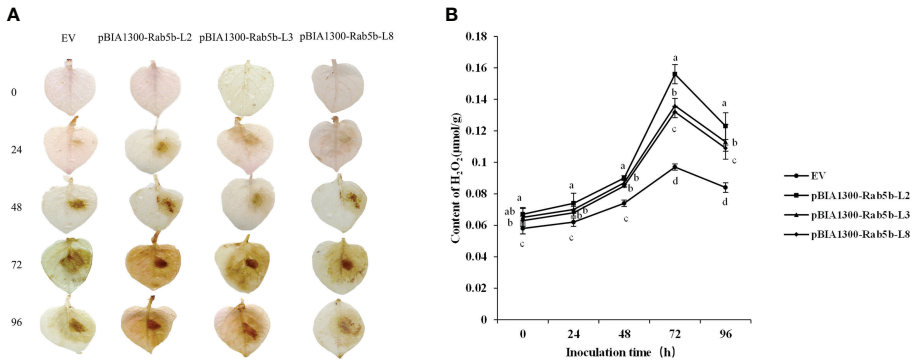

FIGURE 5
Qualitative and quantitative detection of H_2O_2 accumulation after inoculation with *Phytophthora infestans* **(A)** DAB staining to detect H_2O_2 accumulation on transgenic plants. Leaves of transgenic plants were inoculated with *Phytophthora infestans* on the right side, while water was used on the left side as control treatment. Three biological replicates were prepared. Lesion expansion was observed at 0, 24, 48, 72, and 96 hpi. **(B)** Accumulation of H_2O_2 on transgenic plants. Different lower case letters indicate significant differences at $p< 0.05$. Error bars represent the standard deviation (SD) of three biological replicates.

at 48 hpi and 96 hpi (353.1 and 376.8 nmol/min·g), respectively. The expression levels of these four genes (*APX1*, *CAT2*, *POD*, and *SOD*) that encode ROS scavenge enzymes were examined using qPCR after inoculation. The relative expression levels of four genes are consistent with the trend of enzyme activities. The maximum relative expression levels of *APX1*, *CAT2*, *POD*, and *SOD* genes were 5.3, 4.2, 6.2, and 7.5-fold, respectively.

3.7 JA is involved in *StRab5b*-mediated potato resistance to *P. infestans*

Signaling pathways associated with SA, JA, and ET are directly involved in plant resistance to pathogens. To determine whether JA, SA, or ET are involved in *StRab5b*-mediated resistance to *P. infestans*, several marker genes related to different signaling pathways were analyzed at different time points. The results suggested that the expression levels of *LOX* and JA content in three transgenic lines (2, 3, and 8) were induced at 24 hpi and subsequently dropped at 48 hpi, followed by reaching the highest level at 72 hpi (158.2, 140.2, and 124.5-fold expression levels in the *LOX* gene, and 33.2, 24.4, and 20.7 ng/g JA content, respectively); moreover, JA content in transgenic plants was significantly higher than that in EV. Whereas, both *NPR1* and *ACS* showed a different induction pattern compared with the *LOX* gene. They reached the peak at 24 hpi, then decreasing gradually. However, if we compared to the expression of the three marker genes, the induction of *LOX* was much more dramatic than *ACS* or *NPR1*. The induction rate was 67.5-fold at 24 hpi and 141.0-fold at 72 hpi separately, indicating that JA was the dominant molecular signal, which was involved in *StRab5b*-mediated resistance against *P. infestans* (Figure 7).

4 Discussion

4.1 Analysis of *StRab5b* expression profile and induction

Protein StRab5b is an important member of the small G protein Rab family that differs from previously characterized StRab proteins in amino acid structure and domains. The conserved domain motif RabF2 (YYRGA) of the StRab5b was analyzed using multiple sequence alignment. The guanine-binding motif NKAD and the guanine-binding and dissociation motif ETSA were consistent with the expression of TaRab5b protein (Chen et al., 2005). The specific motifs of G1, G3, G5, RabF4, and RabF5 were consistent with those of *Dunaliella salina* and potato StRab proteins. Although the homology of potato *StRab* and *StRab5b* was only 46% at amino acid level, StRab5b showed a structural and functional similarity to the Rab protein (Gao et al., 2012; Yu et al., 2013). Therefore, we replicated the *StRab5b* gene, analyzed its expression profile, and induced expression.

In this study, the relative expression levels of StRab5b were variable among different tissues. The highest expression level was detected in young leaves, followed by stems, old leaves and roots. This is in line with the order of the relative expression levels of another small G protein, *StRac* (Zhang Z et al., 2020). Additionally, the relative expression levels of *StRab5b* among different varieties is also variable, the order from higher to lower is ZHB, DXR, L7, ZS4, DXY, and XPD. It is also partially confirmed by the results that the relative expression of *StBAG3* was also highest in 'Zihuabai' (Lu et al., 2017). Such different expression profiles are likely genotype-dependent. The clear induction pattern of *StRab5b* was also confirmed in 'Desiree', indicating the response of *StRab5b* to *P. infestans* infection.

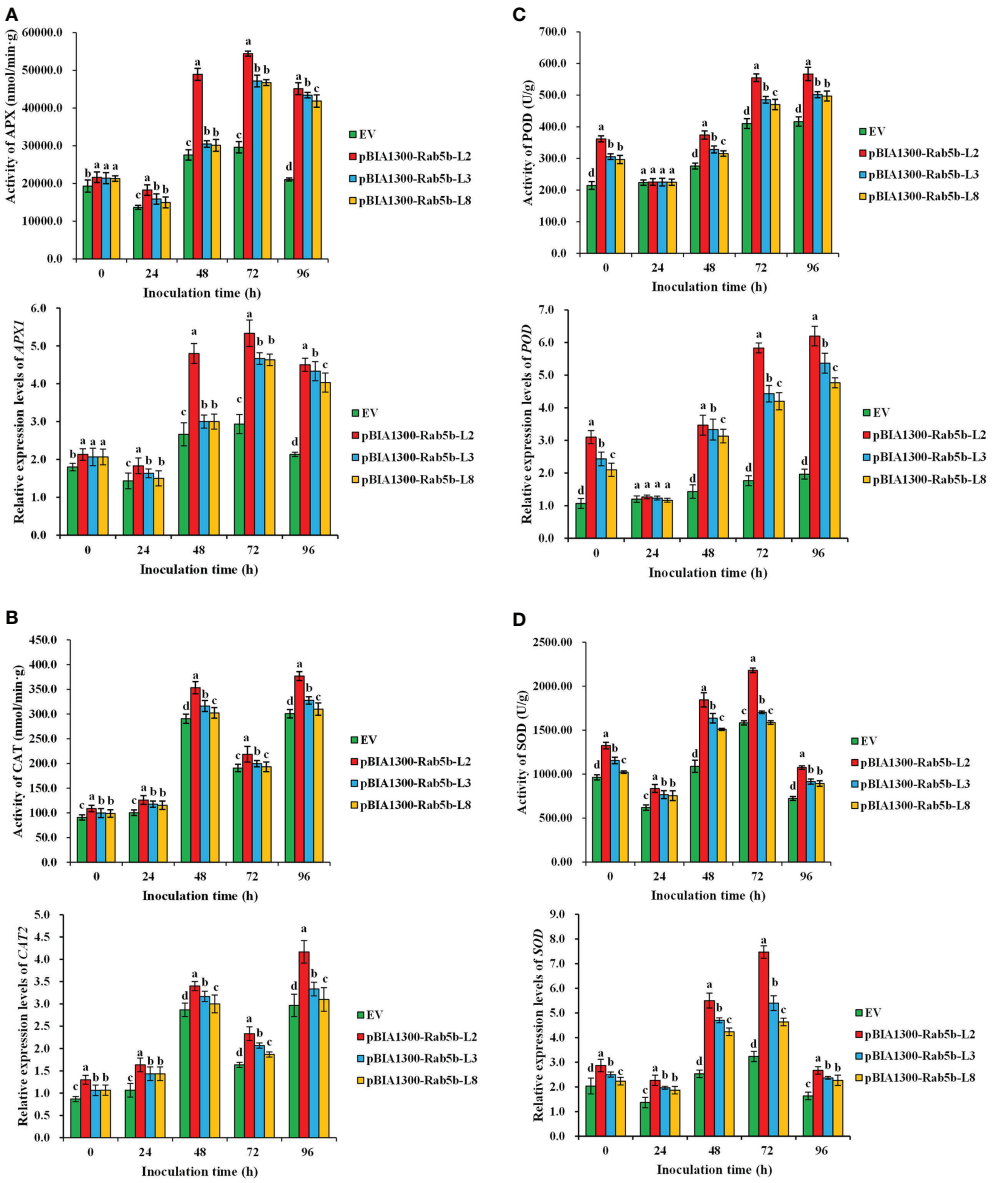

FIGURE 6
Antioxidant enzyme activities and relative expression levels of genes in transgenic plants after inoculation with *Phytophthora infestans* **(A)** The differences of Ascorbate peroxidase activity and expression levels of *APX1* in transgenic plants at 0–96 hpi. **(B)** The differences of Catalase activity and expression levels of *CAT2* in transgenic plants at 0–96 hpi. activity (0–96 h). **(C)** The differences of Peroxidase activity and expression levels of *POD* in transgenic plants at 0–96 hpi. **(D)** The differences of Superoxide dismutase activity and expression levels of *SOD* in transgenic plants at 0–96 hpi. Different lower case letters indicate significant differences at $p < 0.05$. Error bars represent the standard deviation (SD) of three biological replicates.

4.2 Silencing of *Rab5b* gene negatively regulates resistance to *P. infestans*

Virus-induced gene silencing (VIGS) is an important method for studying gene function and has been used routinely in *N. benthamiana, Lycopersicum esculentum*, and *A. thaliana* (Ratcliff et al., 2001; Brigneti et al., 2004; Lacomme and Chapman, 2008; Senthil-Kumar and Mysore, 2014). In the present study, the *StRab5b* silenced leaves were more susceptible to *P. infestans* than control leaves (Figure 3). Similar studies have been reported in *N. benthamiana* and potato. Specifically, small GTPase *NbRanBP1-1*-silencing reduced resistance to *P. infestans* in plants of *N. benthamiana* (Mizuno et al., 2019). Consistently, when silencing *EIN2*, which was involved in pre-invasion phase, facilitated the penetration rate of *P. infestans* (Rin et al., 2017). However, the silencing of

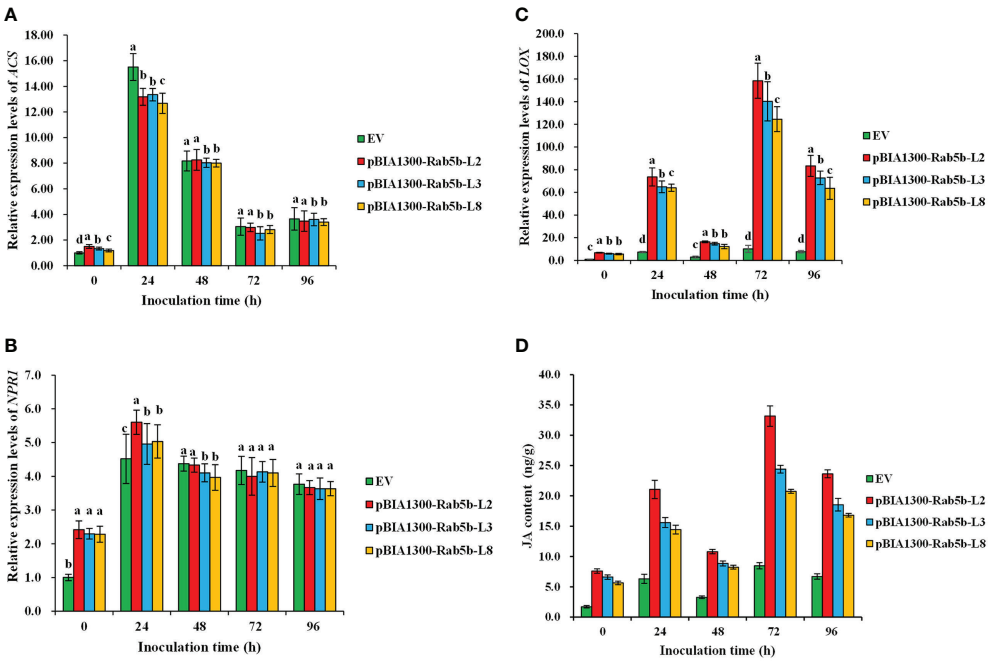

FIGURE 7
Transcriptional levels of jasmonic acid JA-, salicylic acid SA- and ethylene ET-related genes in transgenic plants after inoculation with *Phytophthora infestans*. **(A)** Relative expression levels of *ACS*. *ACS* gene expression levels were examined by qPCR at 0–96 hpi. **(B)** Relative expression levels of *NPR1*. *NPR1* gene expression levels were examined by qPCR at 0–96 hpi. **(C)** Relative expression levels of *LOX*. *LOX* gene expression levels were examined by qPCR at 0–96 hpi. **(D)** Determination of JA content in transgenic potato lines inoculated with late blight. The JA content was examined using ELISA at 0–96 hpi. Different lowercase letters show significant differences at $p< 0.05$. Error bars represent the standard deviation (SD) of three biological replicates.

Matrix metalloprotease 1 made *N. benthamiana* more susceptible to *P. infestans*, but enhanced Nep1-like protein induced cell death (Ha et al., 2017). Silencing five candidate genes *TRV:12* (*sodium dicarboxylate cotransporter*), *TRV:17* (*UDP-arabinose 4-epimerase*), *TRV:21* (*2-oxoglutarate dehydrogenase*), *TRV:22* (Lipoxygenase), and *TRV:48* (*suberization-associated anionic peroxidase*) from *N. benthamiana* facilitated the infection of *P. infestans* (Du et al., 2013). Conversely, silencing *StERF3* in potatoes not only enhanced foliage resistance to *P. infestans*, but also promoted plant tolerance to salt stress (Tian et al., 2015).

4.3 Overexpressing *StRab5b* in transgenic lines regulates the resistance to *P. infestans* by manipulating H_2O_2 accumulation

Currently, small G proteins reportedly respond to abiotic stress factors and regulate plant defense or stress-related gene expression (Miao et al., 2018). The H_2O_2 was previously recognized as a toxic metabolite in plant cells. However, it has been known that H_2O_2 also mediates stimulus responses to plant cells as a signaling factor (Bailey-Serres and Mittler, 2006). According to previous research, small G proteins of the RAB family play important roles in the establishment of plant defense responses (Lamb and Dixon, 1997). In this study, the results confirmed that H_2O_2 accumulation in the transgenic lines might be caused by *StRab5b* expression on the membrane. Previous studies on rice disease resistance have shown that small GTPase *OsRac1* induces hydrogen peroxide products, and enhances resistance to rice blast disease in transgenic plants (Ono et al., 2001). Overexpression of *NtRop* activated H_2O_2 products in Arabidopsis transgenic lines (Cao et al., 2008). In potato, stable expression *DN-AtRop1* led to H_2O_2 accumulation associated smaller lesions on leaves after inoculation with *P. infestans* (Zhang et al., 2014). Among Rab proteins, overexpression of *StRab* in potato reduced the leaf lesion area and increased H_2O_2 production after inoculation with *P. infestans* (Gao et al., 2012). However, less H_2O_2 was observed in miR172a transgenic tomato plants after inoculation with *P. infestans* than in the wild type (Luan et al., 2018). These findings indicate that the involvement of H_2O_2 in disease resistance is a complex phenomenon, and that H_2O_2 accumulation in plant tissues experiences early and late stages. Furthermore, when plants lose the ability for regulation in the late stage, H_2O_2 may be induced in the early

or late stages after inoculation, and may thus increase in concentration, leading to the cell death.

4.4 Antioxidant enzyme activities and key genes are activated in *StRab5b* transgenic lines

In plants, H_2O_2 is mainly produced through NADPH oxidase and polyamine oxidase (PAO) pathways. The production of H_2O_2 in plants generally suffer abiotic stress through the POD pathway. The extracellular PH value temporarily increases, which can activate the peroxidase of cell wall, and reduces O_2 to H_2O_2 (Khokon et al., 2010). The activities of SOD and POD were higher in miR172 transgenic tomato plants after inoculation with *P. infestans*, accompanied by less H_2O_2 (Luan et al., 2018). As shown in Figure 6, the inoculated *StRab5b*-transgenic plants had higher APX, SOD, POD, CAT activities and expression levels of ROS related-genes, and increased H_2O_2 accumulation. These results suggested that *StRab5b* in potato may modulate antioxidants to eliminate redundant H_2O_2 and prevent cellular membrane injury after *P. infestans* infection. Similar results have been found in tomato, and the amounts of H_2O_2 and O_2^- were increased with the continuous salt stress in both roots and leaves. Compared to control, the plants had increased activities of SOD, CAT, POD, and APX after 6 or 12 days of salt stress (Raziq et al., 2022). In tomato, the melatonin-induced H_2O_2 generation through RBOH was attributed to activate some genes expression (*CDPK1*, *MAPK1* and *ERD15*). Conversely, the inhibition of RBOH reduced stress defense response and antioxidant enzyme activity (SOD, CAT and APX) (Gong et al., 2017). In addition, a previous study reported that *APX1* and *SOD* genes showed higher levels in SA- or $CdCl_2$-treated potato plants, when compared to CK plants (Li et al., 2019). Brassinosteroid (BR) activated the expression of the *StPOD* gene, and the expression level was partially increased in potato tubers at the end of wound healing (Han et al., 2022). Some researchers believe that the highly active antioxidant enzymes are used to eliminate active oxygen, and the concentration of H_2O_2 is reduced.

4.5 Potato JA-mediated resistance to late blight is regulated by *StRab5b*

Plant defense responses refer to the resistance of plants to external infection, including the synthesis of pathogenesis-related (PR) proteins. Plant hormones SA, JA, and ET play major roles in the plant immune system (Verma et al., 2016). There are many studies regarding plant resistance and JA signaling pathway. For example, JA may be involved in resistance regulated by AtRop1 (Zhang et al., 2014). Overexpression of *OsRab11* in *Arabidopsis* enhances plant resistance to *Pseudomonas syringae* by inducing the expression of JA-responsive genes, rather than SA-responsive genes (Hong et al., 2013). These results are consistent with those obtained in the present study. Thus, the key enzyme-encoding gene in the JA signaling pathway, i.e., *LOX* and JA content were dramatically induced in *StRab5b* transgenic lines at different inoculation time points, indicating the involvement of JA signaling pathway on potato resistance manipulated by *StRab5b*. This is in contrast with the enhanced late blight resistance observed by the overexpression of LecRK-I.9 in *Arabidopsis* by reducing SA-responsive gene expression (Bouwmeester et al., 2014). SA and ROS were confirmed as the primary signaling molecules that mediated potato resistance to *P. infestans* (Lamb and Dixon, 1997; Na et al., 2012), and the present study clearly highlights the involvement of the JA pathway in potato resistance to *P. infestans*. However, the cross talk of JA and SA signaling pathways were confirmed *via* hormone-responsive transcription factors WRKY70 and PDF1.2, which regulate plant defense responses (Petersen et al., 2000; Li et al., 2004). In other words, the mechanism underlying the involvement of small G proteins on manipulating potato resistance against late blight resistance is rather complex, and different signaling pathways interact with each other through key transcription factors to improve durable resistance in solanaceous crops. Whether the same mechanism occurs in small G protein-mediated establishment of potato resistance remains to be explored. Further experiments are needed to delineate the signaling transduction pathway mediated by small G proteins in potato.

Data availability statement

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

Author contributions

ZT and ZZ conceived the experiments. JuZ designed and guided the experiments scheme. ZT, ZZ, LK and ML completed the experiments. ZT, XG, JY, JiZ and YF provided consumable materials/reagents/software and analyzed the data. ZT wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1065627/full#supplementary-material

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Facilitating effects of the reductive soil disinfestation process combined with *Paenibacillus* sp. amendment on soil health and physiological properties of *Momordica charantia*

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Reductive soil disinfestation (RSD) is an anaerobic and facultative anaerobic microbial-mediated soil management process. The extent of improvement of diseased soil properties by RSD relative to comparable healthy soil is, however, not well characterized. Importantly, how to promote the colonization efficiency of these facultative anaerobic functional species to ensure soil and plant health remain unknown. Here, *Fusarium* wilt-diseased soil of *Momordica charantia* grown under a plastic-shed field (PS-CK) was used to conduct molasses-RSD (MO-RSD) along with *Paenibacillus* sp. (a model of facultative anaerobic species) (MO_{PA}-RSD) treatment, and the soil from a nearby open-air paddy field was considered comparable healthy soil (OA-CK). Both RSD treatments significantly improved the properties of PS-CK soil, and the extent of improvement of soil pH, *Fusarium oxysporum* reduction efficiency (98.36% ~99.56%), and microbial community and functional composition were higher than that achieved for OA-CK soil, which indicated that RSD-regulated most soil properties outperformed those of the comparable healthy soil. The disease incidence and ascorbic acid content of *M. charantia* in MO-RSD- and MO_{PA}-RSD-treated soils were considerably decreased, while the weight and soluble protein contents were correspondingly increased, as compared to those of *M. charantia* in PS-CK soil. Specifically, the changes in these physiological properties of *M. charantia* in MO_{PA}-RSD soil performed well than that in MO-RSD soil. The relative abundances of *Cohnella*, *Effusibacillus*, *Rummeliibacillus*, *Oxobacter*, *Thermicanus*, and *Penicillium* enriched in both RSD-treated soils were positively correlated with *Paenibacillus* and negatively correlated with

F.oxysporum population and disease incidence ($P < 0.05$). Notably, the relative abundances of these potential probiotics were considerably higher in MO_{PA}-RSD-treated soil than in MO-RSD alone-treated soil. These results show that the RSD process with inoculation of *Paenibacillus* sp. could promote the colonization of this species and simultaneously stimulate the proliferation of other probiotic consortia to further enhance soil health and plant disease resistance.

KEYWORDS

soil health, microbial community, *paenibacillus*, probiotic consortia, fusarium wilt

Introduction

Driven by the shortage of land resources and economic benefits, the plastic shed cultivation system has become a popular soil utilization strategy in contemporary agriculture. Unfortunately, the management practices adopted in this cultivation system are usually characterized by overfertilization and intensive cultivation, thereby posing a serious threat to plant health through its adverse effects on soil properties (Fan et al., 2021; Shen et al., 2021). It is accepted that the soil microbial community that dominated by some beneficial functional microorganisms, such as rhizosphere growth-promoting bacteria (PGRP), can directly limit the invasion of pathogens into plant roots by competing for ecological resources, producing antagonistic substances, and inducing plant defense response (Raaijmakers et al., 2009; Mendes et al., 2011; Jin et al., 2022). Soil abiotic properties (e.g., pH and contents of salt, moisture, and nutrients) can indirectly influence plant health through their effects on microbial community composition (Janvier et al., 2007; Fierer, 2017; Liu et al., 2022a). Thus, effective management strategies for soil abiotic and biotic properties should be developed to maintain soil ecosystem health and ensure future food security.

Reductive soil disinfestation (RSD), also known as biological or anaerobic soil disinfestation, is defined as a process in which soil is incorporated with labile organic matter, irrigated to saturation, and covered with a plastic film to establish a strong anaerobic and reductive environment for improving soil physicochemical properties and microbial communities (Blok et al., 2000; Butler et al., 2012; Huang et al., 2016). Organic acids (acetic acid, propionic acid, butyric acid, and isovaleric acid) produced by functional microorganisms (such as *Clostridium*, *Coprococcus*, and *Ruminococcaceae*) during RSD treatment can significantly protect against multiple plant pathogens (Huang et al., 2015; Shrestha et al., 2016; Mao et al., 2022). To date, the RSD practice is widely considered an effective strategy for soil and plant health management and has been popularized in the Netherlands, China, USA, and Japan (Blok et al., 2000; Butler et al., 2012; Momma et al., 2013; Huang et al., 2016). Despite such promising results, the maximum improvement efficiency of soil properties by RSD remains unclear; this is because the current findings are obtained for diseased soils and lack the results for comparable healthy soils. Particularly, these studies on RSD aimed at alleviating the occurrence of plant diseases; however, how RSD affects other physiological properties of plants, especially predicators related to plant defense response, has not been well studied.

Because most fungi grow in an aerobic environment, the functional microorganisms during the RSD treatment process are dominated by anaerobic and facultative anaerobic bacteria such as most members of *Firmicutes* (Mowlick et al., 2013; Hewavitharana et al., 2019). The composition of these functional microorganisms in RSD-treated soils could be affected by the characteristics of organic matter, including decomposability, total carbon input, and carbon/nitrogen (C/N) ratio (Butler et al., 2012; Liu et al., 2016; Shrestha et al., 2021). For example, a higher abundance of *Firmicutes* was observed in RSD soil incorporated with readily decomposable organic matter (such as molasses and ethanol) than in RSD soil incorporated with plant residues, while soil without organic matter input (irrigated alone) showed no influence on *Firmicutes* composition (Liu et al., 2016; Hewavitharana et al., 2019). Additionally, although facultative anaerobic microorganisms continue to be the dominant taxa even after aerobic conditions are restored for RSD-treated soil, their abundance often shows a declining trend as compared to that during RSD treatment (Mowlick et al., 2013; Liu et al., 2018). Therefore, it is important to develop appropriate methods to promote the colonization efficiency of these native functional microorganisms, especially facultative anaerobic bacteria, in RSD-treated soils for maintaining soil and plant health. Our previous studies showed that biocontrol agents such as *Bacillus subtilis* SQR-N1 and *Trichoderma harzianum* SQR-T37 inoculated into the RSD-completed soil (aerobic conditions) could further enhance its resistance to damping-off

and *Fusarium* wilt diseases of cucumber (Huang et al., 2016; Ali et al., 2022). However, we are unsure whether the functional microorganisms inoculated into the strongly anaerobic and reductive environment, which is the RSD treatment process, may improve their colonization efficiencies and plant disease resistance.

Paenibacillus sp. is an important PGRP and has been reported as a common facultative anaerobic species in the RSD practices that treated with different types of organic materials (Huang et al., 2018; Zhao et al., 2020; Liu et al., 2021). Specifically, our previous study has demonstrated that the relative abundance of *Paenibacillus* sp. in the RSD incorporated with liquid-readily decomposable compounds (such as molasses) was significantly higher than that amended with solid plant residues (such as sawdust) (Yan et al., 2022). In the present study, therefore, the diseased soil of *Momordica charantia* severely infected with *Fusarium oxysporum* f. sp. *momordicae* cultivated under the plastic shed field (PS-CK) was used to conduct molasses-RSD (MO-RSD) along with *Paenibacillus* sp. inoculation (MO_{PA}-RSD); soil from the nearby open-air paddy field (OA-CK) was considered a comparable healthy or original soil. The study aimed to address the above-mentioned knowledge gaps: (1) to what extent does RSD improve the properties of PS-CK soil relative to those of OA-CK soil, (2) whether the addition of facultative anaerobic functional microorganisms during RSD treatment is more conducive to manage plant physiological properties, and (3) what are the underlying drivers that could be revealed by deciphering changes in soil microbial communities after treatment?

Materials and methods

Field description

The plastic shed field was located in Nanmiao Town (28°9′ N, 114°42′ E), Jiangxi Province, China. Limited rotation of *M. charantia* and edible amaranth is the primary cultivation pattern in this field, while the incidence of *Fusarium* wilt disease of *M. charantia* has exceeded 30% in recent seasons. The classification of this soil is a Feeralic Cambisol (IUSS Working Group WRB, 2015), with the following initial properties: total organic carbon (TOC) 26.02 g kg^{-1}; total nitrogen (TN) 3.52 g kg^{-1}; and pH 4.11 (Liu et al., 2022b).

Experimental design

Four treatments with a randomized complete block design were conducted in this study: OA-CK, untreated flooded paddy soil in the open-air cultivation system; PS-CK, untreated soil (converted from OA-CK 4 years ago) in the plastic shed field with moisture content maintained at 15~20%; MO-RSD, PS-CK soil amended with 7.5 t ha^{-1} molasses (TOC 347.8 g kg^{-1}, TN 16.8 g kg^{-1}, and C/N ratio 20.7), irrigated to saturation, and covered with a plastic film (transparent, 0.08 mm thickness); and MO_{PA}-RSD, MO-RSD treatment process combined with inoculation of *Paenibacillus* sp. agent (2.5×10^{10} copies g^{-1} m^{-2}) provided by Beihai Ye Sheng Wang Biological Texhnology Co., LTD, Guangxi Province, China (accession number: OP602366). The molasses and *Paenibacillus* sp. agent were diluted to 5% before use. Each treatment contained three replicates, and the area of each replicate covered 60 m^2. The treatment was followed for 18 days at an average greenhouse temperature of 28~40 °C (randomly measured by a thermometer). The additive amount of molasses and incubation period and temperature during RSD treatment were according to our previous study (Yan et al., 2022), which has demonstrated that RSD treatment under the above conditions can significantly improve soil quality by improving soil microbial community and function. The plastic films in the RSD treatments were then removed, and the soils were then drained. Soil samples (0~15 cm depth) in each replicate were collected from 6 soil cores and stored at 4°C for analyzing physicochemical properties and at -20 °C for DNA extraction. Seedlings of *M. charantia* with three leaves were planted in PS-CK-, MO-RSD-, and MO_{PA}-RSD-treated soils at intervals of 50 cm. After 90 days, the disease incidence in each soil was recorded. The fruits, ranked from largest to smallest in weight (Figure S1), were randomly collected from each replicate to detect the physiological properties.

Determination of soil physicochemical and plant physiological properties

Soil pH and electrical conductivity (EC) were measured using the S220K and S230K electrodes (Mettler-Toledo International Inc., Shanghai, China) with a soil: water (w/v) ratio of 1:2.5 and 1:5, respectively. Soil NO_3^--N and NH_4^+-N were extracted using KCl solution (2 mol L^{-1}) with a ratio of soil: solution of 1:5 and detected by a continuous flow analyzer (San++; Skalar, Breda, the Netherlands).

The disease incidence, fruit weight, ascorbic acid content, soluble protein content, and texture properties (hardness and fracturability) of fruits were used to indicate the physiological properties of the plant. Ascorbic acid content was measured with 2,6-dichlorophenol indophenol using the colorimetric method with the measurement of absorbance at 520 nm (Niroula et al., 2021). Soluble protein content was estimated using the Coomassie Brilliant Blue G-250 dye as described by Bradford (1976), and bovine serum albumin was used to generate the protein standard. Hardness and fracturability of the fruit were determined using a 2-mm-diameter stainless steel cylinder probe at the speed of 1 mm s^{-1} on a texture analyzer (TA.XTC-18, BosinTech Co., Ltd., Shanghai, China). All measurements were conducted in triplicate to avoid the randomness of the data.

DNA isolation and microbial quantification

Soil microbial DNA was isolated from each replicate (0.5 g soil) using the FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA), and the quality of the isolated DNA was assessed by a DS-11 spectrophotometer (Denovix Inc., Wilmington, DE, USA). Bacteria, fungi, and *F. oxysporum* were quantified using the QuanStudio 3 Real-Time PCR system (Applied Biosystems, USA). All PCR reaction mixtures contained 1 μL of paired primers (Table S1), 2 μL of template DNA, 10 μL of SYBR Green premix Taq (2×), and 6 μL of sterile distilled water. Amplification protocols, specificity, and standard curves of each gene were established according to a previous study (Yan et al., 2022).

MiSeq sequencing and data processing

The V4–V5 region of bacterial 16S rDNA and the internal transcribed spacer (ITS) region of fungi in all pre-planting soil samples (4 treatments × 3 replicates) were amplified using the paired primers 515F/907R and ITS1F/ITS2R, respectively (Table S1). All PCR reactions were performed using 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 2 μM of paired primers, and 10 ng template DNA. The equimolar concentrations of amplicons were then sequenced by Beijing Novogene Bioinformatics Technology Co. Ltd. (Beijing, China) on an Illumina NovaSeq platform.

Quantitative insights into microbial ecology 2 (QIIME2) (Bolyen et al., 2019) software was used to process the raw sequencing data along with the customized program scripts (https://docs.qiime2.org/2019.1/). Briefly, the files of paired-end FASTQ sequences were imported into the format that could be recognized by the QIIME2 system using the qiime tools import program. Demultiplexed sequences from each sample were quality-filtered, trimmed, de-noised, and merged. The chimeric, contaminating mitochondrial, and chloroplast sequences were then identified and removed using the QIIME2 dada2 plugin to obtain the feature table of amplicon sequence variant (ASV) (Callahan et al., 2016). The high-quality bacterial and fungal ASV sequences were rarefied to 36,429 and 40,584 sequences and then annotated according to pre-trained databases of SILVA (Quast et al., 2013) and UNITE (Kõljalg et al., 2013) at 99% similarity by using the QIIME2 feature-classifier plugin, respectively.

Bioinformatics and statistical analyses

Microbial α diversity was calculated using the core-diversity plugin in QIIME2. Microbial β diversity based on Bray–Curtis distance matrices at the genus level was analyzed by principal coordinate analysis (PCoA) using the Wekemo Bioincloud (https://www.bioincloud.tech). The effect of different treatments on microbial β diversity was calculated by permutational multivariate analysis of variance (PERMANOVA) using the "adonis" function in R "vegan" package (Oksanen et al., 2022). Moreover, because the microbial communities between OA-CK and PS-CK soils or MO-RSD- and MO_{PA}-RSD-treated soils have certain similarities, the interactions of bacterial or fungal taxa between CK- (PS-CK and OA-CK) and RSD- (MO-RSD and MO_{PA}-RSD) treated soils were determined using the co-occurrence network analysis. In brief, bacterial and fungal ASV sequences with abundances lower than 0.15% and 0.1% were filtered, and Pearson's correlations with a cutoff at $|r| > 0.95$ and P-value < 0.01 (corrected by false discovery rate) among the obtained taxa were calculated using R "psych" package (Steinhauser et al., 2007). Gephi (Bastian et al., 2009) software (version 0.9.2) was then used to visualize the network composition and determine the topology parameters. To gain insights into the effect of RSD on soil microbial functions, PICRUSt2 and FUNGuild were used to infer Kyoto Encyclopedia of Genes and Genomes (KEGG) and ecological guild functional profiles based on the bacterial 16S rDNA and fungal ITS ASV sequences, respectively (Langille et al., 2013; Nguyen et al., 2016). The relationships between soil dominant genera and plant physiological properties were visualized by the partial mantel test and network interaction analysis according to Liu et al. (2022a).

Significant differences ($P < 0.05$) between plant physiological properties, soil physicochemical properties, microbial abundance (transformed by $1og_{10}$), and dominant genera in different treatments were determined by one-way analysis of variance (ANOVA) and LSD test using SPSS 22.0 (SPSS Inc., Chicago, IL).

Results

Soil physicochemical properties

The pH of PS-CK soil was significantly decreased ($P < 0.05$) by 0.95 as compared to that of OA-CK soil, while the opposite result was observed following the treatment of PS-CK soil with MO-RSD and MO_{PA}-RSD, wherein the pH of these soils remarkably increased ($P < 0.05$) by 1.16 and 1.23, respectively, and was significantly higher than that of OA-CK soil (Table 1). Compared to OA-CK soil, PS-CK soil showed a significant increase ($P < 0.05$) in EC and NO_3^--N by 0.38 mS cm^{-1} and 134 mg kg^{-1}, respectively (Table 1), while these properties were considerably ($P < 0.05$) decreased in RSD-treated soils (Table 1). Specifically, soil NO_3^--N were at a similar level ($P > 0.05$) between OA-CK and RSD-treated soils. The NH_4^+-N content in the RSD-treated soils was significantly higher ($P < 0.05$) than that in CK soils (Table 1).

TABLE 1 Soil physicochemical properties in different treatments.

Treatment [a]	pH [b]	EC (mS cm^{-1}) [c]	NO_3^-N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})
PS-CK	4.11 ± 0.11c	0.41 ± 0.33a	135.0 ± 2.25a	55.55 ± 16.41b
OA-CK	5.06 ± 0.09b	0.03 ± 0.00d	1.00 ± 0.36b	3.15 ± 0.25c
MO-RSD	5.27 ± 0.07a	0.11 ± 0.01c	0.41 ± 0.11b	88.15 ± 5.27a
MO_{PA}-RSD	5.34 ± 0.08a	0.15 ± 0.02b	0.48 ± 0.14b	101.75 ± 11.79a

[a]PS-CK, untreated soil under the plastic shed field system; OA-CK, untreated flooded paddy soil under the open-air cultivation system; MO-RSD, PS-CK soil amended with 7.5 t ha^{-1} molasses, irrigated to saturation, and covered with a plastic film; MO_{PA}-RSD, MO-RSD treatment process combined with *Paenibacillus* sp. agent inoculation.
[b]Values (means ± SD, n = 3) followed by different letters in each column represent significant differences at $P < 0.05$ according to LSD test.
[c]EC, electrical conductivity.

Populations of soil bacteria, fungi, and *F. oxysporum*

The population of bacteria in PS-CK soil (2.49×10^{10} copies g^{-1}) was significantly decreased ($P < 0.05$) by 0.39-fold as compared to that in OA-CK soil (4.15×10^{10} copies g^{-1}), while the bacterial abundance was considerably increased ($P < 0.05$) by 2.81- or 2.16-fold and by 1.28- or 0.90-fold in MO-RSD-treated soil (9.50×10^{10} copies g^{-1}) and MO_{PA}-RSD-treated soil (7.89×10^{10} copies g^{-1}) when compared with those in PS-CK and OA-CK soils, respectively (Figure 1A). No significant difference ($P > 0.05$) was observed in the population of fungi between PS-CK, OA-CK, and MO-RSD, while the fungal abundance was remarkably decreased ($P < 0.05$) by 65.53% after PS-CK soil (1.51×10^8 copies g^{-1}) was treated with MO_{PA}-RSD (5.21×10^7 copies g^{-1}) (Figure 1B). The population of *F. oxysporum* in PS-CK soil (5.13×10^6 copies g^{-1}) was significantly increased ($P < 0.05$) by 90.43% as compared to that in OA-CK soil (4.91×10^5 copies g^{-1}), whereas the population of this pathogen was remarkably decreased ($P < 0.05$) by 98.36% and 99.56% after PS-CK soil was treated with MO-RSD (8.43×10^4 copies g^{-1}) and MO_{PA}-RSD (2.29×10^4 copies g^{-1}), respectively (Figure 1C). The fungi/bacteria ratio in OA-CK and RSD-treated soils was significantly lower than that in PS-CK soil, while the lowest

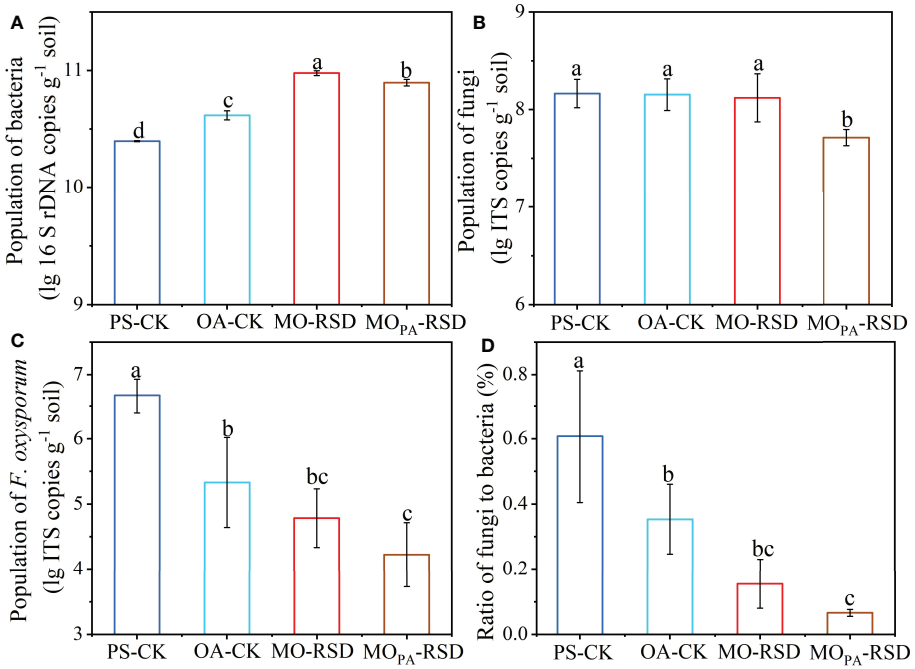

FIGURE 1
The populations of bacteria **(A)**, fungi **(B)**, and *F. oxysporum* **(C)** as well as the ratio of fungi to bacteria **(D)**. Error bars represent standard deviations (SDs). Different letters between various treatments represent significant differences at $P < 0.05$ according to LSD test. The treatment abbreviations are defined in Table 1.

fungi/bacteria ratio was detected in MO_{PA}-RSD soil (Figure 1D). Notably, these parameters of MO_{PA}-RSD-treated soil were lower than those of MO-RSD-treated soil.

Soil microbial α and β diversities

Bacterial richness (observed species) in MO_{PA}-RSD-treated soil was significantly decreased ($P < 0.05$) as compared to that in PS-CK soil, whereas bacterial richness in the remaining soils and fungal richness in all soils were not significantly different ($P > 0.05$) (Figures 2A, B). Bacterial Shannon index between PS-CK and OA-CK soils and fungal Shannon index in all soils showed no significant difference ($P > 0.05$), whereas RSD treatments remarkably decreased the bacterial Shannon index as compared to that in PS-CK soil (Figures 2C, D).

PCoA plots showed that the microbial community was significantly different ($P < 0.01$ according to PERMANOVA) between RSD-treated soils and CK soils (Figures 2E, F). Specifically, the dissimilarities in bacterial and fungal community structures between PS-CK and RSD-treated soils (determined by PC 1 scores: 78.76% and 60.42%, respectively) were larger than those between PS-CK and OA-CK soils (determined by PC 2 and PC 3 scores: 14.75% and 12.27%, respectively). The bacterial and fungal communities in MO_{PA}-RSD-treated soil were slightly changed as compared to those in MO-RSD-treated soil, as determined by PC 3 (2.35%) and PC 2 (15.54%) scores, respectively.

Co-occurrence network of soil microbial community

Co-occurrence network analysis showed that the microbial interactions in RSD-treated soils (MO-RSD and MO_{PA}-RSD) were significantly different as compared to those in CK soils (PS-CK and OA-CK) (Figures 3A-D). Specifically, the interactions of bacterial and fungal species in RSD-treated soil primarily included the members of *Firmicutes* and *Ascomycota* that accounted for >90% of the obtained sequences and more than that in the CK soils, respectively (Figures 3E, F). In addition, compared to CK soils, the RSD-treated soils showed higher topological characteristics of the bacterial network, such as number of nodes and edges, average connectivity, modularity, average path length, and average clustering coefficient; however, the fungal network in both RSD-treated soils showed an opposite trend for number of edges, average connectivity, modularity, and average path length (Table S2).

Soil microbial community composition

The dominant bacterial phyla, accounting for 87.48~96.63% of all sequences, across all treatments were *Firmicutes*, *Acidobacteriota*, *Proteobacteria*, *Chloroflexi*, *Actinobacteriota*, *Gemmatimonadota*, *Desulfobacterota*, *RCP2_54*, *Bacteroidota*, and *Myxococcota* (Figure S2A). The relative abundances of all the phyla, except for *RCP2_54*, were significantly different

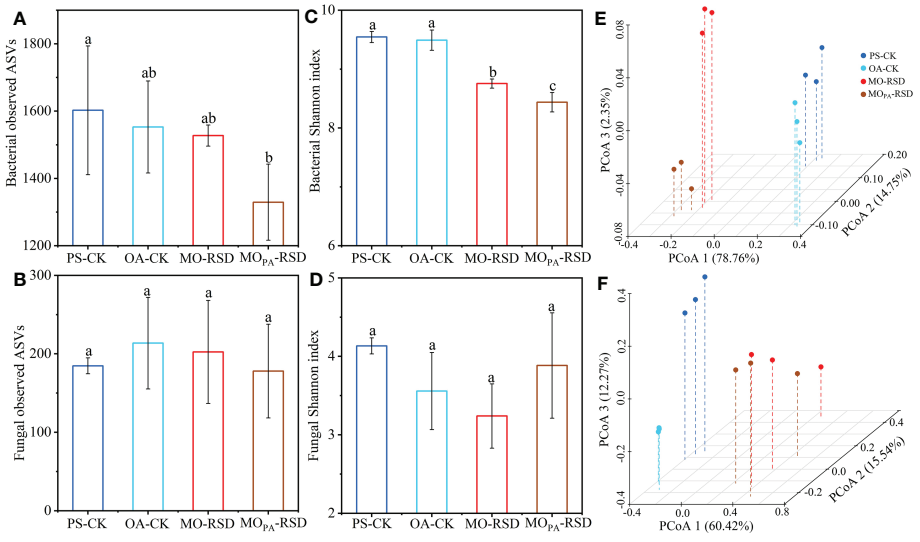

FIGURE 2
The microbial α **(A-D)** and β **(E, F)** diversities in the different treatments. Principal coordinates analyses (PCoAs) of the bacterial **(E)** and fungal **(F)** community structures were determined using the Bray-Curtis distance indices at the genus level. Error bars represent SDs. Different letters between various treatments represent significant differences at $P < 0.05$ according to LSD test. The treatment abbreviations are defined in Table 1.

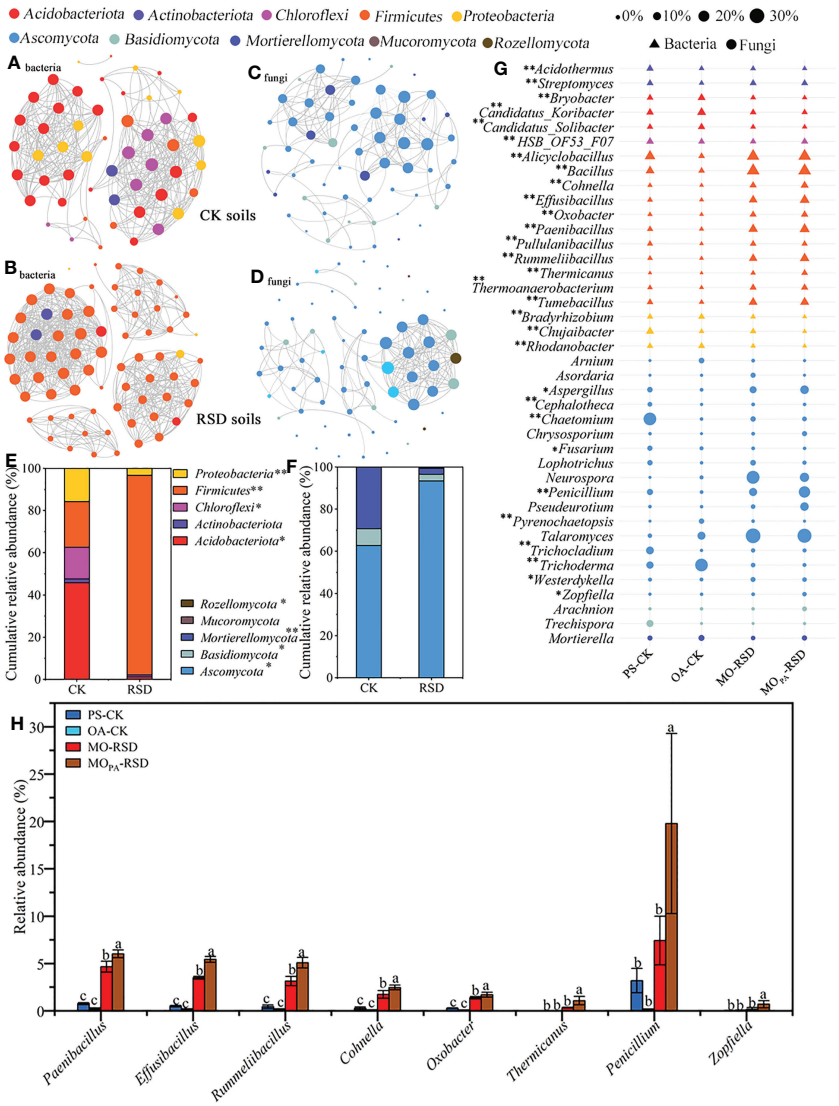

FIGURE 3
Dissimilarities in dominant microbial compositions among the different soils. Co-occurrence networks of bacterial **(A, B)** and fungal **(C, D)** dominant taxa were calculated using the Pearson correlations with a cutoff at $|r| > 0.95$ and P-value < 0.01 (FDR corrected), and the cumulative relative abundances of these nodes at the phylum level were showed in plots **(E, F)**. Relative abundances of the top 20 dominant bacterial and fungal genera **(G)** and that significantly enriched in RSD-treated soils **(H)** were listed. "*" ($P < 0.05$) and "**" ($P < 0.01$) indicate significant differences using the LSD test. Error bars in plot **(H)** represent SDs, and the different letters represent significant differences at $P < 0.05$ according to LSD test. CK or RSD soils indicate the combinations of PS-CK and OA-CK or MO-RSD and MO_{PA}-RSD soils that are defined in Table 1.

($P < 0.05$) between various treatments. Specifically, the relative abundance of *Firmicutes* was remarkably increased ($P < 0.05$) in RSD-treated soils as compared to that in PS- and OA-CK soils, and its relative abundance in MO_{PA}-RSD-treated soil was considerably higher ($P < 0.05$) than that in MO-RSD-treated soil (Figure S2A). Furthermore, the relative abundances (accounting for 99.44~100%) of all dominant fungal phyla showed no significant difference between PS-CK and OA-CK soils ($P > 0.05$), whereas the relative abundance of *Ascomycota* was significantly ($P < 0.05$) increased after PS-CK soil was treated with MO-RSD and MO_{PA}-RSD (Figure S2B).

The compositions of bacterial and fungal genera were clustered into CK- (OA-CK and PS-CK) and RSD- (MO-RSD and MO_{PA}-RSD) groups, indicating most of these genera had a similar level of relative abundance between the groups (Figure S3A, B). However, the relative abundances of the dominant bacterial genera *Candidatus_Solibacter*, *Candidatus_Koribacter*, *Bryobacter*, and *Rhodanobacter* as well as the dominant fungal genera *Arnium*, *Trichoderma*, and *Pyrenochaetopsis* in PS-CK were significantly lower ($P < 0.05$) than those in OA-CK, whereas the genera *Chujaibacter*, *HSB_OF53_F07*, *Cephalotheca*, *Trichocladium*, *Chaetomium*, and *Fusarium*

showed an opposite trend (Figure 3G). The relative abundances of the dominant bacterial genera *Alicyclobacillus*, *Bacillus*, *Cohnella*, *Effusibacillus*, *Oxobacter*, *Paenibacillus*, *Pullulanibacillus*, *Rummeliibacillus*, *Thermicanus*, *Thermoanaerobacterium*, and *Tumebacillus* were considerably increased ($P < 0.05$) in both RSD-treated soils as compared to those in both CK soils (Figure 3G). The relative abundances of the dominant fungal genera *Westerdykella* in MO-RSD-treated soil and *Zopfiella*, *Penicillium*, and *Aspergillus* in MO_{PA}-RSD-treated soil were significantly higher ($P < 0.05$) than those in both CK soils. Interestingly, the relative abundances of *Paenibacillus*, *Cohnella*, *Effusibacillus*, *Rummeliibacillus*, *Oxobacter*, *Thermicanus*, *Zopfiella*, and *Penicillium* in MO_{PA}-RSD-treated soil were remarkably higher ($P < 0.05$) than those in MO-RSD-treated soil (Figure 3H).

Soil microbial functional composition

Compared to OA-CK soil, PS-CK soil showed a significant decrease ($P < 0.05$) in the relative abundances of bacterial functions such as cellular processes and fungal functions such as symbiotroph and pathotroph/saproytroph/symbiotroph; in contrast, the relative abundances of environmental information processing, metabolism, and organismal system in the bacterial function profile and pathotroph and saprotroph in the fungal function profile of PS-CK soil showed a significant upward trend ($P < 0.05$) (Figures 4A, B). Additionally, the relative abundances of bacterial functions such as cellular processes, environmental information processing, and genetic information processing and fungal functions such as saprotroph were remarkably increased in RSD-treated soils as compared to those in PS-CK soil, while the relative abundances of human diseases, metabolism, and organismal systems in the bacterial function profile as well as pathotrophs in the fungal function profile showed an opposite trend (Figures 4A, B). Specifically, the relative abundances of cellular processes and genetic information processing in MO_{PA}-RSD-treated soil were significantly higher than those in MO-RSD-treated soil, while the relative abundances of metabolism and organismal systems were significantly higher in MO-RSD-treated soil (Figures 4A, B).

In the bacterial functional pathway of human diseases (Figure 4C), we found that the relative abundances of drug resistance: antimicrobial and bacterial infectious disease were significantly enriched ($P < 0.05$) in RSD-treated soils, whereas the

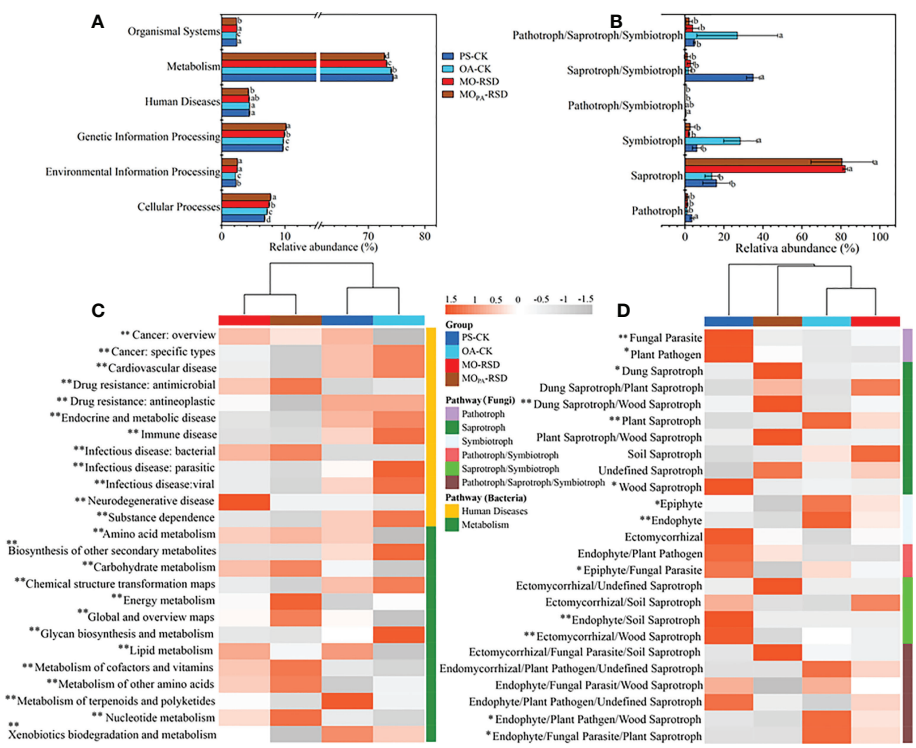

FIGURE 4
Dissimilarities in bacterial KEGG orthologue and fungal ecology guild functional profiles among the different treatments. The bacterial (**A**, level 2; **C**, level 3) and fungal (**B**, level 1; **D**, level 2) functional profiles were predicted by PICRUSt2 and FUNGuild, respectively. The dominant pathway of human diseases and metabolism profiles **(C)** in bacteria were listed. Error bars in plots **(A, B)** represent SDs, and the different letters represent significant differences at $P < 0.05$ according to LSD test. The key from gray to red indicate the least abundant to most abundant in each row for a given functional profile **(C, D)**, and "*" ($P < 0.05$) and "**" ($P < 0.01$) indicate significant differences using LSD test. The treatment abbreviations are defined in Table 1.

relative abundances of other human diseases, i.e., cancer: specific types, drug resistance: antineoplastic, immune disease, substance dependence, etc., were remarkably enriched ($P < 0.05$) in CK soils. Regarding metabolism (Figure 4C), we found that the relative abundances of amino acid metabolism, carbohydrate metabolism, energy metabolism, global and overview maps, metabolism of cofactors and vitamins, metabolism of other amino acids, and nucleotide metabolism were significantly increased ($P < 0.05$) in RSD-treated soils, whereas the relative abundances of biosynthesis of other secondary metabolites and xenobiotic biodegradation and metabolism were higher ($P < 0.05$) in CK soils. In the fungal functional pathway of pathotroph (Figure 4D), we found that the relative abundances of fungal parasites and plant pathogens in PS-CK soil were significantly higher ($P < 0.05$) than those in other soils. For saprotrophs, the relative abundances of dung, dung/wood, and plant/wood saprotrophs in MO_{PA}-RSD-treated soil and the relative abundances of dung/plant, plant, and soil saprotrophs in MO-RSD-treated soil were higher than those in PS-CK soil. For symbiotrophs, we observed that the relative abundances of epiphytes and endophytes in OA-CK soil were considerably higher ($P < 0.05$) than those in other soils.

Plant physiological properties

Compared to PS-CK soil, both MO-RSD- and MO_{PA}-RSD-treated soils showed a significant decrease ($P < 0.05$) in the disease incidence of the *M. charantia* plant by 75.21% and 90.09%, respectively (Table 2). The ascorbic acid content, hardness, and fracturability of *M. charantia* fruits grown in MO-RSD and MO_{PA}-RSD soils were significantly decreased ($P < 0.05$) by 61.18% and 74.34%, 14.73% and 37.98%, and 20.23% and 41.17%, respectively (Table 2). Conversely, the soluble protein content and weight of fruits grown in MO-RSD- and MO_{PA}-RSD-treated soils were significantly increased ($P < 0.05$) by 0.89- and 1.08-fold and by 0.64- and 1.04-fold, respectively, when compared with those of fruits grown in PS-CK soil (Table 2). Notably, the weight and soluble protein content ($P < 0.05$) of fruits grown in MO_{PA}-RSD-treated soil were higher than those of fruits grown in MO-RSD soil, while the disease incidence, ascorbic acid, hardness ($P < 0.05$), and fracturability ($P < 0.05$) of fruits grown in MO_{PA}-RSD-treated soil showed an opposite trend.

Soil microbial composition associated with plant physiological properties

The dissimilarities in the dominant bacterial and fungal genera composition were significantly and positively correlated with the differences in plant physiological properties, and the effects of the dominant bacterial genera composition on plant physiological properties were larger than those of the dominant fungal genera composition (Figure 5A). Specifically, the population of *F. oxysporum*, plant disease incidence, and ascorbic acid content showed a significant and positive interaction with each other, while the opposite trend was observed between these properties and soluble protein content (Figure 5B). Furthermore, the relative abundances of the dominant genera that increased in RSD-treated soils, such as *Alicyclobacillus*, *Bacillus*, *Cohnella*, *Effusibacillus*, *Oxobacter*, *Paenibacillus*, *Rummeliibacillus*, *Thermicanus*, *Thermoanaerobacterium*, *Tumebacillus*, *Penicillium*, *Talaromyces*, and *Zopfiella*, were significantly and negatively ($P < 0.05$) correlated with the populations of *F. oxysporum*, disease incidence, and ascorbic acid content (Figure 5B).

Discussion

RSD-regulated most soil properties outperformed those of the comparable healthy soil

Soil acidification and secondary salinization are the two major degradation characteristics in the plastic-shed cultivation system due to long-term overfertilization (Fan et al., 2021; Zhang et al., 2022), similar to pH and EC of PS-CK soil in the present study. The pH and NH_4^+-N content of RSD-treated soils, especially those of MO_{PA}-RSD-treated soil, were significantly higher than those of CK soils, while the EC and NO_3^--N content of the former group were remarkably

TABLE 2 Plant physiological properties in different soils.

Physiological properties	PS-CK [a]	MO-RSD	MO_{PA}-RSD
Disease incidence (%)	44.83 ± 2.75a	11.11 ± 3.84b	4.44 ± 3.84b
Ascorbic acid (mg kg^{-1})	183.81 ± 32.26a	71.34 ± 15.21b	47.16 ± 1.81b
Soluble protein (mg g^{-1})	1.69 ± 0.15c	3.19 ± 0.09b	3.51 ± 0.17a
Weight (g $fruit^{-1}$)	66.87 ± 15.78b	109.71 ± 13.16a	136.10 ± 25.73a
Hardness (N)	6.61 ± 0.42a	5.63 ± 0.43b	4.09 ± 0.37c
Fracturability (N)	1.70 ± 0.16a	1.36 ± 0.06b	1.00 ± 0.02c

[a]Values (means ± SD, n = 3) followed by different letters in each row represent significant differences at $P < 0.05$ according to LSD test. The treatment abbreviations are defined in Table 1.

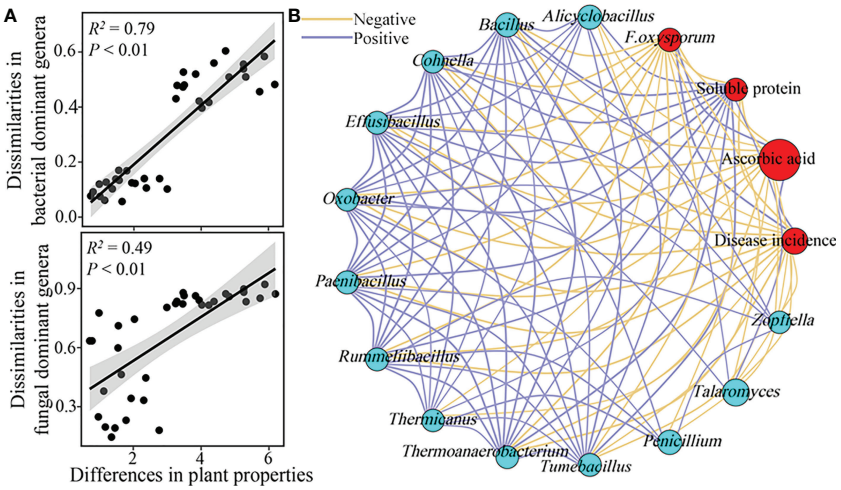

FIGURE 5
Soil microbial composition associated with plant physiological properties. Relationships between the dissimilarities in relative abundances of bacterial and fungal **(A)** dominant genera and the differences in plant properties were calculated using partial mantel test. The differences in plant properties were normalized according to the z-score method. Network interactions among the relative abundances in dominant genera that significantly enriched in RSD-treated soils, *F. oxysporum population*, disease incidence, and contents of soluble protein and ascorbic acid of plants were listed in plot **(B)**.

decreased to a level comparable to those of OA-CK soil; this finding indicates that the restored effects of RSD on soil acidification outperformed that in OA-CK soil. This was mainly due to the reduction and denitrification environment created during RSD treatment, which may be stronger than that in the flooded paddy field, thus leading to rapid consumption of H^+ in the soil (Zhu et al., 2011; Di Gioia et al., 2017).

Microbial abundance, diversity, community, and functional compositions have long been considered the important predictors of soil health (Chaparro et al., 2012; Wang et al., 2019). Although the increase in bacteria and decrease in plant pathogens after RSD treatment are a common phenomenon, these properties showed a better performance than those in OA-CK soil, which was supported by molasses used in RSD treatment that can stimulate the proliferation of beneficial bacteria and produce a high amount of organic acids (Butler et al., 2012; Momma et al., 2013). The microbial communities in healthy soils often have highly connected networks that can directly resist the invasion of plant roots by pathogens (Wang et al., 2019; Wei et al., 2019). Herein, we observed that the bacterial network in RSD-treated soils was more complex and highly connected than that in CK soils, indicating the changes in the bacterial community during RSD treatment play a predominant role in maintaining good soil health. These results were further supported by significant enrichment of the beneficial members of *Firmicutes* in the RSD-treated network and the larger dissimilarities in bacterial communities between RSD-treated soils and PS-CK soils than that between OA-CK and PS-CK soils. Notably, previous studies have revealed that the dominant microbes belonging to *Firmicutes* significantly increased during the RSD treatment can result in a decrease in soil bacterial α diversity (Meng et al., 2019; Huang et al., 2018), which may also be the main reason for the change of bacterial diversity in this study.

The downward trend of the total metabolism activity in RSD-treated soil was contrary to that observed in previous studies (Zhao et al., 2020; Li et al., 2021); this was mainly because that the metabolism of all soil microorganisms was detected in these previous studies by using Biolog EcoPlates based on the carbon source utilization pattern, while the metabolic function of only bacteria was predicted in the present study. Nevertheless, some similar findings were noted, in that the relative abundances of amino acid metabolism, carbohydrate metabolism, and metabolism of cofactors significantly increased in RSD-treated soils. Chen et al. (2021) revealed that RSD treatments can effectively reduce the expression of antibiotic resistance genes (ARGs), including part members of multidrug, beta-lactam, macrolide, and phenicol resistance genes. We observed that most human disease functions significantly decreased during RSD treatments as compared to that in CK soils; this may be associated with the reduction of some ARGs or human pathogenic bacteria, which should be confirmed in the future by using more advanced techniques such as metagenomic sequencing and high-throughput quantitative PCR (Zhu et al., 2017). For fungi, the relative abundance of saprotrophs was also enriched in RSD-treated soils, which seems beneficial for soil quality because the increase of saprophytic fungi can promote the formation of humus and provide the required energy source for improving soil fertility (Li et al., 2021). Overall, most soil properties, such as acidification, number of pathogens, bacterial community, and functional compositions, influenced by RSD treatment were

distributed more reasonably than those in the comparable healthy soil.

RSD process combined with *Paenibacillus* sp. inoculation further enhanced the performance of plant physiological properties

In this study, we observed that RSD treatments effectively decreased the plant disease incidence and increased crop yield, which is consistent with the results of previous studies (Butler et al., 2012; Huang et al., 2016). The plant health status can also directly affect the physiological properties of fruits, such as soluble proteins and ascorbic acid, which in turn are closely related to the host health (Kruijt et al., 2005; Asensi-Fabado and Munne-Bosch, 2010). This is in line with our study finding that ascorbic acid and soluble protein contents significantly interacted with the disease incidence and population of *F. oxysporum*.

Ascorbic acid, as a multifunctional metabolic substance, not only plays a very important role in plant antioxidation and photosynthesis processes but also induces systemic resistance by affecting the biosynthesis of plant hormones (Asensi-Fabado and Munne-Bosch, 2010). For example, the low content of ascorbic acid can effectively promote plant defense response by regulating the biosynthesis of abscisic acid, jasmonic acid, and ethylene, whereas an opposite trend was found in plants with a high content of ascorbate (Kuzniak and Sklodowska, 2001; Conklin and Barth, 2004). Furthermore, soluble proteins, as an important osmotic regulator in plants, can also affect plant disease resistance by participating in various intracellular enzymatic reactions (Kruijt et al., 2005). Wang et al. (2002) and Li et al. (2015) reported that the content of soluble proteins in watermelon and potato tubers is significantly reduced after infection with *F. oxysporum* f. sp. *niveum* and *F. trichothecioides*, respectively. From the present study, for the first time, we found these interesting results that RSD-treated soils can significantly decrease the content of ascorbic acid and considerably increase the content of soluble proteins, which may play important roles in RSD-treated soil to induce plant systemic resistance.

Specifically, RSD-completed soil combined with the inoculation of beneficial species (such as *B. subtilis* SQR-N1 and *Trichoderma* spp.) has attracted increasing attention for enhancing plant disease resistance; this is mainly because RSD alone cannot always perform well to control soil-borne diseases during plant cultivation (Huang et al., 2016; Khadka and Miller, 2021; Ali et al., 2022). For example, our previous study showed that the control efficiencies of RSD on *Fusarium* wilt and *F. oxysporum* in watermelon were unsatisfactory (Liu et al., 2018). In the present study, MO_{PA}-RSD-treated soil showed better performance than MO-RSD-treated soil in terms of these above-mentioned plant properties together with fruit taste (hardness and fracturability) and *F. oxysporum* disinfestation efficiency. This result indicates that the anaerobic and reductive environment, which was created during RSD treatment, combined with *Paenibacillus* sp. inoculation could further enhance the plant health performance from multiple aspects.

Regulation of plant physiological properties by RSD combined with *Paenibacillus* sp. inoculation is closely linked with the proliferation of specific probiotic consortia

Not surprisingly, the effect of RSD on plant health performance is mainly associated with the improvement of microbial communities. Here, the dissimilarities in the relative abundances of the dominant bacterial and fungal genera were significantly and positively correlated with the differences in plant physiological properties; a higher correlation coefficient was observed between the dominant bacterial genera and plant physiological properties. Moreover, the relative abundances of ten dominant bacterial genera and three dominant fungal genera enriched in RSD-treated soils were significantly and negatively associated with the disease incidence or population of *F. oxysporum* (Figure 5B). These results revealed that the soil disinfestation and plant protection processes of RSD treatment were mainly mediated by the bacterial community.

Interestingly, all these dominant bacterial genera belonged to the phylum *Firmicutes*, which may play an important role in reducing soil-borne pathogens, mediating plant immunity, and inducing plant disease resistance by releasing antifungal compounds or stimulating the release of various plant hormones such as cytokinin (Lee et al., 2021; Gupta et al., 2022; Liu et al., 2022a). Herein, for example, the specific bacterial taxa (e.g, *Bacillus*, *Cohnella*, and *Paenibacillus* spp.) have been previously reported as the potential PGRPs, which have the capacity to promote plant growth by establishing defense lines against pathogen invasion in the rhizosphere (Li and Chen, 2019; Premalatha et al., 2021; Ali et al., 2022; Zhou et al., 2022). The species of *Oxobacter*, *Rummeliibacillus*, and *Thermoanaerobacterium* can suppress pathogens by releasing organic acids such as acetic acid, ethanol, and lactic acid during the fermentation of organic materials (Mowlick et al., 2013; Pang et al., 2018; Tan et al., 2019). In addition, the fungal members of *Zopfiella*, *Penicillium*, and *Talaromyces* are known as producers of antibiotics and enzymes, including zopfiellin, xylanolytic enzymes, crude ethyl acetate, and crude methanol (Soytong and Poeaim, 2015; Zhao et al., 2020; Liu et al., 2021), which may act against soil-borne pathogens and promote plant disease suppression.

Some recent studies have reported that the facilitative microbe-microbe interactions are widespread in the soil–plant

system, which is primarily associated with the cross-feeding or production of secondary metabolites (such as siderophores) by microorganisms (Pacheco et al., 2019; Kramer et al., 2020). Undoubtedly, microorganisms do not individually perform suppression of plant diseases; they often look for "helpers" with facilitative interaction to build defense lines to limit the invasion of pathogens (Hu et al., 2021; Li et al., 2021). In the present study, we found a similar phenomenon wherein the relative abundances of *Paenibacillus*, *Cohnella*, *Effusibacillus*, *Rummeliibacillus*, *Oxobacter*, *Thermicanus*, and *Penicillium* in MO_{PA}-RSD-treated soil were significantly higher than those in MO-RSD-treated soil. Importantly, the relative abundance of *Paenibacillus* significantly and positively interacted with the remaining microbial species. These results indicate that *Paenibacillus* sp. inoculation as a step in the RSD treatment process may be helpful to (1) promote its colonization efficiency, (2) stimulate the proliferation of other probiotic consortia, and (3) cooperate to maintain good soil and plant health. Notably, although the microbial community showed a huge effect on plant disease suppression, plants can also reshape their microbial composition by releasing a variety of root exudates (Haichar et al., 2008; Liu et al., 2018). Furthermore, our previous studies reported that the re-degradation of soil abiotic factors during plant cultivation, such as soil pH, can further induce the deterioration of microbial communities regulated by RSD (Liu et al., 2021). In the present study, the biotic and abiotic data were collected after RSD treatment; therefore, how to ensure the continuous colonization of these probiotic consortia during plant cultivation is a major task in future studies.

Conclusions

The present study found that the efficiencies of RSD to improve diseased soil properties under a plastic shed cultivation system, such as soil acidification, pathogen abundance reduction, and bacterial community and functional compositions, were distributed more reasonably than those in the comparable healthy soil from an open-air paddy cultivation field. The anaerobic and reductive environment of the MO-RSD treatment process combined with the inoculation of facultative anaerobic functional species (*Paenibacillus* sp.) can further enhance the performance of soil health and plant physiological properties as compared to MO-RSD treatment alone. Specifically, this integrative RSD practice is not only beneficial for *Paenibacillus* sp. colonization, but it can also stimulate the proliferation of specific probiotic consortia, which ultimately and cooperatively control more soil-borne pathogens and induce plant systemic resistance. The present study contributes to the growing body of knowledge on how to promote RSD efficiency to maintain good soil health and plant disease resistance.

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/, PRJNA890763.

Author contributions

LLL, QS, ZCC, and XQH conceived designed research. YX, XZ, and QQD performed the experiment and collected the data. LLL and QS analysed the data. LLL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1095656/full#supplementary-material

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Nanotechnological approaches for management of soil-borne plant pathogens

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Soil borne pathogens are significant contributor of plant yield loss globally. The constraints in early diagnosis, wide host range, longer persistence in soil makes their management cumbersome and difficult. Therefore, it is crucial to devise innovative and effective management strategy to combat the losses caused by soil borne diseases. The use of chemical pesticides is the mainstay of current plant disease management practices that potentially cause ecological imbalance. Nanotechnology presents a suitable alternative to overcome the challenges associated with diagnosis and management of soil-borne plant pathogens. This review explores the use of nanotechnology for the management of soil-borne diseases using a variety of strategies, such as nanoparticles acting as a protectant, as carriers of actives like pesticides, fertilizers, antimicrobials, and microbes or by promoting plant growth and development. Nanotechnology can also be used for precise and accurate detection of soil-borne pathogens for devising efficient management strategy. The unique physico-chemical properties of nanoparticles allow greater penetration and interaction with biological membrane thereby increasing its efficacy and releasability. However, the nanoscience specifically agricultural nanotechnology is still in its toddler stage and to realize its full potential, extensive field trials, utilization of pest crop host system and toxicological studies are essential to tackle the fundamental queries associated with development of commercial nano-formulations.

KEYWORDS

nanotechnology, plant pathogen, plant health management, nanoformulation, mode of action

1 Introduction

Soil is a reservoir of millions of microorganisms which imparts great impact on agriculture. A majority of microbes are beneficial for soil and plant health. However, some microorganisms pose great threat to crops as they often damage the root and crown tissues of plants thereby causing huge economic loses. Thus, pathogens which persist in the soil matrix or in residues over the soil surface are known as soil-borne plant pathogens (Veena et al., 2014). The soil-borne plant pathogens are distributed widely in soil however, few species exhibit localized distribution pattern. Soil-borne diseases caused by fungi, bacteria, nematodes, oomycetes, protozoa, viruses are considered vital in realization of potential yield in agricultural crops. Once established, these pathogens accumulate through synergistic associations and cause greater economic losses that are difficult to control. The soil-borne plant pathogens *viz*., *Fusarium* spp., *Rhizoctonia* spp., *Pythium* spp., *Sclerotinia* spp., *Verticillium* spp., and *Phytophthora* spp. can cause yield loss upto 50-75% for economically important crops such as wheat, maize, cotton, vegetables and fruits (Mihajlovic et al., 2017). *Fusarium oxysporum* strains alone can infect more than 150 agricultural crop species such as banana, tomato, melon, cotton etc. causing severe vascular wilt disease (Bertoldo et al., 2014). In cucurbitaceous crops, the pathogen is responsible for causing yield losses of around 30-80% (Lü et al., 2011). Fusarium wilt of banana, caused by *F. oxysporum* f. sp. *cubense* is a major threat to banana cultivation worldwide. The race Tropical Race 4 has been causing serious losses in Southeast Asian countries, thereby affecting the lives of small producers. Asides from wilt disease, some other strains of *Fusarium oxysporum* are capable of causing root/foot rot and damping off (Michielse and Rep, 2009). *F. solani* is mainly known to cause collar and root rots in many economically important crops such as beans and peas. *Fusarium* spp. also contaminate cereals and food grains by producing mycotoxins such as fumonisins, trichothecenes, zearalenone, and deoxynivalenol (Nelson et al., 1981). *F. graminearum* and *F. verticillioides* cause cob rot in maize, both species are known to produce mycotoxins. In oil seed rape (*Brassica napus*), the predominant population of *Rhizoctonia solani* AG2-1 isolate causes severe seedling diseases, establishment losses of up to 80-100%, and final yield losses of up to 30%. (Tahvonen et al., 1984; Kataria and Verma, 1992; Khangura et al., 1999). Rhizoctonia produces a variety of symptoms such as stem lesions, damping off, crown rot, root rot, stem rot and aerial web blight. The infection ultimately causes wilting, stunting and finally the death of the plant. The species of Phytophthora and Pythium cause damping off and root rot disease under cool and wet conditions and can affect 5-80% of the seedlings thereby incurring huge economic loss to the farmers (Alcala et al., 2016). Late blight caused by *Phytophthora* spp. is one of the most destructive soil-borne diseases of potatoes and tomatoes worldwide (Son et al., 2008). Worldwide, it causes an estimated loss of $5 billion annually (Latijnhouwers et al., 2004). Among the bacterial soil-borne pathogens, *Ralstonia solanacearum* causing bacterial wilt disease in more than 180 plants of 45 families ranks the first (Tahat and Sijam, 2010). In tomato crop, *R. solanacearum* can cause yield loss of 0-90% depending on the strain of the pathogen, cropping pattern, cultivar and climate (Nion and Toyota, 2015). Root knot nematodes (RKN) represent an important class of soil-borne pathogen that infect more than 5,500 host plants. *Meloidogyne* spp. are polyphagous, obligate sedentary, parthenogenetic and considered the most important plant parasitic nematode group worldwide (Jones et al., 2013). The typical symptoms produced by RKN include gall formation and damage to root system, along with above ground symptoms such as chlorosis, stunting, wilting and yield reduction (Karssen et al., 2013). The effect of RKN on the host plant is further intensified by the attack of secondary plant pathogens such root rot, fungal and bacterial wilt causing pathogens (Back et al., 2002; Karssen et al., 2013). The soil-borne diseases remain unnoticed until the above ground plant parts exhibit symptoms such as chlorosis, stunting, wilting and finally death. The common soil-borne diseases include damping off, root rot, vascular wilt etc. (Hornby et al., 1988). These diseases are often difficult to manage as they have wide host range and can survive for long periods on soil organic matter and plant debris, as free-living organisms or by producing resistant structures like sclerotia, microsclerotia, oospores or chlamydospore even in absence of host plant. Also, its diagnosis is difficult and cumbersome due to similarity in symptoms such as root rot, stunting, chlorosis, seedling damping, root blackening, bark cracking and branch and twig dieback (Patil et al., 2021). The non-specific symptoms and its resemblance with physiological disorders and water stress symptoms makes its timely diagnosis difficult (Åström and Gerhardson, 1988). Thus, the major hinderance in management of soil-borne diseases is its heterogenous incidence and scarce knowledge on the epidemiological aspects of pathogens. The experiences and observations passed on through several generations have given rise to cultural practices that reduce the losses caused by soil-borne plant pathogens but its effective management strategy still need to be explored. The expanding diversity of crops in agriculture emphasizes parallel expansion of strategies and develop novel strategies for effective management of soil-borne plant pathogens.

The farmers use synthetic fumigants and chemical fungicides at regular interval throughout the cropping season to minimize the soil-borne disease outbreak. However, extensive use of fumigants such as methyl bromide and fungicides disrupt the ecological balance, cause human and animal health hazards, damage to aquatic ecosystem and beneficial organisms in soil (Panth et al., 2020). The cultural practices such as crop rotation, biofumigation, anaerobic soil disinfestation, soil solarization, soil steam sterilization are mainly adopted by farmers to minimize the losses but these methods give inconsistent results and are less effective than chemical control methods. The mounting environmental constraints and ineffective management options emphasizes need of alternative sustainable and effective management strategy.

Nanotechnology emerged as one of the most rapidly advancing science of twenty first century. Diversified application of nanotechnology in various fields have been found to uplift the entire scenario of industry and agricultural sector including information technology, medicine, disease detection and diagnosis, food safety and security, pest and disease management, environmental science and many more. From agriculture point of view, the major concern related to soil and environmental health includes: increased pesticide residue in soil and water bodies, decline in soil beneficial organisms, alteration of soil physical and chemical properties, pesticide resistance in pathogens and many more (Sharma et al., 2019). Nanotechnology can address most of these concerns and can bring revolutionary changes. It possesses marvelous application as antimicrobial and therapeutic compounds, targeted drug delivery, high sensitivity disease detection and diagnosis and thus likely to enhance agricultural productivity due to decline in cost associated with agricultural production practices (Dutta et al., 2021; Dutta et al.,

2022). The small size of nanoparticles (<100 nm), greater surface area to volume ratio and high reactivity favors its wide-scale application in the field of human and plant pathology (Jeevanandam et al., 2018). The use of nano-encapsulated fertilizers and pesticides can reduce the amount of chemical fumigants and pesticides reaching the soil surface as compared to conventional formulations and also prolongs protection to plants against various phytopathogens. Nano-based materials can also act as cargo molecule and can release the active ingredients owing to its greater surface area to volume ratio. The effect on non-target organisms can also be reduced as they are highly target specific (Din et al., 2017). The disease tolerance ability of plants can also be enhanced thereby improving plant health (Figure 1). Thus, it may be predicted that integrity between timely and accurate disease diagnosis and management can be established in near future by exploiting the science of nanotechnology (Mahmood et al., 2017). Recent studies have revealed that nanoparticles show promising results as potential antimicrobial agent and biosensor for detection of plant pathogens especially against soil-borne plant pathogens. This review, focuses on all aspects of nanotechnology for management of soil-borne plant pathogens, thereby condensing scattered literature together at one place.

2 Nanotechnology in agriculture

In the field of agriculture, nanoscience is explored in delivery of plant hormones, seed germination, transfer of genes of interest, water management, nano-biosensors, nanobarcoding and controlled release of agro-chemicals (Hayles et al., 2017). Furthermore, nanoparticles are engineered with desired properties (i.e., size, shape, surface area etc.) for its use as protectant, therapeutant or site specific delivery of active ingredients such as fungicides *via* conjugation, adsorption or encapsulation (Khandelwal et al., 2016). The nano-based materials can be applied to plants as seed treatment, root dip treatment, soil application and foliar spray. The metallic oxides, nonmetals, metalloids, polymeric and carbon nanomaterials exhibit disease suppressing and growth promoting activities in plants (Elmer et al., 2018). The most popularly explored nanoparticles include silver, gold, copper, zinc oxide, iron and many more. The three main mechanisms involved in use of nanoparticles include: (a) nanoparticle as biosensor, (b) nanoparticle as protectant or therapeutant, and (c) nanoparticle as smart delivery vehicle of fungicides or actives such as target genes. Nanoparticles such as quantum dots and metallic nanoparticles can be functionalized with biological markers for *in situ* and rapid detection of soil-borne pathogens. The nanoparticles have the potential to serve as protective or therapeutic agents against a variety of soil-borne pathogens, *viz*., *Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani, Sclerotinia sclerotiorum* (Kaman and Dutta, 2019; Abdelrhim et al., 2021) *Ralstonia solanacearum* (Khairy et al., 2022), soil-borne viruses *viz*., Barley yellow mosaic virus (BaYMV) (Aref et al., 2012), etc. The main mechanism of action against microorganisms include agglutination and cell membrane disruption, inhibition of synthesis of RNA, proteins, toxins, enzymes such as H+-ATPase and blockage of flow of nutrients (Dakal et al., 2016; Malerba and Cerana, 2016). Nanoparticles also acts as carrier molecule and allow target-specific release of active ingredients into the plant system, thereby reducing the load of chemicals into the environment. The nano-based formulations provide several benefits such as improved water solubility of pesticides, site specific delivery and uptake by target sites, increased shelf life, reduced effect on non-target organisms and residual effect on environment (Hayles et al., 2017). Also, the stability and activity of nano-based formulations are greater as compared to conventional pesticides even under unfavorable environmental conditions (rainfall and UV exposure), thereby reducing the number of applications, toxicity and overall costs.

Another important aspect of plant health management is the use of fertilizers. Nanofertilizers bear the potential to increase the release and uptake efficacy of nutrients thereby boosting plant disease resistance. It has widely been explored in plant/disease systems *viz*., Fusarium wilt in tomato, chrysanthemum, root and crown rot in asparagus, red root rot in tea, verticillium wilt in brinjal (Elmer et al., 2018).

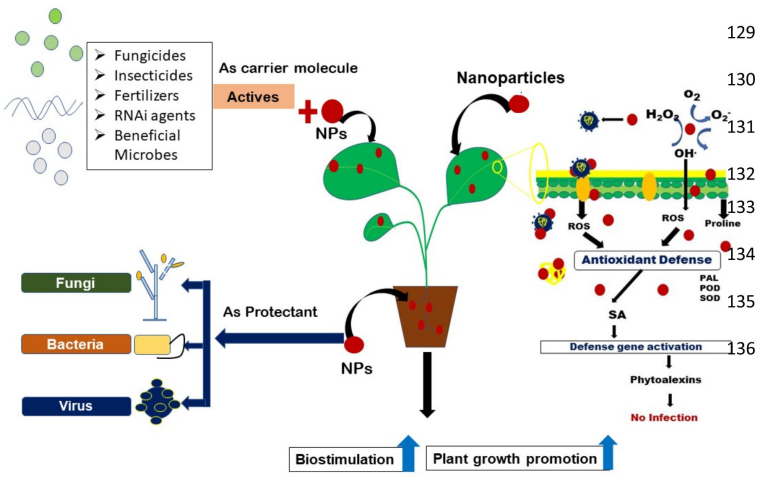

FIGURE 1
Schematic representation of different approaches of nanoparticles for management of soil borne plant pathogens.

3 Nanotechnology based diagnosis of soilborne pathogens

Rapid detection and diagnosis of soil-borne pathogens is fundamental for its effective and timely management. A number of immunological, serological, nucleic acid-based detection assays have been developed for accurate detection of plant pathogens. Over the past two decades, numerous efforts have been made to develop methods for diagnosing and monitoring plant infections using biochemical assays utilizing specific proteins, toxins, ELISA, nucleic acid probe technologies, and PCR amplification of nucleic acid sequences (McCartney et al., 2003; Sundelin et al., 2009; Kashyap et al., 2013a; Kumar et al., 2013; Kumar and Kashyap, 2013; Singh et al., 2014). These biochemical assays and nucleic acid-based methods are sensitive, exact, and useful for verifying visual scouting, but they are unsuitable as screening tests to check on the health of plants before symptoms manifest. They necessitate intricate sampling techniques, costly infrastructure, and might mask the true state of pathogen infections. Unfortunately, only a small number of plant diseases can be effectively detected using these assays. However, the majority of these techniques are ineffective for on-site disease detection in crop fields. The use of molecular approaches is further constrained by the high cost and limited shelf life of molecular biology reagents like enzymes and primers. Therefore, the introduction of low-cost techniques to increase the precision and speed of plant pathogen diagnostics is required.

Recent advancement in nanotechnology has led to development of functional nanoparticles (electronic, optical, magnetic, or structural) which can be covalently attached to biological molecules including nucleic acids, peptides and proteins. Quantum dots (QD), one of the most promising nanomaterials, have been extensively exploited in a wide range of bio-related applications, including the quick and precise detection of a specific biological marker (Kashyap et al., 2015).Tools for high-throughput analysis, high-quality monitoring, and crop protection such as biosensors, quantum dots, nanostructured platforms, nanoimaging, and nanopore DNA sequencing have the potential to increase the sensitivity, specificity, and speed of disease detection (Khiyami et al., 2014). Additionally, nano-diagnostic kit tools are rapid and simple to use in identifying potential plant pathogens, enabling specialists to assist farmers in the prevention of epidemic illness. The ability of QD-based nanosensors to simultaneously probe several enzyme activities has been demonstrated by Knudsen et al. (2013). CdTe quantum dots have been utilized as biosensors by coating with specific antibodies against the glutathione S-transferase (GST) protein of *Polymyxa betae*, the vector of BNYVV causing rhizomania disease in sugar beet (Safarpour et al., 2012). In order to detect the harmful fungus *Sclerotinia sclerotiorum*, Wang et al. (2010) used indirect stimulation to construct a sensitive electrochemical sensor employing a modified gold electrode with copper nanoparticles. They used this sensor to successfully and precisely quantify salicylic acid in oilseeds to detect the pathogen. It is necessary to conduct more research on related sensors and sensing systems to detect pathogens, their byproducts, or to track physiological changes brought on by infections in plants. Schwenkbier et al. (2015) created a helicase-dependent isothermal amplification (HDA) in conjunction with on-chip hybridization for identifying *Phytophthora* species. With this method, the target gene locus for the yeast GTP-binding protein (Ypt1) can be amplified effectively at a single consistent temperature in a tiny heating unit. By using on-chip DNA hybridization and subsequent silver nanoparticle deposition, the assay's specificity was established. The silver deposits act as reliable endpoint signals, enabling both electrical and optical readout. These developments suggest that the combined techniques will soon be applied on-site for the accurate identification of several soil-borne pathogens. Hervas et al. (2011) established a "lab-on-chip" method for the rapid, sensitive, and selective quantification of zearalenone generated by *Fusarium* sp. that incorporates an electrokinetic magnetic bead-based electrochemical immunoassay on a microfluidic chip. Rispail et al. (2014) studied the effects of superparamagnetic nanoparticles and quantum dots on *Fusarium oxysporum*. The presence of the pathogenic fungus was quickly identified by interactions between nanomaterials and the fungal hypha, though their internalization patterns varied. This study showed viability of new nanotechnology-based systems for the early detection and eventual control of harmful fungi which is the first study on the effects of quantum dots and superparamagnetic particles on fungal cells. Hashimoto et al. (2008) created a novel biosensor system comprising two biosensors for rapid detection of soil-borne pathogens. Here, equal amounts of two distinct microorganisms, each immobilized on an electrode, were used to build the system.

However, the science of nanodiagnostics is still in the toddler stage for accurate detection of pathogens and toxins in agricultural field. Extensive research is needed to optimize the diagnostic assays for detection of precise signals emitted from low level of pathogens. Also, efforts must be channelized towards development of portable, cheap, efficient and hand held nanodevices for *in situ* detection of soil borne pathogens.

4 Nanotechnology for management of soil-borne pathogens

4.1 Nanoparticles as protectants

Nanoparticles alone can be directly utilized as antimicrobial agent and have been found effective against numerous soil-borne pathogens. It can be applied to soil, seed, root, foliage for providing protection against pests and pathogens such as fungi, bacteria and viruses. Nanoparticles penetrate the plant system and directly acts against the pathogen or it behaves as elicitor molecule for inducing local and systemic defense responses in plants. Metallic nanoparticles such as gold, silver, titanium-oxide, zinc-oxide, copper oxide are most intensely studied nanoparticles and known to exhibit antifungal, antibacterial and antiviral properties (Gogos et al., 2012; Kah and Hofmann, 2014; Kim et al., 2018). Several studies reported nanoparticles as an effective antimicrobial agent to curb the menace caused by soil-borne phytopathogens.

Kaman and Dutta (2019) studied the antifungal activity of biogenically synthesized silver nanoparticles (AgNPs) against soil-borne phytopathogens *viz*., *Sclerotium rolfsii*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* at 100 ppm

AgNP concentration. Desai et al. (2021) reported the antifungal activity of AgNPs against the pathogen *Sclerotium rolfsii* in wheat plant. They observed 100 per cent mycelial growth inhibition and sclerotial germination inhibition under both *in vivo* and *in vitro* condition at 100 ppm AgNP concentration. However, the root of wheat plants exhibited phytotoxic effect at this concentration. Thus, 50 ppm AgNP concentration was inferred best in terms of disease management and plant growth. Another study conducted by Zaki et al. (2022) revealed antifungal activity of mycogenically synthesized Zinc oxide nanoparticles (ZnONPs) mediating *Trichoderma* spp., against soil-borne pathogens *viz*., *Rhizoctonia solani, Macrophomina phseolina* and *Fusarium fujikuroi*). Significant antifungal effect was recorded under *in vitro* condition as well as on cotton seedlings. Also, inhibitory effect of AgNPs was reported in a dose dependent manner against mycelial growth of *R. solani*, *S. sclerotiorum* and *S. minor* (Min et al., 2009). The mode of action of AgNPs as revealed from microscopic observations indicates fungal cell disintegration, separation of layers of hyphal wall and ultimately hyphal collapse and death. Tomah et al. (2020) determined the antifungal activity of AgNPs synthesized mediating *Trichoderma* spp. against *Sclerotinia sclerotiorum. In vitro* antifungal assays reported 93.8%, 100% and 100% inhibition of sclerotial formation, myceliogenic germination and hyphal growth at 200 µg/mL AgNP concentration respectively. The SEM and EDS study indicated direct interaction of nanoparticles and fungal cells including AgNP contact and accumulation within fungal cells, micropore or fissure formation on fungal cell wall and lamellar fragment production. Similar results were obtained by Guilger-Casagrande et al. (2021) against *Sclerotinia sclerotiorum.* Chen et al. (2020) evaluated the effect of Magnesium oxide nanoparticles (MgONPs) against *Thielaviopsis basicola* and *Phytophthora nicotianae. In vitro* studies revealed inhibition of fungal growth, spore germination and impediment of sporangium development. Direct interaction, adsorption of nanoparticles by fungal hypha and cell morphological changes were the underlying mechanism involved in antifungal effect as confirmed by SEM, TEM and EDS. Under greenhouse conditions, 42.35% and 36.58% decline in tobacco black root rot and black shank disease respectively was observed at 500 µg/ml of MgONP testifying suppression of fungal invasion through root irrigation. Juan-ni et al. (2022) observed similar results for copper oxide nanoparticles (CuONPs) against *Phytophthora nicotianae.* They observed 33.69% increase in control efficacy and tobacco black shank disease suppression without inducing phytotoxicity at 100 mg L^{-1} of CuONPs treatment under pot condition. Additionally, they reported increased SOD enzyme activity and intracellular ROS accumulation as antifungal mechanisms of the used nanoparticles. Exposure of tobacco plants to CuONPs also significantly activated cascade of defense enzymes, resistance genes and Cu-content in leaves and root of treated plants. Encinas et al. (2020) showed silver-chitosan nanoparticles significantly inhibited mycelial growth of *Fusarium oxysporum* upto 70% and reduced the severity of the disease in *Fusarium oxysporum* inoculated tomato seedlings after 14 days post inoculation. Further, nanoparticles did not exhibit any negative impact on vegetative development of the seedlings upto 2000 ppm concentration.

Jiang et al. (2021) tested three metal oxide NPs *viz*., ZnO, FeO and CuO NPs against the tomato bacterial wilt pathogen *Ralstonia solanacearum*. The results showed nanoparticles especially CuONPs significantly reduced incidence of tomato bacterial wilt disease caused by soil-borne bacterium *Ralstonia solanacearum*. Also, significant improvement in morpho-physiological parameters of infected plants, diversity and richness of rhizospheric bacterial community were observed (Table 1).

Alkubaisi and Aref (2016) studied the effect of gold nanoparticles (AuNPs) on soil-borne Barley yellow dwarf virus (BaYDV) using TEM. They observed that dual existence of AuNPs *in vivo* and *in vitro* affected the configuration of capsid protein of virus after 24 and 48 hours of incubation period. Also, the size of nanoparticles plays critical role in reducing virus infectivity. The AuNPs of size 3.151 and 31.67 nm caused deterioration of virus particle by 75.3% and 24.7% respectively. A high yield of ruined virus like particles (VLPs) were observed in the local cultivar *Hordeum vulgare*. After 48 hours, completely lysed VLPs and some deteriorated VLPs were observed.

The increased potential and application of metallic nanoparticles indicates greater exposure of biological systems to metallic nanoparticles. Thus, understanding the interaction of metallic nanoparticles with plants, animals, human as well as environment is of utmost importance. Natural nanoparticles are constantly present in the environment, thus relationship between nature and nanoparticles is quite ancient. Biological beings have evolved both genetically and phenotypically under different types of stress conditions and possess defense mechanisms to counteract these adversities. However, engineered nanoparticles presents new concern to the ecological balance. The heavy metal nanoparticle mediated stress in plants leads to generation of variety of reactive oxygen species (ROS) which induce different responses in plants such as oxidative damage, lipid peroxidation, alteration in ion transport across cell membrane, malfunctioning of mitochondrial DNA, proteins and chloroplast. Plants have developed mechanisms to defuse these radicals however, balance between ROS generation and detoxification is essential for protection of plants. Gopalakrishnan Nair et al. (2014) reported increase in hydrogen peroxide content and lipid peroxidation in Mung bean plant exposed to CuONPs. Another study reported iron oxide phytotoxicity in *Lemna minor* plant which caused enhanced production of ROS and malondialdehyde in a dose-dependent manner (Souza et al., 2019). Plants also possess ROS scavenging mechanism that aids plants in overcoming these stresses (Czarnocka and Karpiński, 2018). The antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, ascorbate peroxidase etc. The antioxidant mechanism and ROS production depends on plant species, concentration, type of nanoparticles and the duration of exposure. Several studies as discussed in later part of the article reported increase in radical scavenging enzymes in nanoparticle treated plants which in turn enhances plant growth, development and yield. Thus, the relationship between plant adaptability and phytotoxicity is still debatable and needs further research to gain deeper insights. Another major concern is the accumulation of metallic nanoparticles in ecosystem that presents continuous threat to human and ecological health. Metallic nanoparticles are known to interact with cell membrane, damage membrane permeability, DNA, proteins and can easily enter the into the bloodstream and accumulate into the vital organs thereby causing toxicity (Hsin et al., 2008). The nano-size allows particles to gets easily absorbed, 15-20 times greater than bulk counterparts into any system including biological systems.

They also get absorbed in the soil, water and air ecosystem and may enter the food chain and deleteriously affect the natural fauna including beneficial organisms and microbes. Aquatic ecosystem is another habitat vulnerable to nanotoxicology due to accumulation of nanoparticles through surface run off. Several toxicity studies have been conducted on aquatic species but genotoxicity is not well established in these species. (Baalousha et al., 2011). Also, the engineered nanoparticles are analogous to heavy metal oxides and their behaviour and fate are affected by aggregation processes. Nanoparticles tends to aggregate and settle down leading to water decontamination due to loss of pollutants. On the other hand, nanoparticles have also been reported to exert toxicity on aquatic organisms including algae, plants, microorganisms, invertibrates and vertibrates (Zhang et al., 2018). Thus, the research on nanotoxicology have although increased in the recent decade however more studies need to be channelized towards this area to understand the environmental fate, transformation, bioavailability, transport, relevant toxicity and draw conclusive remark.

Polymeric nanoparticles such as cellulose, lignin and chitosan constitute important group of antimicrobial compounds possessing antifungal, antibacterial properties as well as plant growth promoting abilities. These compounds are considered as environment friendly as these are biodegradable and are abundantly found in nature. Dawwam et al. (2022) green synthesized cellulose nanocrystals (CNs) using agro-wastes obtained from palm sheath fibers and ZnONPs were synthesized using sono-co-precipitation method. The CNS-ZnO bio-nanocomposite were evaluated for antibacterial activity against gram negative (*Escherichia coli* and *Salmonella*) and Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) bacteria which possess ability to persist in soil. They observed MIC value of the CNS-ZnO nanocomposite were in the range 0.5-1.0 µg/ml against *E. coli* and *L. monocytogenes* while the MIC values against *Salmonella* and *S. aureus* were 0.25 – 1 µg/ml indicating influence of CNS-ZnO at low concentration. Also, the virulence and toxin associated genes of the bacterial pathogens were found to be downregulated suggesting anti-toxigenic properties of the CNS-ZnO nanocomposites. In another study, Schiavi et al. (2022) synthesized cellulose nanocrystals (CNCs) from the wastes obtained from olive pruning using chemical bleaching method. The synthesized nanocrystals exhibited inhibition of bacterial pathogen causing olive knot disease (*Pseudomonas savastanoi* pv. *savastanoi*) under *in vitro* condition. An inhibition of bacterial biofilm formation and reduction in bacterial epiphytic survival with no adverse effect on leaf development and root uptake were reported from their study. Lignin is another important aromatic polymer obtained from natural sources which possess antimicrobial activity and can be used as nanocarriers. Paul et al. (2021) synthesized lignin nanospheres using combination of solvent displacement method with sonochemistry and reported increased inhibition of Gram-positive *Bacillus megaterium* and gram-negative *E. coli*. Chitosan nanoparticles (ChNPs) evolved as a promising antimicrobial and plant growth promoting compound having potential to be used in management of soil-borne pathogens. The biopolymer based ChNPs were found effective against numerous pathogens *viz*., *Rhizoctonia solani* (Saharan et al., 2013; Boruah and Dutta, 2021), *Fusarium oxysporum* (Muthukrishnan and Ramalingam, 2016; Boruah and Dutta, 2021), *Sclerotium rolfsii* (Boruah and Dutta, 2021). Kheiri et al. (2016) reported synthesis of ChNPs of different molecular weight and demonstrated its antifungal activity against Fusarium head blight pathogen *Fusarium graminearum*. The per cent mycelial growth inhibition was recorded as 77% at 5000 ChNP concentration. The greenhouse trial indicated decline in AUPDC in treated plants. The ChNPs were also found effective against *Macrophomina phaseolina* and *R. solani* and showed inhibition of radial growth of the pathogen in a dose dependent manner (Saharan et al., 2013). Suarez-Fernandez et al. (2020) reported that root exudates from chitosan treated tomato plants inhibited soil borne fungal pathogen *F. oxysporum* f.sp. *radicislycopersici* and root knot nematode *Meloidogyne javanica*. Two-fold reduction in mycelial growth of fungal pathogen was observed with respect to control and 1.5-fold reduction in hatching of *M. javanica* eggs were recorded after 72 hours. Khairy et al. (2022) reported that ChNPs were effective against bacterial wilt of tomato and potato caused by *Ralstonia solanacearum*. *In vitro* assay indicated highest zone of inhibition at 200 µg/ml concentration. *In vivo* assay exhibited decline in disease incidence and severity after foliar application of ChNPs in wilt affected plants. The ChNPs were found to directly interact with bacterial cell wall causing alteration in shape, loss of flagella and ultimately cell lysis. The results obtained from RAPD-PCR revealed differences in genotype of exposed *Ralstonia solanacearum* as compared to untreated ones. Kim et al. (2013) synthesized Chitosan-lignosulfonate (CS-LS) nanohybrid and reported inhibition of *Bacillius subtillis, S. aureus* and *E. coli* at a rate higher than CS and LS alone.

From the above discussion, we can infer that nanoparticles have been widely studied for its antimicrobial activity against soil-borne pathogens more specifically fungal pathogens. The literature on effect of nanoparticles on soil-borne bacterial and viral pathogens is scarce. The metallic nanoparticles such as Ag, ZnO, and CuO NPs have been found to produce promising results in management of most of the soil-borne pathogens. The positively charged metallic nanoparticles can easily be adsorbed on the surface and penetrate into the cell as compared to its bulk counterparts (Gupta and Rai, 2017). Also, the mode of action of nanoparticles as established from previous studies include nanoparticle contact, accumulation, cell wall disruption, membrane leakage, inhibition of RNA and protein synthesis, inhibition of ATPase activity, intracellular ROS production and increased SOD activity (Dakal et al., 2016) (Figure 2). The antiviral activity includes direct interaction of nanoparticles with capsid protein and degradation of virus particle (Alkubaisi and Aref, 2016). More research is needed especially against soil-borne bacteria and viruses to determine its exact mode of action. Further, *in vivo* trials are needed to validate the results obtained from *in vitro* assays. However, the use of heavy metal nanoparticles raises toxicity concerns and its persistence and accumulation in the food chain as well as ecosystem. Extensive research is needed in this regard to obtain accurate conclusions. Natural polymeric nanoparticles such as chitosan, lignin and cellulose could prove as a suitable alternative as these are biodegradable and environment friendly in nature.

4.2 Nanomaterials as carrier of antimicrobial agents

Nanotechnology can meet the need of sustainable agriculture by reducing the load of chemical fungicides and pesticides in the

TABLE 1 Use of Nanoparticles for management of soil-borne plant pathogens.

Nanomaterial	Type of Pathogen	Target Pathogen	Crop	Effect	Reference
As Protectant					
Silver Nanoparticles (AgNPs)	Fungi	*Rhizoctonia solani, Fusarium oxysporum, Sclerotium rolfsii* and *Sclerotinia sclerotiorum*	Cereals, Pulses and vegetables	Mycelial growth inhibition at 100 ppm AgNP	Kaman and Dutta, (2019)
AgNPs	Fungi	*Fusarium fujikuroi, Rhizoctonia solani and Macrophomina phseolina*	Cotton (*Gossypium herbaceum)*	Reduction in mycelial growth and illness of cotton seedlings	Zaki et al. (2022)
AgNPs	Fungi	*Sclerotinia sclerotiorum*	Mustard (*Brassica juncea*)	Inhibition of hyphal growth, sclerotial formation and myceliogenic germination of sclerotia	Tomah et al. (2020)
Capped AgNPs	Fungi	*Sclerotinia sclerotiorum*	Vegetables	Inhibition of mycelial growth and sclerotia germination	Guilger-Casagrande et al. (2021)
Magnesium oxide nanoparticles (MgONPs)	Fungi	*Thielaviopsis basicola* and *Phytophthora nicotianae*	Tobacco (*Nicotiana tabacum)*	Inhibition of fungal growth, spore germination and impediment of sporangium development	Chen et al. (2020)
MgONPs	Fungi	*Phytophthora infestans*	Potato (*Solanum tuberosum*)	Inhibition of *Phytophthora infestans* by cell membrane distortion, oxidative stress, disruption of metabolic pathways and membrane transport activity with no harmful effect on potato	Wang et al. (2022)
Copper oxide nanoparticles (CuONPs)	Fungi	*Phytophthora nicotianae*	Tobacco *(Nicotiana tabacum)*	33.69% increase in control efficacy and tobacco black shank disease suppression without inducing phytotoxicity at 100 mg L^{-1} of CuO NPs treatment	Juan-ni et al. (2022)
Chitosan NPs	Fungi	*Rhizoctonia solani, Fusarium oxysporum, Sclerotium rolfsii*	Cereals, Vegetables	Reduction in mycelial growth	Boruah and Dutta, 2021
Carboxymethyl cellulose coated core/shell SiO_2@Cu nanoparticles	Fungi	*Phytophthora capsici* Host: Black pepper	Black pepper (*Piper nigrum)*	Antifungal activity against *P. capsici* with MIC 75 ppm	Hai et al. (2021)
AgNPs	Fungi	*Macrophomina phaseolina* and *Fusarium solani*	Strawberry (*Fragaria ananassa)*	The nanoparticle showed broad spectrum antagonism against *M phaseolina* (67.05%) and *F. solani* (83.05%)	Paola et al. (2018)
AgNPs	Fungi	*Phomopsis* sp. Host: Soybean seeds	Soybean (*Glycine max)*	Absolute inhibition of the pathogen was observed 270 and 540 ppm concentration	Mendes et al. (2014)
Zinc oxide (ZnO), Iron oxide (FeO) and Copper oxide (CuO) nanoparticles	Bacteria	*Ralstonia solanacearum* Host: Tomato	Tomato *(Solanum lycopersicum)*	Reduced incidence of tomato bacterial wilt disease	Jiang et al. (2021)
Gold nanoparticles (AuNPs)	Virus	Barley yellow dwarf virus (BaYDV)	Barley (*Hordeum vulgare*)	A high yield of ruined virus like particles (VLPs) were also observed	Alkubaisi and Aref (2016)
Copper/Iron NPs	Nematodes	*Meloidogyne incognita* and *M. javanica*	Tomato *(Solanum lycopersicum)*	Nematicidal activity such as paralysis, biological cycle arrest, reduction in number of galls with lowest EC50 value as compared to commercial nematicides	Gkanatsiou et al. (2019)
AgNPs	Nematodes	*Meloidogyne javanica*	Tomato *(Solanum lycopersicum)*	Nematicidal activity on egg hatchability and juvenile mortality. Reduction in number of galls, egg masses, number of females per root/plant and mortality of juveniles.	Ghareeb et al. (2020)
As Carrier of Actives					
Pectobacterium cypripedii nanoghost loaded with tebuconazole	Fungi	*Leptosphaeria nodorum, Pyrenophora teres*	Barley (*Hordeum vulgare*) and Wheat (*Triticum aestivum*)	The efficacy of the loaded bacterial ghost for resistance to rainfall and the protective and curative effects against the pathogens increased	Hatfaludi et al. (2004)

(Continued)

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TABLE 1 Continued

Nanomaterial	Type of Pathogen	Target Pathogen	Crop	Effect	Reference
Lignin-modified polymer nanocapsule loaded with pyraclostrobin	Fungi	*Fusarium. oxysporum* f. sp. *radicis-lycopersici*	Tomato *(Solanum lycopersicum)*	The nanocapsules lead to rapid release of actives and increased its efficacy against the pathogen and soil mobility. Also, residue development in soil was reduced	Luo et al. (2020)
Carbendazim-loaded polymeric nanoparticles	Fungi	*Fusarium oxysporum* and *Aspergillus parasiticus*	*Cucumber (Cucumis sativa), Maize (Zea mays)* and Tomato *(Solanum lycopersicum)*	Increased rate of fungal inhibition	Kumar et al. (2017)
lecithin/chitosan-encapsulated kaempferol	Fungi	*Fusarium oxysporum*	Vegetables and stored food products	67% inhibitory efficiency after 60 days of storage on a Petri dish with *Fusarium oxysporum*-infected fungus	Ilk et al. (2017)
Combination of AgNPs and Fluconazole	Fungi	*Phoma glomerata, Phoma herbarum, Fusarium semitectum, Trichoderma* sp., *and candida albicans*	Pulses, Vegetables	Enhanced antifungal activity of fluconazole against *Phoma glomerata, Trichoderma* sp., *and candida albicans*	Gajbhiye et al. (2009)
SLNs loaded with essential oil of *Zataria multiflora*	Fungi	*Rhizoctonia solani*	Cereals, Pulses, Post-harvest pathogens	Stabilization of essential oil of *Zataria multiflora*, thereby increasing its efficacy	Nasseri et al., 2016
Alginate-gelatin nanocomposite encapsulated *Pseudomonas fluorescens* (VUPF5 and T17-4 strains)	Fungi	*Fusarium solani* Crop: Potato	Potato (*Solanum tuberosum*)	The encapsulated *Pseudomonas fluorescence* strains showed enhanced shelf life than non-coated bacteria. Also, the green house experiment revealed increased control efficacy against *Fusarium solani* causing disease in potato plants.	Pour et al. (2019)
T. asperellum and nanochitosan based formulation	Fungi	*Rhizoctonia solani, Fusarium oxysporum, Sclerotium rolfsii*	Cereals, Pulses, Vegetables	Enhanced reduction in mycelial growth of the pathogens	Boruah and Dutta (2021)
Encapsulated *Streptomyces fulvissimus* Uts22 strain based on alginate–Arabic gum and nanoparticles (SiO_2 and TiO_2)	Fungi	*Pythium aphanidermatum* Crop: Cucumber	Cucumber (*Cucumis sativus*)	Encapsulated bacteria resulted in a 95% reduction in damping-off disease of cucumber and showed more potential effects on increasing plant growth traits than free bacteria under green-house condition.	Saberi Riseh et al. (2022)
Bacillus subtilis Vru 1 encapsulated in alginate-bentonite coating and enriched with titanium nanoparticles	Fungi	*Rhizoctonia solani* Crop: Bean	Bean (*Phaseolus vulgaris* L.)	Vru 1 nanocapsules showed 90% inhibition of the pathogen as compared to 60% inhibition by free Vru 1. Also, vegetative growth parameters in bean plant were significantly enhanced	Saberi-Rise and Moradi-Pour (2020)
Nanoparticles as stimulator of plant growth and development					
AgNPs	Fungi	*Rhizoctonia solani* Crop: Rice	Rice (*Oryza sativa*)	Inhibition of mycelial growth and sclerotial germination Reduction in per cent disease incidence, enhanced plant growth parameters and secondary metabolites *viz*., phenols, flavonoids, terpenoids and TSS	Dutta et al. (2021)
Silicon dioxide nanoparticles (SiO_2 NPs)	Fungi	*Rhizoctonia solani*	Wheat (*Triticum aestivum*)	Increased the amount of chlorophylls, carotenoids, defense-related stimulants (particularly salicylic acid), POD, SOD, APX, CAT, and PPO enzymes, phenolics and flavonoids antioxidant defense mechanisms.	Abdelrhim et al. (2021)
ZnO-NPs	Fungi	*Fusarium oxysporum*	Brinjal (*Solanum melongena*)	Increased plant height, root length, plant fresh biomass, chlorophyll a, chlorophyll b, total soluble carbohydrates, total soluble protein, phenol, antioxidant activity, and isozymes	Abdelaziz et al. (2022)

environment. The nanoparticles as carrier molecule allows slow, timely and targeted release of active ingredients in the environment thereby increasing its efficacy. The soil-borne pathogens or its propagules do not invade the roots of the plant at the same time, thus persistent and slow release of active ingredients is essential to provide protection to the crop throughout the growing season (Khan et al., 2011). Nanocarriers reduce the rate of application as the chemical actually effective against the pathogen is 10-15% lesser than the chemicals required with conventional formulations. The nanomaterials having small size, greater kinetic stability, low viscosity, and optical transparency can prove better and smart delivery vehicle (Xu et al., 2010). The nanoformulations as carrier

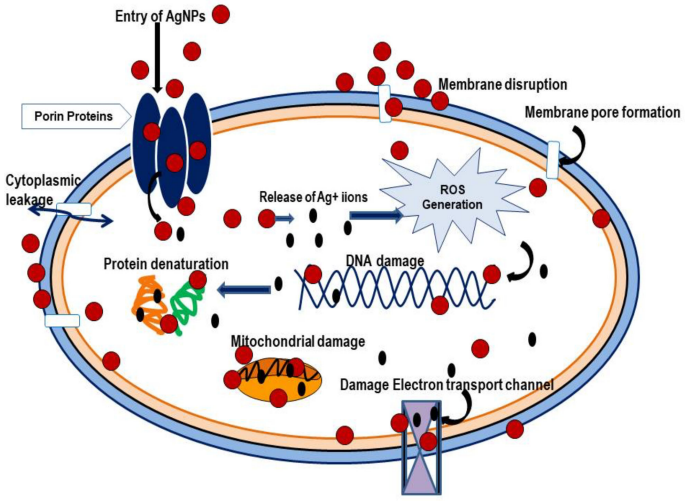

FIGURE 2
Mechanism of antimicrobial action of nanoparticles on Microorganisms.

of active antimicrobial agents can enhance the solubility, wettability, dispersion and bioavailability of chemical fungicides and pesticides (Bergeson, 2010a). Nanomaterials exhibit unique properties such as solubility, thermal stability, permeability, crystallinity and biodegradability (Bordes et al., 2009; Bouwmeester et al., 2009) essential for nanopesticide development. The common nano-based delivery systems that potentially be effective in plant protection strategies include nanoencapsulates, nanotubes, nanowires, nanoemulsions and nanocages (Bouwmeester et al., 2009; Lyons and Scrinis, 2009; Bergeson, 2010b). Ghormade et al. (2011) summarized the studies on use of nano-based smart delivery systems for agrochemicals viz., pesticides, fertilizers and plant growth promoters. Nanoencapsulates, primarily nano-clay provides interactive surfaces having aspect ratio for encapsulation of agrochemicals (Table 1).

In 1997, the first research on nano-based fungicides was carried out, and efforts were made to embed fungicides into solid wood (Liu et al., 2001; Liu et al., 2002). Since then, numerous investigations using a variety of nanoparticles have been undertaken using regular fungicides (20 studies) and biocides possessing antifungal capabilities (6 studies). A variety of essential oils not covered by the fungicide groups were investigated, along with nine Fungicide Resistance Action Committee (FRAC) groups. Polymer blends, silica, and chitosan were the most frequently researched nanoparticle carriers. To assess the effectiveness of the nanofungicide, a variety of fungi were used. However, there weren't many toxicity studies, nor were many plants studied. Nanosized bacterial ghosts, which are non-attenuated void cell envelopes of Gram-negative bacteria, were utilized by Hatfaludi et al. (2004) to improve the low water-solubility of tebuconazole and boost adhesion to the leaf surface. *Pectobacterium cypripedii* as the nano-bacterial ghost was chosen due of its capacity to stick to plants. When exposed to severe simulated rain under glasshouse settings, fluorescently labelled ghosts stuck to rice leaves the best (retaining 55%) and soya leaves the least (10%) of the six plants examined (rice, soya, cabbage, cotton, barley, and maize). All six plants were tested against a variety of fungi using ghost-loaded tebuconazole or two different commercial tebuconazole treatments (WP 25 and EW 250). Plants that weren't exposed to rain had protection that was at par with or better than that of commercial treatments with one exception. None of the groups outperformed the commercial solutions when the plants were exposed to intense rain and fungus one hour after treatment except one. However, ghost-loaded tebuconazole was equivalent to, or larger than, WP 25 treatments when rinsed 24 hours after treatment, although EW 250-treated controls were often more effective. Luo et al. (2020) attempted to develop a nanoscale delivery system for low water soluble fungicide pyraclostrobin. The active ingredient was encapsulated using lignin-modified polymer nanocapsule to enhance soil mobility. The nanocapsules allowed rapid release of actives and increased the distribution and accumulation of active ingredient on the surface of target organisms thereby increasing its efficacy and also soil mobility. The pot trials revealed nanoemulsions in water improved control efficacy against tomato crown and root rot caused by *Fusarium. oxysporum* f. sp. *radicis-lycopersici* as compared with micron-grade microcapsule suspension (CS) of pyraclostrobin. Also, lower fungicide residue in soil was evident than CS treatment. Xu et al. (2014) conducted similar study where pyraclostrobin was encapsulated using chitosan–lactide copolymer nanoparticles. Initially, the nanofungicide was less efficient in inhibition of *C. gossypii* as compared with commercial pyraclostrobin. However, seven days post treatment, an increase in pathogen inhibition was observed greater than the active alone. In a different experiment, lecithin/chitosan-encapsulated kaempferol (another low-soluble fungicide) demonstrated 67% inhibitory efficiency after 60 days of storage on a Petri dish with *Fusarium oxysporum*-infected fungus (Ilk et al., 2017). Another study was conducted by Qian et al. (2011) where nanosized calcium carbonate carrying the active molecules was used to achieve slow release of the compounds. The effectiveness of validamycin loaded nanoparticles was inferior to validamycin alone against *Rhizoctonia solani* during first week of treatment, However, post two weeks the nanoparticle formulation demonstrated marginally superior outcomes compared to the active alone,

emphasizing the longevity of the nanoformulation's effectiveness. Kumar et al. (2017) observed that carbendazim-loaded polymeric nanoparticles exhibited increased rate of fungal inhibition against *Fusarium oxysporum* and *Aspergillus parasiticus* as compared to carbendazim alone. Phytotoxicity assays verified that the nanoformulated carbendazim had no adverse effect on plant germination and root development of *Zea mays*, *Lycopersicum esculentum*, and *Cucumis sativa* seeds. Santiago et al. (2019) reported that ChNp loaded with AgNP showed antibacterial activity against tomato bacterial wilt pathogen *Ralstonia solanacearum* and suggested AgNP entrapped chitosan is a suitable alternative to chemical antibiotics/bactericides.

Botanical extracts and essential oils having antimicrobial properties are most studied compounds in the era of organic farming. Several studies have been conducted to encapsulate the extracts and essential oils derived from plants to prevent its volatilization and to enhance the shelf life. Janatova et al. (2015) effectively encapsulated five distinct essential oil components into MSN and demonstrated greater antifungal activity 14 days post *Aspergillus niger* infection. Similar to this, SLNs have also been utilized to stabilize the essential oil of *Zataria multiflora*, offering defense against six fungi including soil-borne pathogen *Rhizoctonia solani* (Nasseri et al., 2016).

The major concern related to soil health is the leaching of chemicals through soil but limited studies have been conducted in this aspect. Wanyika (2013) encapsulated metalaxyl using MSNs and compared leaching in soil between encapsulated metalaxyl (11.5%) and free metalaxyl (76% release) for a period of 30 days. The relevance of conducting tests in a farming context was demonstrated by the encapsulated metalaxyl, which had a 47% higher release rate in water than in soil. Campos et al. (2015) investigated the cytotoxicity of carbendazim and/or tebuconazole placed onto two different types of nanoparticles, solid lipid or polymeric. In preosteoblast and fibroblast mouse cell lines, toxicity of the insecticides with nanoparticles was found to be reduced. Most of the studies have been conducted to develop nano-encapsulated fungicides however, the literature on encapsulation of antibiotics and antiviral agents against phytopathogens is rare. Thus, it indicates that more research in needed in this area.

4.3 Nanoparticles for induction of plant defense mechanism

4.3.1 Antioxidant system

Plant health is disrupted severely by numerous soil-borne pathogens such as fungi, bacteria and viruses. In order to respond to vast array of biotic and abiotic stresses, plants have developed multifaceted defense systems which included both inducive and constitutive defenses. Constitutive mechanism presents first line of defense against the pathogen. Upon invasion of the pathogen into roots of plants bypassing constitutive defense mechanisms, leads to activation of induced defense mechanisms. For any management practices, induction of plant defenses is an important aspect. The production of ROS, which inhibits pathogen transmission and triggers local and systemic defense responses such as the release of pathogenesis-related (PR) proteins, is a component of the plant defense response to diverse stressors. Oxidative products are produced and the equilibrium between ROS and antioxidants is upset when the quantity of ROS exceeds the threshold. The antioxidant system in plants works to counteract the effects of oxidants. Superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and guaiacol peroxidase (GPX) are a few of the enzymes that make up the antioxidant system (Tan et al., 2018). Nanoparticles alter cellular redox equilibrium by increasing or decreasing oxidative stress (Soares et al., 2018). According to previous studies, depending on the needs of the host plant, nanoparticles can either stimulate the generation of ROS, or suppress the oxidative burst by production of antioxidant enzymes and secondary metabolites. Abdelrhim et al. (2021) observed that Silicon dioxide nanoparticles (SiO_2 NPs) activated antioxidant system and innate defense responses in wheat seedlings against the pathogen *R. solani*. SiO_2 NP application increased the amount of photosynthetic pigments (chlorophylls and carotenoids), prompted the accumulation of defense-related stimulants (particularly salicylic acid), and reduced oxidative stress by activating both enzymatic (POD, SOD, APX, CAT, and PPO) and non-enzymatic (phenolics and flavonoids) antioxidant defense mechanisms. Plants exposed to nanoparticles showed increased expression of superoxide dismutase (SOD), which catalyzes the detoxification of O_2 into either regular molecular oxygen (O_2) or H_2O_2, and ascorbate peroxidase (APX), which detoxifies peroxides like H_2O_2 utilizing ascorbic acid (Asc) as a substrate (Fu et al., 2014). The enzymes that control the cellular Asc redox state, dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDAR), were downregulated (Fu et al., 2014). SOD, APX, and glutathione-S-transferase (GST) were found in greater abundance in AgNP treated *O. sativa* roots using proteomic analysis (Mirzajani et al., 2014). Additionally, these nanoparticles dramatically increased the activity of SOD and APX in *Pisum sativum* L. seedlings while inhibiting glutathione reductase (GR) and DHAR (Tripathi et al., 2017). When wheat roots were exposed to 500 mg/kg CuONPs, catalase (CAT), another enzyme that shields cells from oxidative damage, was noticeably increased (Dimkpa et al., 2012). When examined after 10 days, maize plants growing on soil supplemented with 0, 400, and 800 mg/kg Cerium dioxide nanoparticles (CeO_2NPs) demonstrated a concentration-dependent increase in the buildup of H_2O_2 after 10 days, but 20th day showed no difference (Zhao et al., 2012). Lignin nanoparticle primed maize seeds showed positive effect on seed germination, radicle length in the initial stages. At later stages, increased biomass and biochemical parameters such as total soluble protein, total chlorophyll, carotenoid and anthocyanin were evident (Del Buono et al., 2021). Seed treatment of chickpeas with ChNPs exhibited increased germination percentage, biomass and seed vigor index (Saharan et al., 2013). ChNPs conjugated with rhizobacteria (PS2 and PS 10) showed increased seed germination, leaf area, plant height and chlorophyll content in maize plant. The stress tolerance mechanism in maize plant was attributed to greater production of antioxidant enzymes such as alkaline phosphatase, dehydrogenase and fluorescein diacetate hydrolysis (Khati et al., 2017). Abdelaziz et al. (2022) observed that ZnO-NPs prompted the healing of *F. oxysporum* infected eggplant by increasing morphological and metabolic markers such as plant height (152.5%), root length (106.6%), plant fresh biomass (146%), chlorophyll a (102.8%),

chlorophyll b (67.86%), total soluble carbohydrates (48.5%), total soluble protein (81.8%), phenol (10.5%), antioxidant activity, and isozymes in comparison to infected control. It is becoming clear that the induction of antioxidant machinery by nanoparticles may foster plant growth as confirmed in a few investigations (Sharma et al., 2012; Burman et al., 2013; Kumar et al., 2013) as long as a harmful level of ROS is not reached in the cells. However, once this level is breached, this may result in impaired organ growth, development and induce phytotoxicity (Mittler, 2017).

4.3.2 Phytohormones and plant signaling molecules

The signaling molecules salicylic acid (SA), Jasmonic acid (JA), and ethylene (ET) cause the proper defense reactions to be triggered. Gibberellin (GA), cytokinin (CK), auxin [indole-3-acetic acid (IAA)], abscisic acid (ABA), brassinosteroids (BRs), and strigolactone (SL) are other plant growth-regulating hormones that have the capacity to control defense responses Crosstalk between various plant hormones controls the balance between plant's defenses and growth. Nanoparticles are known to affect the balance of plant hormones (Rastogi et al., 2017). Zahedi et al. (2019) reported accumulation of stress signaling molecules indole-3-acetic acid and abscisic acid, in strawberry plants treated with selenium nanoparticles (SeNPs). Additionally, increased levels of organic acids (such as malic, citric, and succinic acids) and sugars (such as glucose, fructose, and sucrose) in the fruits of strawberry plants treated with Se-NPs under saline conditions demonstrated the benefits of Se-NPs on the improvement of fruit quality and nutritional values. Azhar et al. (2021) examined phytohormone signaling when different metallic nanoparticles (ZnO, SiO2, and ZnO/SiO2 composite NPs) were exposed to Arabidopsis. They discovered that nanoparticle accumulation in plant tissue altered the expression level of genes associated with the cytokinin signalling pathway (ARR7 and ARR15), which suggested the significance of cytokinin in the plant's response to nanoparticles. Shang et al. (2020) studied the effect of CuONPs on the soil-borne pathogen *Gibberella fujikuroi* that causes Bakanae disease in rice plants. They reported that seed treatment with copper sulphide nanoparticles significantly increased *in planta* JA content and shoot ABA content to levels equivalent to healthy control plants, while no difference in SA content was observed as compared to healthy and diseased control. Also, the level of sakuranetin (SN), an important phytoalexin in rice, was found to increase by 96.4% relative to diseased control when CuONPs were applied using foliar spray and seed treatment. Ma et al. (2021) studied the effect of nanoscale hydroxyapatite (nHA) on tomato plants infected with *Fusarium oxysporum* f.sp. *lycopersici*. They revealed that exposure to nHA significantly enhanced phenylalanine ammonialyase activity (30-80%) and total phenolic content (40-68%) in infected plants. The level of SA in shoots also increased by 10-45%, indicating a relationship between phytohormones and antioxidant pathways in nHA promoted defense against the fungal pathogen in the host plant. Another essential component of a plant's immune system is PR proteins, which serve as a part of the diagnostic biomarkers of plant defense signaling pathways. The activation of the PR1, PR2, and PR5 genes indicates that the SA signaling pathway has increased (Ali et al., 2018). Healthy tobacco plants (*N. benthamiana*) when treated with SiO_2 NPs and ZnONPs upregulated PR1 and PR2 genes that are SA-inducible, and treatment with magnetite nanoparticles (Fe_3O_4NPs) had a similar impact (Cai et al., 2020). As a result, the changed levels of phytohormones and PR proteins in plants exposed to nanoparticles suggest activation of the plant's defense system. Grodetskaya et al. (2022) studied the effect of CuONPs on infection of downy birch micro-clones with soil-borne pathogens, *viz*., *Fusarium oxysporum* and *Fusarium avenaceum*, and assessed the level of expression of genes associated with defence responses in plants induced by microorganisms. CuONPs significantly suppressed the infection of *Fusarium avenaceum*, while no effect was observed against *Fusarium oxysporum*. Also, *a* decline in the expression of MYB46, PR-1, and PR-10 genes by 5.4 times was observed and could be due to a reduction in the pathogenic load caused by the effect of nanoparticles and the simultaneous stimulation of clones."

5 Conclusion and future prospects

Soil borne plant pathogens represent diverse group of microorganisms such as fungi, bacteria, viruses, nematodes dwelling in soil and cause huge economic loss by affecting the root and collar region of plants. These pathogens are difficult to manage using conventional strategies as chemical pesticides can hardly reach into the soil system and large amount of these chemicals upsets the soil and environmental health. Nanotechnology has emerged as one of the potential management strategy to curb the menace of soil-borne plant pathogens. Nanomaterials can be effectively utilized to manage soil-borne pathogens owing to their versatile antimicrobial properties, including generation of ROS, cell membrane, organelles and other macromolecule destabilization and toxicity due to nanoparticles. The smaller size and greater surface area to volume ratio of nanoparticles allow for greater penetration potential and better interaction with soil-dwelling microbes thereby increasing their control efficacy. Nanomaterials can also be used as smart delivery vehicle for pesticides, fungicides and fertilizers to reduce the chemical load on the environment. Also, nanodiagnostics have emerged as a potential science to overcome the difficulties associated with the detection of soil-borne pathogens. Nanotechnology have been utilized to develop affordable biosensor systems for the early and sensitive detection of soil-borne pathogens based on the qualitative and quantitative detection of specific metabolites secreted by them. The indirect mechanism of management of soil-borne pathogens involves activation of plant defense mechanism and promotion of plant growth. To use nanomaterials wisely in soil-borne pathogen protection, it is imperative to understand their ecotoxicity, phytotoxicity, cytotoxicity, genotoxicity and interactions with the soil system and the soil residents. The nanomaterials upon reaching the soil, directly interact with soil particles affecting physicochemical properties, fertility and beneficial organisms. The effect of nanoparticles on soil structure, soil functioning, organic matter, siderophore production, nitrogen fixation, phosphate and potassium solubilization, and related processes need to be assessed to develop a holistic idea of the behaviour and fate of nanoparticles in soil. Also, the repercussions of the use of nanoparticles on beneficial microorganisms need to be examined. The mechanism involved in the interaction of nanoparticles with the rhizospheric microbiome needs to be elucidated further. Integration of nanomaterials with biological control agents and organic additives would prove beneficial

in mitigating soil-borne diseases along with crop produce intensification. However, the technology is still in its infancy and demands extensive research in this arena. Further, nanocomposites can be developed using active ingredients and fertilizers for holistic development of agricultural crops.

Nanopesticides would require more than a decade to reach the market and its end users. Most of the nano-formulations or products are still in the laboratories or start-up phase and fewer products have reached the market till date. In 2019, the global market of nano-based materials was estimated as 8.5 billion US dollars and it was anticipated to flourish at an annual rate of 13.1% from 2020 to the year 2027 (Anonymous, 2016). The increase in market share of nanomaterials in different field is attributed to increase in social acceptance and demand and adoption in different arenas such as medical, food industry, agriculture, sports, aerospace, energy sectors and many more. However, acceptance of nano materials in agriculture is quite low as it is closely related to food and human health. The high initial production cost and complexity in production process makes its use in agriculture debatable. It is well known that nanomaterials synthesized through physical and chemical methods are enormously expensive and pose environmental risk. The green synthesis methods are comparatively cost effective and profitable in long run, less hazardous to the ecological health and have faster reaction rate. Efforts are made globally for regulating secure manufacturing and applications of nanomaterials and nanodevices by supervision and advices or by legislations (Choi et al., 2009). Till date, there is no single law fully committed to offer guidelines related to use of nanotechnology in any country across the globe. There is a need for guidelines and directives to assess impending hazards and for suggestions to ensure safe utilization of nanotechnology and few organizations are working actively in this field such as International Standard Organization (ISO), Organization for Economic Cooperation and Development (OCED) or US Food and Drug Administration (FDA) (Coles and Frewer, 2013). The cautious assessment of benefits of use of nanoparticles throughout its lifecycle addressing environmental, social and economic implications as well as occupational safety and health hazards are need of the hour. The effect of nanoparticles on agricultural, industrial and non-industrial workplaces and measurement of exposure of workers in the workplaces is important. Also, the toxicological properties of nanoparticles should be characterized and gathered information must be stored in database which can be readily accessed by researchers. In nanoparticle manufacturing plants, safety measures should be prioritized to eliminate occupational hazards. Also, safety guidelines should be established in laboratories concerning safe handling, use and disposal of nanoparticles and related waste materials. Proper care should be taken while promoting the benefits of use of nanoparticles in agricultural field so that no adversities result from their use.

Further research is needed to determine the practicability, sustainability, efficiency, applicability and releasability of nanotechnology-based products under field conditions as well as to validate these technologies in comparison to present technologies. Also, more *in vivo* and field trials are to be conducted for pesticide loaded nanomaterials or nanopesticides. To get comprehensive idea on efficacy of these pesticides, long term trial data is required which is constraint at present. Unlike chemical pesticides, nanomaterilals and nanopesticides lacks a clear definition by regulatory authorities. Kookana et al. (2014) reviewed into great detail about how the effects of nanopesticides, in contrast to conventional pesticides, may rely on the uptake, bioavailability, concentration, and toxicity of the nanoparticles as well as the ratio of the active coupled to them. There is also little information available on the problem of pesticide resistance and potential ways that adding nanoparticles could lower its prevalence. The creation of regulatory standards for risk assessment is not possible without the use of extensive analytical techniques. The application of pesticides in field undergoes rotation and revision by regulatory bodies periodically restricts and ban the use of chemical pesticides thus, broad range of nanoformulations need to be available for future applications. Using new tools and methods to produce solid data for analysis, characterization, and risk assessment may be the key to receiving approval from regulatory authorities.

Thus, for logical selection of appropriate nanomaterials and nanopesticides, a thorough knowledge of the structural characteristics of the nanoparticles, including their shape, size, functional groups, and active adsorption/loading capacity is essential. In order to undertake biocompatibility and efficacy investigations at the cell, organism, and pest-host ecosystem levels under as-close-to-field circumstances as feasible, it is also crucial to choose a trustworthy and reproducible system. Further, wholesome development of nanoscience requires integration of different sciences such as biologists, agricultural engineers, plant pathologists, biotechnologists and soil microbiologists. Therefore, efforts must be directed toward creating a soil disease control strategy that is long-lasting, safe, effective, and environmentally benign.

Author contributions

PD was responsible for writing guidance and manuscript preparation. AK was responsible for writing the first draft of the article. PD revised the article with other coauthors. 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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Ralstonia solanacearum – A soil borne hidden enemy of plants: Research development in management strategies, their action mechanism and challenges

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Plant pathogens present in soil cause severe losses to plants every year. Among them, *Ralstonia solanacearum*, because of its destructive nature, is the world's second most damaging bacterial phytopathogen. Over 310 species of plants belonging to 42 plant families are infected by this deadly pathogen. Around the world, the bacterial wilt (BW) disease causes yield losses that range from 20 to 100%. Control measures for managing this pathogen comprises several diverse approaches. Regardless of whether several control methods are developed to manage the BW disease, efficient management strategies with eco-friendly effects and the desired level of effective control is still awaited and there is need to developed effective management methods to eliminate this fetal disease in several crops under field conditions. An analysis of development in the management strategies will provide an effective way to search and develop control methods with desirable level of effectiveness. In this review, we discussed and analyzed the information reported on the development of various management strategies for the management of *R. solanacearum* along with the comprehensive presentation on action mechanism of these management strategies. We have also made an effort to summarize the challenges that make hurdle in the effective management of this deadly pathogen. The analysis of the information in this review article will assist in future implications of management strategies and help in developing effective control measures with more efficacy.

KEYWORDS

IDM, management, soil pathogen, plants, bacteria

1 Introduction

Pathogens in the soil are a key cause of crop losses in many important plants (Elphinstone et al., 2005). *Ralstonia solanacearum*, the soil-borne bacterium that causes bacterial wilt (BW), is the world's second most damaging phytopathogen (Mansfield et al., 2012). Ralstonia spp. are Gram-negative, oxidaseand catalase-positive, aerobic, bacilli, thriving in soil and water (Lampropoulos et al., 2021). This plant disease is among the most damaging disease of several agriculturally important crop plants such as eggplant, tomato, pepper, potato, ginger that affects the quality and quantity of crops globally (Cai et al., 2021). Because of its high destructive nature, *R. solanacearum* is currently one of the most intensively studied plant pathogen.

Over 310 species of plants belonging to 42 plant families are infected by *Ralstonia* (Genin, 2010; Genin and Denny, 2011; Paudel et al., 2020). *Ralstonia solanacearum* Species Complex, or RSSC, is the name given to the pathogen, which is made up of three distinct species: *Ralstonia pseudosolanacearum*, *R. solanacearum*, and *R. sygzii*, having same core genome. Since the first two species have been the most thoroughly studied causes of wilt disease, we will refer to strains of these species as *Ralstonia* in this article. Although the virulence nature of the pathogen is also reported in cooler temperatures, the pathogen thrives mostly in humid and hot climates. During high infection, *Ralstonia* population can reach 10^3–10^6 and 10^8 cfu/gram of soil and of plant tissue, respectively. The bacterium can remain viable for years in water or soil (Alvarez et al., 2010).

Ralstonia typically colonizes the root xylem tissues and infect the roots of both susceptible and resistant plants through small wounds following quick move to stem tissues. The complex water-transporting tissue, xylem is made up of a variety of cell types, both dead (tracheids) and living (parenchyma). Exopolysaccharide, a substance produced by *Ralstonia*, causes blockage in xylem that lead to the appearance of wilting in plants (Ingel et al., 2021). Bacteria move from plant roots to soil when plants wilt. In various infection states, variety of virulence factors are triggered by *Ralstonia* to promote disease (de Pedro-Jové et al., 2021). These comprise exopolysaccharide, genes that aid in movement and swimming, T-3 secreted effector, and enzymes secretions like DNAases, cell wall-degrading enzymes, and enzymes that detoxify reactive oxygen species. The diversity of the species complex, in which individual strains differ not only in host range but also in traits related to virulence and physiology, is likely the cause of pathogen's wide host range.

Around the world, the BW pathogen causes damages about 20-60%. The bacterium severely affects the tomato crop in Uganda, resulting in 88% yield losses, and BW in pepper crop was prevalent in Ethiopia at about 100% disease incidence. BW in potato is the second-most significant disease after late blight by *Phytophthora infestans*, and losses can range from 45 to 80% (Felix et al., 2010). *R. solanacearum* is reported to affect potatoes in 1.6 MH fields in 78 countries, causing an annual loss of $848 million (Charkowski et al., 2020).

Control measures for managing this pathogen comprises several diverse approaches. Regardless of whether several control methods are developed to manage the BW disease, efficient management strategies with eco-friendly effects and the desired level of effective control is still awaited and there is need to developed effective management methods to eliminate this fetal disease in several crops under field conditions. An analysis of development in the management strategies will provide an effective way to search and develop control methods with desirable level of effectiveness. Here, we discussed and analyzed the information reported on the development of various management strategies for the management of *R. solanacearum* along with the comprehensive presentation on action mechanism of these management strategies (Figure 1). We have also made an effort to summarize the challenges that make hurdle in the effective management of this deadly pathogen.

2 Biological approaches

Biological approaches are those which employ natural agents, enemies, agents, or bio-based (animal, plant or microbial) products to control the pests (Figure 2). The ultimate aim of biological approaches is to reduce or eliminate the use of pesticides while controlling plant pests. Biological approaches are selected on the type of host, environmental conditions and target pest and its life cycle pattern. For the control of *R. solanacearum*, two general types of biological approaches i.e. the use of biocontrol agents and animal/plant base organic products were reported. Below is the detail description of these two approaches.

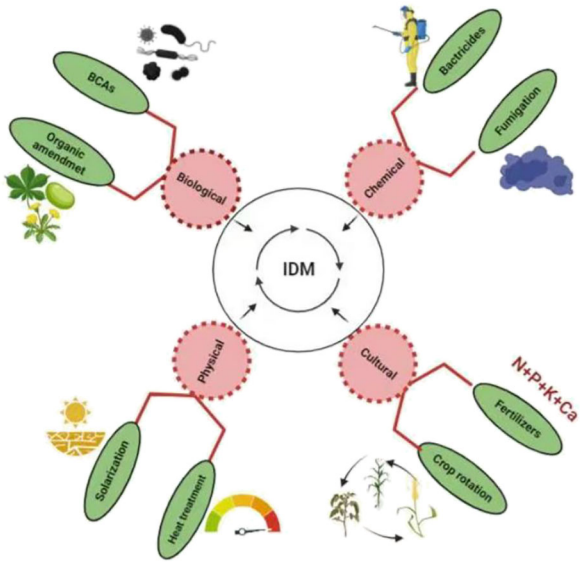

FIGURE 1
Different management strategies used for the management of *R. solanacearum*.

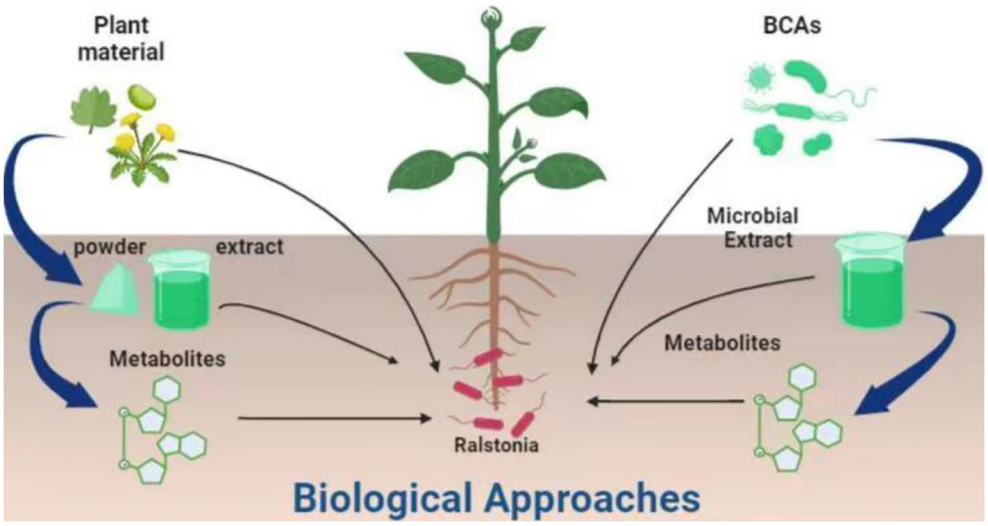

FIGURE 2
Biological approaches used for the management of *R. solanacearum*. IDM, Integrated Disease Management.

2.1 Biocontrol agents

Several beneficial characteristics that biological control agents possess have led to an increase in their use as opposed to chemical control approaches. The long-term disease suppression effect, reduction in input cost of nonrenewable resources and the self-sustaining ability are the key such features (Whipps, 2001; Wang et al., 2021). Due to the lack of effective biocontrol agents, the use of biocontrol microbes to manage bacterial wilt is less common. However, numerous reports have examined the antibacterial potential of various microbes, including bacteria, fungi, and bacteriophages, against *R. solanacearum*. Recently reported biocontrol agents and their action mechanisms are presented in Table 1.

2.1.1 Bacteria

According to earlier studies, bacteria predominated BCAs (90%), followed by fungi (10%). Researchers have looked into the potential efficacy of several potential bacterial BCAs. The endophytic bacterial isolate of *Enterobacter cloacae* from potato plant showed 26.5% bacterial wilt disease suppression. *Paenibacillus polymyxa*, a biocontrol bacterium in potato soil, caused 80% reduction in bacterial wilt. Several researchers have reported that endophytic and rhizobacteria belong to *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Serratia* spp. are potent BCAs against BW. A significant reduction in chilli bacterial (86%) wilt was achieved by the application of seed endospheric bacteria *Bacillus subtilis* of chili variety Firingi (Dowarah et al., 2021). In another study, two *Pseudomonas* species *P. aeruginosa* and *P. syringae* managed bacterial wilt in tomato through host resistance and antibiosis (Mohammed et al., 2020). Two endophytic isolates *Bacillus velezensis* and *Staphylococcus warneri*, among 40 that were obtained from *Gnetum gnemon* plant exhibited antibiosis activity against *R. solanacearum* and promote plant growth in tomato plants under BW stress (Agarwal et al., 2020). Yang et al. (2012) obtained a large number (420) of bacterial strains and 19 strains exhibited biocontrol effect in ginger crop. *Bacillus cereus* AR156, a forest rhizospheric soil showed 62.2% biocontrol effect against tomato BW (Wang et al., 2019). In tobacco plants, bacterial wilt was managed through the application of antagonistic *Bacillus amyloliquefaciens* strain ZM9 obtained rhizosphere of tobacco plant. Field applications of biocontrol bacteria in some cases also gave encouraging results regarding the suppression of bacterial wilt disease. Two strains and *Pseudomonas* sp. Y8 and *B. amyloliquefaciens* Y4 caused 3-4-fold reduction in tobacco bacterial wilt disease in field application. *B. amyloliquefaciens* FZB42 and *Bacillus artrophaeus* LSSC22 produced volatile compounds (VCs) against *R. solanacearum* (Tahir et al., 2017).

Screening of 109 bacterial isolates for their antibacterial effect against *R. solanacearum* showed that 18 active strains were belong to *Pseudomonas* spp. and 2 were belong to *Bacillus* sp. Kurabachew and Wydra (2013) obtained 13 active isolates among 150 rhizobacterial isolates that were belong to *Bacillus* spp., *Serratia marcescens*, and *Pseudomonas* spp. Studies related to *B. amyloliquefaciens* utilization as biocontrol agent against *R. solanacearum* are becoming more common increasing (Chen et al., 2020; Ding et al., 2013). The antibacterial potential of actinobacteria against *R. solanacearum* is also evident from various studies. *Actinobacteria* were reported to have several action mechanisms including induction of host resistance, production of extracellular degrading enzymes and siderophore (Di Francesco et al., 2016). Five endophytic bacteria isolated from ginger rhizomes i.e. *Acinetobacter calcoaceticus*, *P. aeruginosa*, *Enterobacter* sp., *Stenotrophomonas maltophila*, and *Klebsiella* sp. showed significant *in planta* inhibitory activity against bacterial wilt disease.

A recent development in the biocontrol management of plant diseases is the use of effective apoplastic microbes of plants. Using centrifugation and vacuum infiltration, isolated 87 isolates from ginger apoplastic fluid and tested their biocontrol effect against *R. pseudosolanacearum*. The active isolates were belonging to Enterobacteriaceae, Bacillaceae, Staphylococcaceae, and Pseudomonadaceae families. Through soil drenching and seed

TABLE 1 Recently reported biocontrol agents and their action mechanisms against *R. solanacearum* and suppression of bacterial wilt disease in various plants.

BCA	Strain No.	Host	Action mechanism	Reference
B. velezensis	FJAT-46737	*In vitro*	Lipopeptides production	Chen et al., 2020
P. aeruginosa,	—	Tomato	Lipase, protease and α-amylase enzymes	Mohammed et al., 2020
P. syringae	—	Tomato	Lipase, protease and α-amylase enzymes production	Mohammed et al., 2020
P. fluorescens	—	Tomato	Lipase, protease and α-amylase enzymes production	Mohammed et al., 2020
Bacillus sp.	G1S3 G4L1	Tomato	Induction of salicylic acid-, jasmonic acid-, and ethylene-dependent defenses	Fu et al., 2020
Bacillus methylotrophicus	DR-08	Tomato	Difficidin and oxydifficidin production	Im et al., 2020
Paenibacillus polymyxa	IMA5	Eggplant	Lipopeptide production	Abd Alamer et al., 2020
Trichoderma harzianum	—	Tomato	Metabolites	Im et al., 2020
Black Soldier Fly Pupal	—	Tomato	Chitin and Chitosan production	Kemboi et al., 2022
Bacteriophages	—	Tomato	—	Thapa Magar et al., 2022
Bacillus velezensis	YYC	Tomato	Host resistance	Yan et al., 2022
Myxococcus xanthus	R31	Tomato	secreted proteins	Dong et al., 2022
Bradyrhizobium japonicum	—	Tomato	Abscisic acid-production	Chattopadhyay et al., 2022
Streptomyces	—	Solanum melongena	Induces host resistance	Kaari et al., 2022
B. amyoliquefaciens	ZM9	Tobacco	Regulate soil physicochemical properties, promoting beneficial bacteria and antagonistic bacteria of rhizopshere soil	Hu et al., 2021

priming application methods, *Bacillus licheniformis* was found to have a maximum effective strain that reduced 67% disease reduction. Evaluation *in planta* during pathogenic inoculation also revealed a 71% disease decrease. Following a field test that involved solarization of soil and *B. licheniformis* application, the population of *R. pseudosolanacearum* was significantly reduced, dropping from 10^8 to 10^3. The disease was completely suppressed after additional field testing in farmer plots in endemic bacterial wilt regions. This integrated approach, which combines solarization technique and application of *B. licheniformis*, is a successful method of controlling ginger bacterial wilt. The bacterium is currently being advertised as a potential treatment for ginger's bacterial wilt, and the product is called "Bacillich" (Suseela Bhai et al., 2019).

2.1.2 Fungi

Aside from bacteria, some fungal strains have been reported to have a biocontrol effect on *R. solanacearum*. On the basis of findings from *in vitro* and greenhouse testing, it was reported that *Trichoderma viridae* has antibacterial potential against *R. pseudosolanacearum*. Following *Glomus versiforme* inoculation, *R. solanacearum*population on root surface as well as in the rhizosphere and xylem of tomato plants significantly reduced. Both *R. solanacearum* and *Glomus versiforme* colonization of plants caused higher concentration of roots phenols, that may have resulted in the induction of host resistance. In another study, the fungus, *Pythium oligandrum* was shown to have the biocontrol potential to suppress bacterial wilt disease and the induction of host resistance to *R. solanacearum*was attributed to cell wall proteins along with the regulation of signaling pathway for ethylene (Hase et al., 2006). *In vitro* growth of *R. solanacearum* was found to be inhibited by secondary metabolites from Trichoderma spp. (Khan et al., 2020a). Moreover, a lichen fungus *Parmotrema tinctorum* (Gomes et al., 2003) and three other fungi *Scutellospora* sp., *G. margarita*, and *G. mosseae* have been recognized as potent biocontrol fungi against bacterial wilt disease (Tahat et al., 2012).

2.1.3 Bacteriophages

Bacteriophages have been reported for their biocontrol effect against BW. The biocontrol potential of bacteriophages was

evaluated for the management of tobacco bacterial wilt. The host range was limited. Ramírez et al. (2020) reported the control of *R. solanacearum* in banana through lytic bacteriophages. These phages were found banana cultivated soil. Recently, a waterborne phage was succefully used to control *R. solanacearum* in irrigation water (Álvarez et al., 2019). Researchers are now investigating molecular mechanism used by phages for the biocontrol of *R. solanacearum*. Biosca et al. (2021) conducted genomic analysis of the phage having depolymerase activity. These genomic data will contribute to a better understanding of the abilities of these phages to damage host cells and, consequently, to an improvement in the biological control of *R. solanacearum*. Yamada et al., 2007 isolated several different phages and characterize them on the basis of infection they caused to specific biovar or strain of the *R. solanacearum*. Rhizosphere bacteriophages that infect *R. pseudosolanacearum* collected from ginger soil were tested for their host specificity and discovered that the isolated bacteriophage was active against the pathogen of same geographical area from which it was isolated (Yamada et al., 2007).

2.2 Organic approaches

Organic matter is made up of recently living organisms and decomposes or results from decomposition. It is divided into simple organic carbons or of animal and plant origin. Crop productivity has been positively impacted by the use of organic matter to manage *R. solanacearum* by improving the soil physical, chemical, and biological characteristics, which favorably affects growth of plant (Bailey and Lazarovits, 2003; Ahmad et al., 2022). Different OM, including animal waste (10%), plant residue (80%), and simple OM (10%), were reported to have suppression effect against bacterial wilt. Information about the suppression of bacterial wilt disease by plant residues are summarized in Table 2. Several studies showed that organic matter could effectively combat bacterial wilt in field and greenhouses. For instance, in a greenhouse evaluation, fresh parts of crotalaria and pigeon pea were used; 45 days later, they caused 100% suppression in bacterial wilt in tomato (Cardoso et al., 2006); However, because of its high application dose, this OM is not practical in field.

Plant base products in various forms such as green manure, dried powder and plant base oil were effectively reported to control *R. solanacearum* in infested soil. Considerable suppression in soil pathogen count (race 4) was reported by the application of essential oils from palmarosa and lemongrass. But given the high cost involved, using these oils in the field is not practical. Therefore, it is necessary to conduct field research before burying fresh crop manure (Nelson, 2013). Pathogen count have been found to be decreased and plant growth have been improved by the application of a number of medicinal plants. With the help of green manure made from various *Cajanus cajan* and *Crotalaria juncea* parts, tomato bacterial wilt was successfully controlled (Cardoso et al., 2006). Din et al., 2016 suggested that soil application with dried powder from *Adhatoda vasica, Tagetes patula*, and *Calotropis procera*, could be used as an effective management measure against bacterial wilt of tomato.

Management of plant diseases by using animal wastes is evident from many studies and some of them also suggested the suppression of bacterial wilt disease by this method. Pig slurry, for example, suppressed the pathogen count in soil. Poultry and farmyard manure in another study suppressed bacterial wilt by increasing microbial activity and increasing the population of cultural fungi and bacteria (Islam and Toyota, 2004). Pathogen's poor survival was also linked to the reduction in disease index. Although, organic base management approaches gave encouraging results, however, there are drawbacks to their widespread use. Major determinants of organic matter's efficacy in the suppression of plant pathogens are application rate, amendment type, decomposition stage and host-pathogen interaction (Janvier et al., 2007).

In pot experiments, the effects of simple organic substances, such as sugars on tomato BW were assessed. Lysine was added to soil and a pumice culture medium to reduce bacterial wilt in tomatoes by 70-90%, and 62-90%, respectively. Riboflavin, on the other hand, caused a number of reactions related to plant immunity and metabolic reactions in pathogen reduction, defending host plant. In tomato plants, aminobutyric acid increased polyphenol oxidase activity while decreasing catalase activity, suggesting the induction of bacterial wilt resistance. A different investigation revealed that methyl gallate had potent bactericidal effects on *R. solanacearum* (Fan et al., 2014).

3 Breeding and genetic engineering approaches

The most efficient, economical, and pollution free approach of pathogen management is to cultivate the highly resistant cultivars against bacterial wilt (Yuliar et al., 2015). Several important crop plants including vegetables and field crops are subjected to breeding for resistance against bacterial wilt disease. Factors that frequently had an impact on this management strategy include the diversity and availability sources for resistance, the genetic relationships between agronomic and resistance traits, host pathogen interaction mechanism (Elphinstone et al., 2005). By electrically fusing mesophyll protoplasts, somatic hybrids of *Solanum melongena* and two varieties of *Solanum aethiopicum* were created, and it was discovered that they were resistant to *R. solanacearum*. By introducing genotype CF6 of potato to phureja, bacterial wilt severity has been reduced by 90–100%. Resistant plants even severely invaded by *R. solanacearum* exhibited no wilt symptoms. It is reported limited pathogen movement from the protoxylem to other xylem tissues prevented bacterial multiplication in the stems of resistant tomato plants. The correlation between yield and quality of many crops and resistance to bacterial wilt has typically been negative. Due to other agronomic traits and the potential for poor release, resistant cultivars may not be well-received by farmers or consumers. In the future, it is anticipated that greater efforts will be made to genetically enhance bacterial wilt resistance using biotechnology approaches in order to increase crop yield.

Tomato variety with the *NPR1* gene from Arabidopsis showed significantly reduced bacterial wilt. (Lin et al., 2004). By inducing systemic acquired resistance and induced systemic resistance, the

TABLE 2 Plants tested for their antibacterial potential against *R. solanacearum* and management of BW disease in different hosts.

Scientific name	Common name	Family	Plant part used	Host	Reference
Psidium guajava	Lemon guava	Myrtaceae	Leaves	*In vitro*	Acharya and Srivasta, 2009
Piper guineense	Benin pepper	Piperaceae	Leaves	*In vitro*	Acharya and Srivastava, 2009
Tagetes patula	French marigold	Asteraceae	Leaves	*In vitro*	Din et al., 2016
Calotropis procera	Giant milkweed	Apocynaceae	Leaves	*In vitro*	Din et al., 2016
Adhatoda vasica	Malabar nut	Acanthaceae	—	*In vitro*	Din et al., 2016
Xanthium strumarium	Rough cocklebur	Asteraceae	Shoot	Tomato	Khan et al., 2020b
Allium sativum	Garlic	Amaryllidaceae	—	Tomato	Abo-Elyousr and Asran, 2009
Datura	Datura	Solanaceae	Leaves	*In vitro*	Abo-Elyousr and Asran, 2009
Nerium oleander	Oleander	Apocynaceae	Shoots	*In vitro*	Abo-Elyousr and Asran, 2009
Amanita phalloides	Mushroom	Amanitaceae	Shoots	Potato	Erjavec et al., 2016
Clitocybe geotropa	Trooping funnel	Tricholomataceae	Seeds	Potato	Erjavec et al., 2016
Hibsicus sabdariffa	Roselle	Malvaceae	—	Potato	Hassan et al., 2009
Punica granatum	Pomegranate	Lythraceae	Leaves	Potato	Hassan et al., 2009
Eucalyptus globulus	Blue gum	Myrtaceae	—	Potato	Hassan et al., 2009
Syringa oblata	Broadleaf lilac	Oleaceae	Stem	Tobacco	Bai et al., 2016
Punica granatum	Pomegranate	Lythraceae	—	Potato	Farag et al., 2015
Acacia	Wattles	Fabaceae	—	Potato	Farag et al., 2015
Eichhorina crassipes	Water hyacinth	Pontederiaceae	Leaves	Tomato	Derib et al., 2013
Mimosa diplotricha	Nila grass	Fabaceae	—	*In vitro*	Derib et al., 2013
Lantana camara	Lantana	Verbenaceae	—	*In vitro*	Derib et al., 2013
Prosopis juliflora	Mesquite	Fabaceae	Leaves	*In vitro*	Derib et al., 2013
Burcea antidysenterica	Burcea	Burcea	Leaves	Hot pepper	Yihune and Yemata, 2019
Eucalyptus citriodora	Lemon gum	Myrtaceae	Leaves	Hot pepper	Yihune and Yemata, 2019
Justicia schimperiana	Agewgna	Acanthaceae	Leaves	Hot pepper	Yihune and Yemata, 2019
Lantana camara	Lantana	Verbenaceae	Leaves	Hot pepper	Yihune and Yemata, 2019
Melia azedarach	Chinaberry	Meliaceae	Leaves	Hot pepper	Yihune and Yemata, 2019
Ricinus communis	Castor bean	Euphorbiaceae	Leaves	Hot pepper	Yihune and Yemata, 2019
Curcuma amada	Mango ginger	Zingiberaceae	Rhizome	*In vitro*	Karthika et al., 2017
Ocimum gratissimum	Clove basil	Lamiaceae	—	Tomato	Kumar et al., 2017
Tylophora asthmatica	Antamool	Apocynaceae	Leaves	Tomato	Kumar et al., 2017
Calotropis gigantea	Crown flower	Apocynaceae	—	Tomato	Kumar et al., 2017
Ocimum sanctum	Holy basil	Lamiaceae	Leaves	Tomato	Kumar et al., 2017
Tylophora asthmatica	Indian ipecacuan	Asclepiadaceae	Leaves	Tomato	Kumar et al., 2017
Nigella sativa	Black cumin	Ranunculaceae	Shoot	Tomato	Kumar et al., 2017
Ruta graveolens	Rue	Rutaceae	—	Tomato	Kumar et al., 2017

NPR1 gene plays a crucial part in the reaction of host to pathogen infection. It also serves as the main key in the facilitation of cross-talk between jasmonic acid and salicylic acid responses. *NPR1* expression in *Arabidopsis thaliana* ensures a quick reaction to salicylic acid (SA) (Cao et al., 1998). *R. solanacearum*-infected resistant plants showed vascular tissues' tolerance to the BW. Even though cultivar resistance has demonstrated excellent qualities in managing bacterial wilt, acceptance from public is required before such genetically modified crops can be used commercially. Additionally, the quality and quantity of the crops

have mostly been inversely correlated with the reduction of BW in many cases (Yuliar et al., 2015).

Although breeding for resistance is a key disease management tactic, some crops that are severely affected by bacterial wilt disease still lack resistant varieties. For example, none of the ginger cultivars that have been made available are resistant to BW. Since ginger is a crop that is vegetatively propagated, this may be because there is little genetic variation among the accessions (Prasath et al., 2011). Among several varieties that were tested for their response to BW infection, and it was discovered *Curcuma amada* was highly resistant. By using gamma rays to induce mutations in ginger, Prasath et al. (2011) were able to create mutants that were resistant to bacterial wilt. These mutants are currently being tested in the field.

4 Physical approaches

Variety of physical control methods were reported for have good control effect against bacterial wilt disease. These techniques include soil disinfection, hot water treatment, and soil solarization (Yuliar et al., 2015). This section comprises the detail discussion of a number of physical methods in which *R. solanacearum* has been successfully controlled.

The selection of planting and propagating material free from infection must be given the utmost importance in order to stop field outbreaks of BW disease (Kumar and Hayward, 2005). Prior to planting, 30 days' soil solarization was recommended to lower the soil pathogen inoculum resulting in the reduction of disease severity and enhancement in yield and seed germination (Kumar and Hayward, 2005). As a hydrothermal process, soil solarization eliminates the majority of harmful organisms, including the weeds seed without leaving any toxic residues behind. Several physical approaches were reported for soil solarization. Trapping sunlight for increasing soil temperature is one of the useful techniques for soil solarization (Kumar and Hayward, 2005). It is suggested the covering of soil with a plastic cover during protracted periods of high temperature. This aids in capturing the sun's energy to warm the soil which suppress the pathogen population in soil. It is reported that exposing the soil to sunlight suppressed tomato BW significantly. Another study found that sterilizing ginger seeds with microwaving at 42°C completely controlled the BW disease. Before tomato plants were planted, the infected soil was subjected to heat treatment at 52°C for 3 days which resulted in the reduction of bacterial population by 50-86%. Seed pathogens can also be killed using heat treatment. After being exposed to hot air for 28 minutes, ginger seeds that are already infected by the pathogen, produced rhizomes free from infection, and the procedure had no negative effects on growth and sprouting (Kumar and Hayward, 2005).

Cold temperature treatments can occasionally be as effective as those using heat. Due to the low temperature in Queensland, *R. solanacearum* rarely affects tobacco plants in April. However, the disease did arise during high temperature in September, especially in previous occurrence of the disease and the absence of crop rotation. Incubation at low temperature and moisture decreased disease severity and adversely affected *R. solanacearum* population (Islam and Toyota, 2004).

Attention has recently been drawn to the biofumigation which uses plant VC emitted by plant residues to manage plant diseases. The process of biofumigation is known as soil bio-disinfection, and the release of antimicrobial metal ions as well as organic acids cause pathogen suppression. A variety of phytopathogens in soil including *R. solanacearum*, were successfully eradicated by biofumigation using molasses or wheat bran (Nion and Toyota, 2015).

5 Cultural approaches

Cultural approaches include agricultural practices that increase crop yield and quality and lessen the impact of pathogens (Ajilogba and Babalola, 2013). Crop rotation is a low-cost strategy for controlling plant pathogens that entails growing various crops on the same land during different growing seasons (Ajilogba and Babalola, 2013). Similar crops being grown repeatedly may result in the establishment of specific pathogen populations; for instance, planting tomatoes on same location every year will promote the growth of pathogens in the soil. Crop rotation reverses this negative trend and lowers the prevalence of diseases brought on by pathogens in soil (Janvier et al., 2007). It has been demonstrated that growing potatoes in rotation with other crops such as sorghum, carrots, millet increases potato production in comparison to mono-cultured tubers while reducing the bacterial wilt occurrence (Katafiire et al., 2005).

Efficient management of soil plant diseases through crop rotation can be achieved by complete removal of infested soil and replacement with healthy soil. Crop rotation has several advantageous effects such as organic matter and structural management in soil and reducing soil erosion, which is frequently brought on by mono-cropping for long time (Janvier et al., 2007). Crop rotation prevents the establishment of particular plant pathogenic populations, whereas continuous cropping with the same susceptible host plant has the opposite effect and is frequently linked to a decline in plant diseases (Janvier et al., 2007). For instance, when a susceptible tomato variety was grown after corn, lady's fingers, cowpea, or resistant tomato, the development of bacterial wilt was postponed by 15-20 days with 30-40% reduction in overall disease severity (Nion and Toyota, 2015). Two to four times higher potato yield with 50-80% reduction in disease severity was achieved when potato crop was rotated with millet, phaseolus beans, sorghum, and maize comparing to mono-cultivation (Katafiire et al., 2005). In a case study of multi-cropping and revealed that the reduction in the severity of bacterial wilt disease is because of inhibition effect of root exudates of *Allium tuberosum* against *R. solanacearum*.

Researchers reported the reduction of in the occurrence of bacterial wilt through proper fertilizer applications. The most investigated fertilizer for preventing plant diseases is calcium. Plants with more Ca content showed reduced pathogen population and disease severity in tomato stem. In addition, a rise in Ca uptake by tomato shoots was associated with a decline in

pathogen population (Nion and Toyota, 2015). The application of different fertilizers N + P + K decreased disease by 60% resulted in enhanced yield of potato. According to Hacisalihoglu et al. (2007) tomato leaves' distribution of nutrients changed as a result of bacterial wilt infection. The combination of organic fertilizer and rock dust application managed bacterial wilt in tomato.

A number of components in plant cell walls affect how susceptible or resistant they are to pathogen infections, and silicon is regarded as being advantageous for both plants and animals. The use of silicon and chitosan together decreased the frequency of tomato BW by fostering resistance. The use of silicon fertilizer has also been shown to enhance tomato yield while lowering bacterial wilt populations and incidence (Hartmann, 2002). When compared to control soil, soil treated with farmyard manure caused significant increase in yield of tomato and reduction in BW severity. This may be because the physicochemical properties of the organically treated soil have improved, which is advantageous for crop growth.

Grafting is an asexual method of propagating plants that involves attaching pieces of different plants together so that they will eventually fuse and develop into a single plant. Consequently, a grafted plant is a component combination of different plants (Hartmann, 2002). It entails grafting a plant's top portion of a required trait onto a disease resistant rootstock. Several vegetable crops resistant to soil pathogens including *Ralstonia* has been developed through grafting (King et al., 2008; Louws et al., 2010).

6 Chemical approaches

Chemical approaches include the utilization of synthetic chemicals for the control of plant diseases. Over the years, bacterial wilt has been managed using a variety of chemical techniques. However, no method is effective when used alone due to the pathogen's complexity (Yuliar et al., 2015). To prevent bacterial wilt in ginger, methyl bromide use for soil treatment was suggested (Ishii and Aragaki, 1963). Following the methyl bromide ban, chloropicrin's ability to lessen bacterial wilt of ginger in China was assessed. Chloropicrin covered in polyethylene film decreased *R. pseudosolanacearum* soil count and increased plant yield. The most effective rhizome protectant, oxytetracycline at 400 ppm, was reported to increase yield and decrease plant mortality against BW disease. Plant mortality rates of 29.32% and 41.26%, respectively, were found to be effective with streptocycline and oxytetracycline at lower rate. Ginger plant mortality was reported to be reduced by carbendazim and copper oxychloride but not as effective as antibiotics (Suseela Bhai et al., 2019).

These commercial chemical substances have had limited effectiveness in controlling bacterial wilt in the field. In order to manage BW economically a technology that combines soil solarization and soil improvement with CaCl has been developed (Suseela Bhai et al., 2019). The field population of *R. pseudosolanacearum* was significantly reduced in subsequent field testing of CaCl. Additional field trials in the endemic BW regions showed complete control on BW. The study's findings can be used to develop a practical and efficient integrated control measure for the treatment of ginger's bacterial wilt (Suseela Bhai et al., 2019). Although not always, it has been shown that using pesticides to combat BW results in a greater net benefit. Some pesticides may persist in the environment for long time, produce pollution in water and soil, and cause toxicity to consumers due to their ignorance and improper application (Dasgupta et al., 2007). Due to the negative effects on human and environmental health and the emergence of pathogen resistance, the use of chemicals like antibiotics to manage diseases has been criticized.

7 Nano technological approaches

Nanotechnology developed as one of the 21st century's most quickly progressing sciences. Nanotechnology offers a viable solution to the difficulties involved with the identification and control of soil pathogens, such as *R. solanacearum* (Khan et al., 2021; Guo et al., 2022). According to recent research, nanoparticles act as a possible biosensor and antibacterial agent for detecting plant infections, particularly soil-borne pathogens. Several metallic oxide nanoparticles have been investigated for their potential to act as protective agents against *R. solanacearum* (Khairy et al., 2022). NPs of ZnO, FeO, and CuO were recently investigated against the tomato bacterial wilt pathogen Ralstonia solanacearum. The findings demonstrated that NPs, particularly CuONPs, greatly decreased the disease incidence on tomato. There was also a considerable improvement in the morpho-physiological characters of plants (Jiang et al., 2021). ChNPs were found to be effective against tomato BW (Khairy et al., 2022). The maximum zone of inhibition was found in an *in vitro* testing at a dose of 200 g/ml. *In vivo* assays revealed a reduction in disease severity following foliar application of ChNPs to pathogen infected plants. The ChNPs were discovered to interact directly with the cell wall of bacteria, producing shape changes, flagella loss, and lysis. According to Santiago et al. (2019), ChNPs loaded with AgNP demonstrated antibacterial efficacy against *R. solanacearum*.

8 IDM approaches

In integrated disease management (IDM) various management techniques are used to achieve the highest possible level of control. The main objectives of an IDM program, in accordance with Agrios (2005), is the reduction of initial inoculum and its effectiveness, boost host resistance, boost host resistance, and postpone disease development. Because of the wide range of hosts and the complexity of the pathogen, a single management approach could not work well. But when several approaches were used in combination, the effectiveness of control enhanced up to 100% (Wu et al., 2020). To get rid of BW disease, many methods such as cultural practices, soil amendments, use of resistant cultivars and organic formulations were used in combination (Wu et al., 2020). Generally, combine use of 2-3 methods from biological, cultural or chemical approaches can reduce the BW disease by 30-90%. For example, the rate of BW in tomatoes was tracked in soil with *R. solanacearum* after adding a mixture of agricultural and organic industrial wastes along with a

chemical pesticide Actigard. The disease was reduced by 29% when the organic mixture was used, and by 6% when Actigard was used. Adding the organic mixture and Actigard, on the other hand, caused 53% disease reduction. Similarly, using biocontrol agents along with their substrates like sucrose, lysine work better together to stop bacterial wilt in tomatoes. The addition of substrates makes it easier for BCAs to colonize the roots of tomatoes (Nion and Toyota, 2008).

9 Action mechanism of different management approaches

Different action mechanisms were reported for different management approaches used to control bacterial wilt or its pathogen *R. solanacearum*. Some approaches use direct killing or suppression of the pathogen while other approaches support indirect action mechanisms. Breakdown organic matter (OM) may directly affect the soil pathogens by producing substances that stop them from living. This limits the nutrients that are available. It could also make microorganisms work more, making competition effects more likely. Soil amended with organic matter have a direct effect on the production. They are helpful in improving soil's chemical and physical properties, which can help plants grow (Bailey and Lazarovits, 2003; Yi et al., 2021). The soil breakdown of organic matter releasing natural chemicals with different inhibitory properties (Bailey and Lazarovits, 2003). Carbon from OM increases microbial activity in soil and makes it more likely that competition effects will happen in the soil (Bailey and Lazarovits, 2003). It has been shown that adding organic matter to soil makes microorganisms that fight pathogens work more (Akhtar and Malik, 2000). Also, OA often have molecules that are biologically active, which can affect the microbes in the soil. The increasing activities of dehydroascorbate, monodehydroascorbate, peroxidase were recently reported to enhance the host resistance against BW. It is reported that adding microorganisms to natural soil with bio-amendments worked most of the time. The effects on the soil microbial communities were very different depending on the combinations of organisms and their number and type. A new strategy to combat BW is to use a rhizosphere biofilm that only forms in organic hydroponic system (Avinash et al., 2017).

Most researchers reported that the plant residues work by killing microbes and then indirectly stopping the pathogen by making the soil properties better (Cardoso et al., 2006). The 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl were found to be the antimicrobial compounds in *T. patula* that stopped *in vitro* growth of *R. solanacearum*. *Cryptomeria japonica* released ferruginol, and *Cyphomandra betacea* had a protein that inhibit glycosidase activity which stopped *in vitro R. solanacearum* growth. Lansiumamide B, which was found in *Clausena lansium* seeds stopped tobacco bacterial wilt better than the antibiotic. It found that the anti-disease effects of compost made from olive waste seemed to come from a combination of the effects of nutrients competing and antagonistic microbes. Although, it is not clear how pig slurry makes the population of *R. solanacearum* go down faster and stops diseases from spreading, but shifts in the bacterial community profiles have been suggested as a possible explanation.

Another study revealed that the way farmyard manure and poultry stopped BW was linked to the activities of a greater number of fungi and bacteria (Islam and Toyota, 2004). The pathogen suppression was linked to the decreased disease severity in this study. In pot evaluation, organic acids, amino acids, and sugars were tested to see how well they stopped bacterial wilt in tomatoes. When lysine was added to a pumice medium and soil, there was a higher drop in tomato wilt (Nion and Toyota, 2008). This wasn't because of the ISR, but because of changes in the microbial community in soil that made the pathogen die more quickly. Riboflavin, on the other hand, caused a number of defense mechanisms in cell suspensions which made tobacco resistant to R. solanacearum. Aminobutyric acid also made polyphenol activity go up and catalase activity go down in tomato plants. This suggests that aminobutyric acid made the tomato plants resistant to bacterial wilt. Methyl gallate was also reported to kill *R. solanacearum* very well (Fan et al., 2014).

Enterobacter cloacae stopped bacterial wilt disease in potatoes and raised yields by using antibiosis and inducing plant systemic resistance. The effective control of BW by *P. aeruginosa* and *P. syringae* was also attributed to mechanism of antibiosis (Mohammed et al., 2020). In another study, the screening of a large number of endophytic isolates showed the antibiosis activity of *S. warneri* and *B. velezensis* against *R. solanacearum* (Agarwal et al., 2020). Through a process called niche exclusion, *B. amyloliquefaciens* manage tobacco BW very well. The combine effect of niche exclusion, gene copy number reduction in the rhizosphere soil, and direct antagonism were reported for BW disease suppression of *B. amyloliquefaciens*. Production of antibacterial volatile compounds that act as plant growth inducer as well as have direct killing effect against *R. solanacearum*were also reported. According to research by Tahir et al. (2017), VOCs produced by *B. amyloliquefaciens* and *B. artrophaeus* were found to kill *R. solanacearum* while also promoting plant growth. By enhancing the improvement in soil's properties soil amendment with *B. amyloliquefaciens* and marigold powder together reduced the prevalence of tobacco bacterial wilt disease. The action mechanism responsible for physical approaches mainly includes the use of high or low temperature. The investigation on finding the action mechanism of soil solarization in reducing BW disease revealed that soil solarization caused reduction in K, Na, zinc and overall pH which ultimately affect the survival of *R. solanacearum* (Baptista et al., 2006).

10 Future prospects and challenges

Cultural practices have an impact on soil characteristics, which affect the distribution and viability of pathogens. More studies are being done to determine how these practices affect microbial communities or how effective they are at preventing the spread of pathogens in soil. Risk forecasting and technical guidance may benefit greatly from indicators of soil health.

Preventive measures are important for keeping fields free of soil pathogens. *R. solanacearum* can live in plant materials, water, and soil for extended periods of time. Thus, in order to boost agricultural output by preventing this disease, it is important to disinfect seeds, soil, water.

It is necessary to examine the pathogen's dynamics and genetic diversity in order to understand the influence of geographic and environmental variables on the population structure of *Ralstonia* spp.

The understanding of virulence mechanisms of pathogen, complex regulatory networks, and pathogenicity determinants should be advanced through research and analysis.

Early identification of *R. solanacearum* in soil and water is important for avoiding its spread to new locations. Recently, a sensitive quantitative technique for detecting *Ralstonia solanacearum* in soil based on the most probable number analysis of PCR findings was developed. This technique allows the detection of pathogens at very low concentrations.

Complicated interactions occur between soil-borne pathogens such as *R. solanacearum* and plants, involving both abiotic and biotic and variables. Prior research focused mostly on the biotic variables that regulate BW. Future investigations should also be focused on abiotic factors that help in reducing bacterial population.

Researchers have treated bacterial wilt a variety of management strategies; however, few studies, particularly economic assessments, have assessed the efficacy of these methods to increase crop output.

To be able to forecast outbreaks of bacterial wilt, it will be necessary to do more research to determine the severity of crop damage caused by *Ralstonia* spp. and to understand the disease's epidemiology.

Author contributions

ZW and SC: Conceptualization, Literature survey, Writing major original draft. WL and HZ: Writing- review and editing. JZ and ZZ: Figure designing, Tabulation, Writing- review and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Disentangling the resistant mechanism of Fusarium wilt TR4 interactions with different cultivars and its elicitor application

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Fusarium wilt of banana, especially Tropical Race 4 (TR4) is a major factor restricting banana production. Developing a resistant cultivar and inducing plant defenses by elicitor application are currently two of the best options to control this disease. Isotianil is a monocarboxylic acid amide that has been used as a fungicide to control rice blast and could potentially induce systemic acquired resistance in plants. To determine the control effect of elicitor isotianil on TR4 in different resistant cultivars, a greenhouse pot experiment was conducted and its results showed that isotianil could significantly alleviate the symptoms of TR4, provide enhanced disease control on the cultivars 'Baxi' and 'Yunjiao No.1' with control effect 50.14% and 56.14%, respectively. We compared the infection processes in 'Baxi' (susceptible cultivars) and 'Yunjiao No.1' (resistant cultivars) two cultivars inoculated with pathogen TR4. The results showed that TR4 hyphae could rapidly penetrate the cortex into the root vascular bundle for colonization, and the colonization capacity in 'Baxi' was significantly higher than that in 'Yunjiao No.1'. The accumulation of a large number of starch grains was observed in corms cells, and further analysis showed that the starch content in 'Yunjiao No. 1' as resistant cultivar was significantly higher than that in 'Baxi' as susceptible cultivar, and isotianil application could significantly increase the starch content in 'Baxi'. Besides, a mass of tyloses were observed in the roots and corms and these tyloses increased after application with isotianil. Furthermore, the total starch and tyloses contents and the control effect in the corms of 'Yunjiao No.1' was higher than that in the 'Baxi'. Moreover, the expression levels of key genes for plant resistance induction and starch synthesis were analyzed, and the results suggested that these genes were significantly upregulated at different time points after the application of isotianil. These results suggest that there are significant differences between cultivars in response to TR4 invasion and plant reactions with respect to starch

accumulation, tyloses formation and the expression of plant resistance induction and starch synthesis related genes. Results also indicate that isotianil application may contribute to disease control by inducing host plant defense against TR4 infection and could be potentially used together with resistant cultivar as integrated approach to manage this destructive disease. Further research under field conditions should be included in the next phases of study.

KEYWORDS

cultivar, Fusarium wilt of banana (FWB), Tropical Race 4 (TR4), induced resistance, elicitor, starch granule, isotianil

1 Introduction

Bananas, the most traded tropical and subtropical fruit (Li et al., 2019; Zou and Fan, 2022), are also fourth staple crop after wheat, corn and rice (Nayar, 2010), providing food source for approximately 400 million population worldwide (Dusunceli, 2017). However, banana industry is seriously threatened by Fusarium wilt of banana (FWB) which is a soil-borne vascular bundle disease caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) (Ploetz, 2006a; Ploetz, 2015; Dita et al., 2018). On the basis of difference in pathogenicity of *Foc*, it can be divided into 4 physiological races (*Foc* 1, *Foc* 2, *Foc* 3 and *Foc* 4) and *Foc* 4 can be divided into subtropical race 4 (STR4) and tropical race 4 (TR4) (Ploetz, 2006b; Karangwa et al., 2018). In the 1950s', the FWB caused by Foc1 was successful controlled by replaced the 'Gros Michel' (disease-susceptible cultivar)with the 'Cavendish' (disease-resistant cultivar) (Ploetz, 2006b). In the 1990s, the banana industry was again in crisis with the advent of TR4 (Ploetz, 2006b). In the past decades, TR4 gradually has spread to the surrounding countries such as the Philippines and Malaysia (Hwang and Ko, 2004; Ploetz, 2006b). Then it expanded to countries and regions such as Australia, the Middle East, India and Africa (Thangavelu and Mustaffa, 2010; Butler, 2013; Ploetz et al., 2015). In recent years, it has been found in Jordan (García-Bastidas et al., 2014), Lebanon (Ordoñez et al., 2016), Israel (Maymon et al., 2018), Mozambique (García-Bastidas et al., 2014), Pakistan (Ordoñez et al., 2016), Puerto Rico (Garcia et al., 2018), Miyako Island in Okinawa, Japan (Nitani et al., 2018), India (Thangavelu et al., 2019), Mayotte (Aguayo et al., 2020), Colombia (Bastidas et al., 2020) and Peru (Acuña et al., 2021). As TR4 continues to spread rapidly around the world (Dita et al., 2018; Zheng et al., 2018; Pegg et al., 2019), it is essential to take actions to stop its further spread and to have comprehensive management approaches. Nowadays, although historical experience has shown that disease-resistance breeding is a particularly effective way to control FWB (Li et al., 2015; Bubici1 et al., 2019; Zorrilla-Fontanesi et al., 2020), no completely immune TR4 cultivar has been incorporated into agricultural production, because of the major challenge faced to breed disease-resistant cultivars in traditional ways due to the peculiarities of triploids of banana plants.

In the natural environment, in order to prevent pathogenic infection, plants not only form a physical barrier on the surface, but also have various internal immune responses. Plants are able to induce broad defense reactions by pathogens in their surroundings (Choudhary et al., 2007). So, activating its inherent defense by specific elicitors would be an effective way to protect plants from disease (Ward et al., 1991; Pieterse et al., 1998b). Therefore, most researchers prefer the plant-induced resistance as a new type of plant disease control strategy (Eschen-Lippold et al., 2010; Kurth et al., 2014; Dorneles et al., 2018; Sopeña-Torres et al., 2018), which may also become a new sustainable plant protection approach in the future (Roberts and Taylor, 2016).Today, many bacterial, fungal and chemical inducers that induce plant defenses to control crop disease have been commercialized (Verhagen et al., 2004; Takahashi et al., 2006). However, so far there is no study on exogenous inducers on FWB. Whether these exogenous elicitors can induce bananas to acquire systemic resistance to FWB is still unknown.

According to the molecular mechanism of induction, induced resistance is divided into systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse et al., 2009). SAR, which depends on salicylic (SA) and its associated systemic immune responses have been confirmed in some plants (Pieterse et al., 2002; Fu and Dong, 2013; Bektas and Eulgem, 2014), such as, enhances the expression of pathogenesis-related (*PR*) genes (Loon et al., 2006). *PR* proteins, with antibacterial activity outside the cell, can directly act on pathogen (Loon et al., 2006). *NPR1*, a key gen regulator for transducing the SA signaling and activating PR gene expression in the pathway (Dong, 2004; Grant and Lamb, 2006), and both exogenous SA application and pathogen infection may lead to enhanced expression of the NPR1 gene of the SAR pathway in plants (Cao et al., 1997; Ryals et al., 1997). In contrast to SAR, ISR relies primarily on jasmonic acid (JA) and ethylene (ET) pathways (Loon et al., 1998; Pieterse et al., 1998a; Pieterse et al., 2002; Choudhary et al., 2007; Pieterse et al., 2012; Pieterse et al., 2014). Although SAR and ISR are significantly different, studies have shown that ISR also requires NPR1 (Pieterse et al., 2014; Nie et al., 2017). ET is synthesized from the amino acid methionine by a pathway requiring *SAMS* (S-adenosylmethionine synthetase), *ACS* [1-aminocyclopropane-1-carboxylic acid (ACC) synthase] and *ACO* (ACC oxidase), and ACS is a key synthetase gen in this pathway (Sauter et al., 2013; Wang et al., 2013; Dubois et al., 2018).

In general, when ISR is activated, the expression of genes involved in ET biosynthesis (e.g., *ACS*) and signaling (e.g., *ERF1*, ethylene response factor 1) is usually upregulated (Shoresh et al., 2005; Ribaudo et al., 2006; Poupin et al., 2016). The basic helix-loop-helix (*bHLH*) transcription factor (TF) *MYC2* as a major regulator in the JA pathway, coordinates plant resistance to pathogens through JA-mediated defense responses (Lorenzo et al., 2004; Kazan and Manners, 2013; Du et al., 2017; Liu et al., 2019). In addition, both ET and JA pathways activation synergistically induce plant response to pathogens by ERF1 transcription factor (Zhou et al., 2022).

Starch is a decisive factor for plants to adapt to abiotic stress (Thalmann and Santelia, 2017), and often shows very obvious plasticity when different plant tissues face stresses (Cuellar-Ortiz et al., 2008; Yin et al., 2009; Morais et al., 2019). Bananas plants with high starch content in corm are more resistant to FWB than those with low starch content (Dong et al., 2019). It is well known that ADP-glucose pyrophosphorylase (*AGPase*), Starch branching enzyme (*SBE*) and granule-binding starch synthase (*GBSS*) are key enzymes in the starch biosynthesis pathway, and *AGPase* plays an important role in crop heat tolerance (Saripalli and Gupta, 2015).The activity of *GBSS* within granules is the main determinant of amylose content (Seung, 2020). *SBE* is a key enzyme in pullulan synthesis (Li and Gilbert, 2016).

Isotianil is one such elicitors which acts as a salicylic acid (SA) mimic, with proven activity against rice blast (Bektas and Eulgem, 2014) and wheat blast (Portz et al., 2020). It was discovered by Bayer in 1997 (Toquin et al., 2012). Although isotianil does not have any direct antimicrobial activity against bacteria and fungi, it can induce the defense response of various plants to pathogens. For example, isotianil treatment can induce the expression of some defense-related genes, such as *NPR1* and *PR1* in the SA signaling pathway (Yoshida and Toda, 2013; Bektas and Eulgem, 2014). There is only one paten report on the application of isotianil in FWB before (Gilbert et al., 2019), and the specific mechanism of isotianil inducing plant resistance is also unclear yet. Therefore, this study aims to explore whether isotianil could induce plant resistance and alleviate the infection of FWB in different two cultivars. Besides, the interaction between TR4 and bananas have been explored by confocal laser-scanning microscope (CLSM) and molecular approaches have been used to analyze the mechanism of action mode of isotianil on banana plants (Figure 1).

2 Materials and methods

2.1 Plant materials

In this study, two cultivars of Cavendish were used, 'Baxi' (*Musa* spp. AAA, Susceptibility cultivar) and 'Yunjiao No.1' (*Musa* spp. AAA, moderately resistant cultivar), and the banana plantlets were propagated by plant tissue culture. The tissue plantlets were cultured at 25°C, under a 16 h/18 h (light/dark) photoperiod until new roots grew and then transplanted into an aperture disk (32 tray specification, capacity 110 mL), filled with coconut bran and seedling substrate. Each banana plantlets about 15 cm high with 5 leaves were transplanted into the plastic pot with 25 cm in the diameter containing garden soil and substrate. All banana plants were grown in a solar greenhouse with isotianil watering and fertilization management.

2.2 Isotianil application and pathogen inoculation

TR4 labeled with green fluorescent protein (GFP) was used to explore the infestation process of pathogens in plants (Zhang et al., 2018a). After TR4 was grown on PDA medium at 28 °C for 7 day, the spores were collected by rinsing the plates with sterile water, and the concentration of suspension was 1×10^7 spores/mL measured by hemocytometer. Isotianil as the main compound of Routine® product was provided by Bayer AG, Crop Science Division company. Routine® is a suspension concentrate (SC) containing 0.2 g/mL isotianil and was applied when the banana plants had 6-7 leaves. The applied diluent was prepared by dissolving 0.035 mL of the original product in 100 mL of tap water per banana plant, applied by either drenching the roots or spraying the leaves (0.07 mg/mL isotianil, 100 mL/plant). The applications were performed once every 28 days, and the applications were performed three times in total. Seven days after the second application of Routine®, 100 mL of 1×10^7 spores/mL of TR4 was drenched for root inoculation, and the control treatment was applied with tap water. Before TR4 inoculation, the roots were treated with two wounds around plants, using a shovel. Banana roots and corms were taken 0 d, 1 d, 7 d, 14 d, and 62 d after TR4 inoculation as samples for subsequent TR4 content detection, microscopic observation and gene expression determination.

2.3 Experimental treatment design

Four treatments were set of each banana cultivar which contain: control (CK), inoculated with pathogen alone (TR4), banana leaves sprayed Routine® and inoculated with TR4 (TR4+R1) and Banana root drenched with Routine® and inoculated with TR4 (TR4+R2) (Table 1). Three biological replicates were designed for per treatment and 45 plantlets were prepared for per replicate.

2.4 Disease index investigation

The banana corms were dissected to investigate the disease index after 62 days post inoculated TR4. After plant corms were dissected, the degree of lesions of each plant corm was investigated according to five grades from 0 to 4. The five grades of 0, 1, 2, 3 and 4 represent no lesions of the corm, the area of corm lesions is 1-10%, the area of lesions in the corm is 11-30%, the area of corm lesions is 31-50% and the area of corm lesions is more than 50%, respectively. The formula for calculating the disease index and control effect is as follows (Zuo et al., 2018; Chen et al., 2019; Fan et al., 2021).

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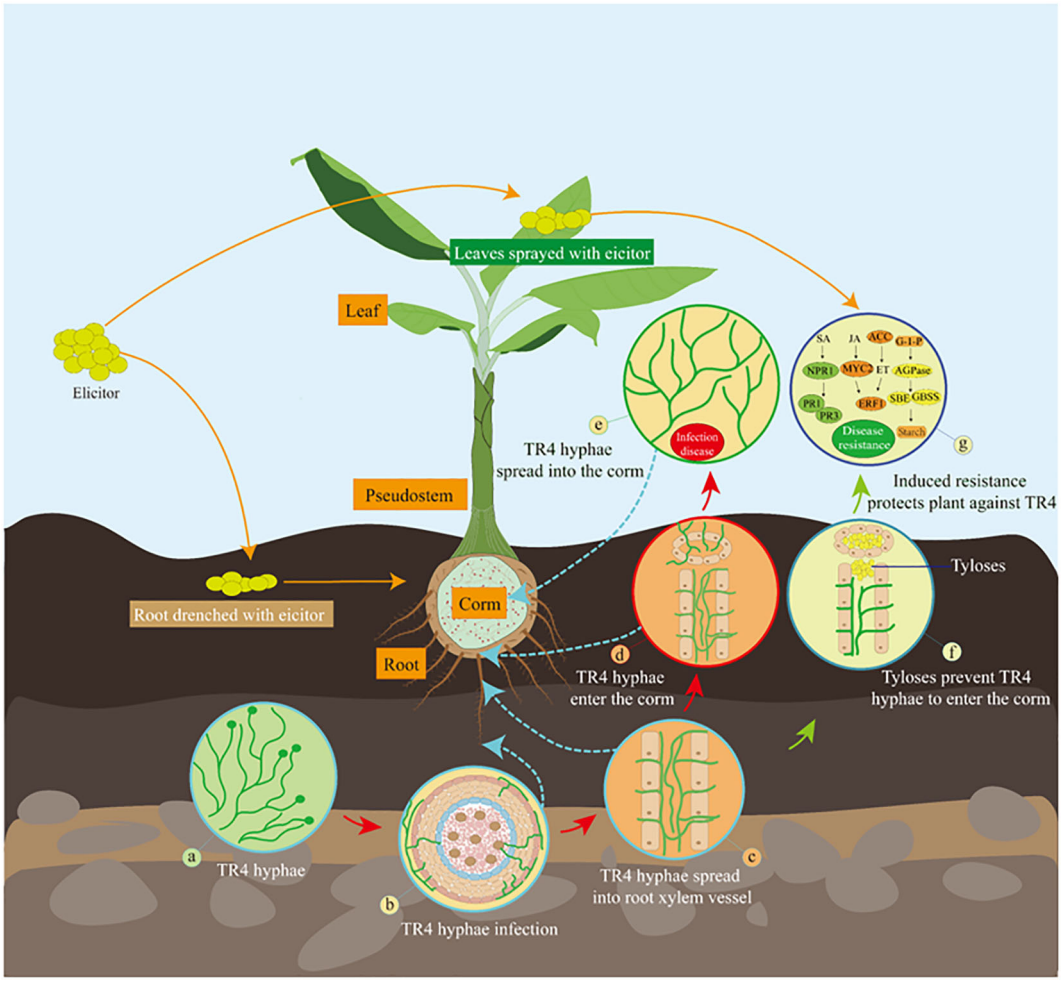

FIGURE 1
Scheme illustrating that isotianil-induced multi-resistance in bananas to prevent TR4 infection. **(A)** TR4 hyphae in the soil; **(B)** TR4 hyphae are accumulated in the rhizosphere and infestation into the plant; **(C)** TR4 hyphae enter plant root xylem vessel and spread further; **(D)** TR4 hyphae spread into the xylem vessel that connects the root to the corm; **(E)** TR4 hyphae spread into the corm and multiplies; **(F)** TR4 hyphae is blocked to enter the corm by elicitor -induced tyloses in the xylem vessel connecting the root to the corm; **(G)** After elicitor applicated plants by drenching roots or spraying leaves, multiple defense systems in the banana plant are activated to prevent further spread of TR4 in the corm. SA, salicylic acid; *NPR1*, nonexpressorofpathogenesis-relatedgenes1; *PR1*, pathogenesis-related 1 genes; *PR3*, pathogenesis-related 3 genes; JA, jasmonic acid; *MYC2*, basic helix-loop-helix transcription factor; *ERF1*, ethylene response factor 1; ET, ethylene; *ACC*, 1-aminocyclopropane-1-carboxylic acid synthase; *AGPase*, ADP-glucose pyrophosphorylase; SBE, Starch branching enzyme; *GBSS*, granule-binding starch synthase.

$$Disease\ \ index\ \ (\%) = \sum \frac{Grade \times Number\ \ of\ \ plants\ \ in\ \ the\ \ grade}{4 \times Total\ \ number\ \ of\ \ assessed\ \ plants} \times 100$$

$$Control\ \ effect\ \ (\%) = \frac{Control\ \ disease\ \ index - Treatment\ \ disease\ \ index}{Control\ \ disease\ \ index} \times 100$$

2.5 Detection of TR4 content in banana roots and corms by qPCR

The roots and corms of bananas plants after inoculation with TR4 were frozen in liquid nitrogen immediately after being collected at 4 different time points, and then stored at -80°C for later use. Genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method (Tamari et al., 2013). Fungal biomass determination is basically genomic quantification of gene copy numbers by qPCR based on our previous established protocol (Zhang et al., 2018b). Three plants were prepared for each treatment as one biological replicate, and each treatment was repeated with three replicates. The quality of the resulting standard curve can be used for data analysis (efficiency, 90% to 110%; Correlation coefficient, R^2>0.99).

2.6 Confocal laser scanning microscope observation

For examined infection process and colonization of TR4, the roots and corms of the banana plants were collected after inoculated

TABLE 1 Different treatments in pot experiment.

Treatment code	Cultivar	Description
CK	Baxi	Control
TR4	Baxi	Inoculated with pathogen TR4 alone
TR4+R1	Baxi	Banana leaves sprayed Routine® and inoculated with TR4
TR4+R2	Baxi	Banana root drenched with Routine® and inoculated with TR4
CK	Yunjiao No.1	Control
TR4	Yunjiao No.1	Inoculated with pathogen TR4 alone
TR4+R1	Yunjiao No.1	Banana leaves sprayed Routine® and inoculated with TR4
TR4+R2	Yunjiao No.1	Banana root drenched with Routine® and inoculated with TR4

with TR4. Three biological replicates were designed for per treatment and 3 plantlets were prepared for per replicate. The samples were washed in sterile water and 75% alcohol, and cut into transverse and longitudinal thin slices with an ultra-thin blade. The slices were placed on the microscope slide with MQ water droplets, and then cover the sample with a glass cover slip. The processed samples were microscopically observed under a confocal laser scanning microscope (Lecia TCS-SP, Wetzlar, Germany). The spectral parameters of GFP fluorescence and plant autofluorescence in this confocal laser scanning microscope are (excitation wavelength 488 nm, emission wavelength 500-560 nm) and (excitation wavelength 561 nm, emission wavelength 570-670 nm), respectively.

2.7 Determination of starch contents in corms

Fresh corms of different treatments were collected at 1 d, 7 d, 14 d and 62 d after inoculation TR4. Three plants were prepared for each treatment as one biological replicate, and each treatment was repeated with three replicates. The corms were frozen and ground into powder in liquid nitrogen immediately after collection, and the starch content was determined using the Plant Starch Content assay Kit (Comin Biotechnology Co Ltd. Suzhou, China) according to the manufacturer's instructions.

2.8 Analysis of key genes expression related to starch synthesis and plant defense by quntitative real-time PCR

Three key genes (*AGPase*, *GBSS*, *SBE*) of banana in the starch synthetic pathway were selected for expression study at 1 d, 7 d, 14 d and 62 d post inoculation with TR4. Six defense-related gens *NPR1*, *PR1*, *PR3*, *MYC2*, *ERF1* and *ACC* were also selected for this study. In each treatment, 9 corms were collected to detect the expression of related genes, and three technical replicates and three biological replicates were performed for each analysis. The collected corms sample were immediately frozen in liquid nitrogen and stored at -80°C, and then the total RNA was extracted using the Omega Plant RNA Extraction Kit according to the manufacturer's protocols. The A260/A280 and A260/A230 of total RNA were 1.9 to 2.1 and 2.0 to 2.4, respectively, and can be used for further experiments. Additionally, cDNA was synthesized by the Prime Script RT Master Mix Kit (TaKaRa), and Reverse-transcription quantitative PCR (RT-qPCR) was performed using the iTaq Universal SYBR Green Supermix Kit (BIO-RAD) according to the manufacturer's protocols. Relative changes in gene expression levels were calculated by the $2^{-\triangle\triangle}$CT method (Zhao et al., 2013; Dong et al., 2019). Relevant primer sequences for RT-qPCR analysis are listed in Supplementary Table 1 (Dong et al., 2019; Dalio et al., 2020). Moreover, *Musa25SrRNA* was used as the reference gene (Berg et al., 2007; Wu et al., 2013). In the preparation of the standard curve for real-time PCR amplification, each cDNA is diluted according to a gradient of 1-2-4-8-16-32-64-128, and then the corresponding standard curve is established. The R^2 and amplification efficiencies of the standard curve were greater than 0.99 and between 90%-110%, respectively, and the next step of the experiment can be continued.

2.9 Data analysis

Data were analyzed by using SPSS 25 and were graphed using Origin2018 (Graph Pad Software). All values are expressed as mean ± standard deviation, and statistically significant differences were determined using the Duncan multiple range tests ($P< 0.05$).

3 Results

3.1 Isotianil application can significantly induce resistance of banana to Fusarium wilt

Banana corms were split at 62 dpi to investigate the disease severity, the symptoms of banana plants with TR4 infection were recorded. The untreated control treatment showed no symptoms or

phytotoxicity. Compared with the control plants, the corms after TR4 inoculation showed obvious symptoms, and the color of the corms showing brownish-black zones of infection (Figure 2). However, the application of isotianil alleviated the symptoms caused by the TR4. Disease investigation showed that the disease indexes following isotianil application ('Baxi' 25.52% and 'Yunjiao No.1' 11.98%) were significantly lower than those where there was only TR4 inoculation ('Baxi' 51.56% and 'Yunjiao No.1' 27.08%) (Figure 3A). Among them, there was no significant difference between leaves sprayed (TR4+R1) or roots drenched (TR4+R2) with isotianil, but the disease indexes in 'Yunjiao No.1' were significantly lower than in 'Baxi'. The control effects of isotianil in 'Baxi' and 'Yunjiao No.1' to FWB in greenhouse experiments were 50.14% and 56.14%, respectively (Figure 3B). For the control effects, there was no significant difference between leaves sprayed (TR4 +R1) or roots drenched (TR4+R2) with isotianil. The results showed that 'Yunjiao No.1' is more resistant against TR4 than 'Baxi', and isotianil could significantly induce the resistance of both cultivars to FWB.

3.2 Determination of pathogen biomass in different tissues of banana plants

The pathogen biomass was measured by qPCR in different plant tissues at different time points (Figure 4). The results showed that the pathogen biomass of 'Baxi' (susceptible cultivar) was significantly higher than that of 'Yunjiao No.1' (resistant cultivar) in banana corms (Figure 4B). The pathogen biomass of corms in 'Baxi' and 'Yunjiao No.1' ranged from 139.58 ± 31.48 copies/g to 10905.68 ± 1745.75 copies/g and 104.65 ± 8.81 copies/g to 3540.11 ± 2184.47 copies/g, respectively. In addition, the content of TR4 in corms was significantly lower than root in all times points (Figures 4C, D). The pathogen biomass of corm and root in 'Baxi'

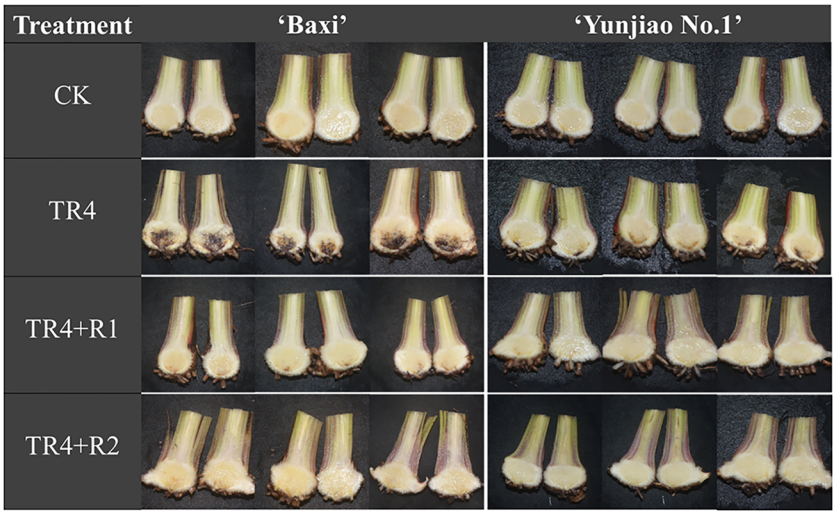

FIGURE 2
Corm dissection to show effect of isotianil application on banana plants after inoculated TR4. CK: control plants; TR4: plants inoculated with TR4; TR4+R1: plants applied with isotianil in leaves and inoculated with TR4; TR4 + R2: plants applied isotianil in roots and inoculated with TR4.

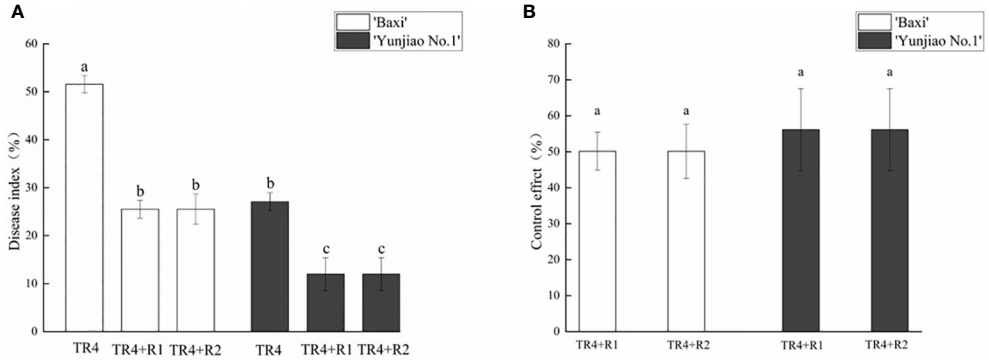

FIGURE 3
Effect of isotianil application on banana plants after inoculated TR4. **(A)** Disease index of all treatments. **(B)** Control effect of application with isotianil. The data represents three independently repeating values. Based on Duncan's multiple range test, a significant difference was determined at P<0.05. Error bars represent ± standard deviation.

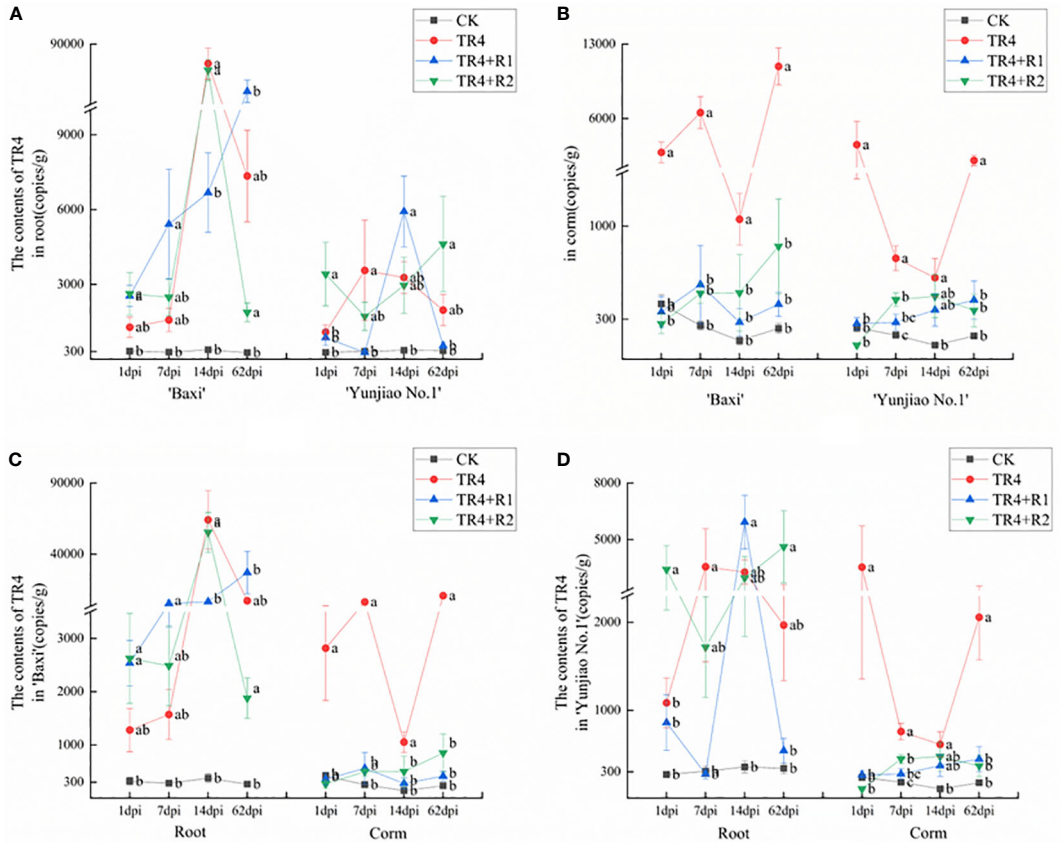

FIGURE 4
Biomass of TR4 in different tissue of banana cultivars at different time points after TR4 inoculation. **(A)** Differences of TR4 content in two cultivars in roots; **(B)** Differences of TR4 content in two cultivars in corms; **(C)** Differences of TR4 content in different tissue in 'Baxi'; **(D)** Differences of TR4 content in different tissue in 'Yunjiao No.1'. The data represents three independently repeating values. Based on Duncan's multiple range test, a significant difference was determined at $P<0.05$. Lowercase letters a, b, c, d mark significant differences: the same letter means no difference during the same period, while different letters indicate a significant difference. Error bars represent ± standard deviation.

ranged from 262.2 copies/g to 64,159.7 copies/g and 139.5 copies/g to 10,905.6 copies/g, respectively. There was no significant difference between TR4 inoculated plants (TR4), isotianil applied plants and inoculated with TR4 (TR4+R1, TR4+R2) in roots. However, the plants inoculated with TR4 (TR4) had significantly more pathogenic biomass than the control plants (CK) and the plants applied with isotianil and inoculated with TR4 (TR4+R1, TR4+R2) (Figure 4B).

3.3 Differences in the infection process of TR4 in banana plants

In order to explore the infection difference in different tissue, we used confocal laser-scanning microscope to monitor the infection colonization process of TR4 in banana tissue. The results showed that TR4 mainly existed in the form of hyphae in banana plants, and spores were hardly found. The detailed observation results were presented in Supplementary Table 2.

3.3.1 TR4 infection in roots

The hyphae could penetrate the vascular bundle tissues of all treatments, but some differences were observed in their infection process. At 1 day post inoculation (dpi), the hyphae were observed in the vascular bundle tissues only in cultivar 'Baxi' inoculated TR4 treatments, but not observed in other treatments. At 7 dpi to 62 dpi, a large number of hyphae were observed in the vascular bundle of root and it multiplies in large numbers in all treatment (Figure 5).

3.3.2 TR4 infection in corms

To accurately understand the infection mechanism of pathogens, TR4 hyphae in the corms from 1dpi to 62 dpi were further monitored. Few TR4 hyphae were discovered in the cortex vascular tissues at 7 dpi and then expanded to the central cylinder in cultivar 'Baxi' inoculated TR4 treatments at 14 dpi. However, hardly any TR4 hyphae were observed in the cortex and central cylinder in other treatments. At 14 dpi, a mass of hyphae was found in the cortical root vessels of corms in the cultivar 'Baxi' inoculated TR4

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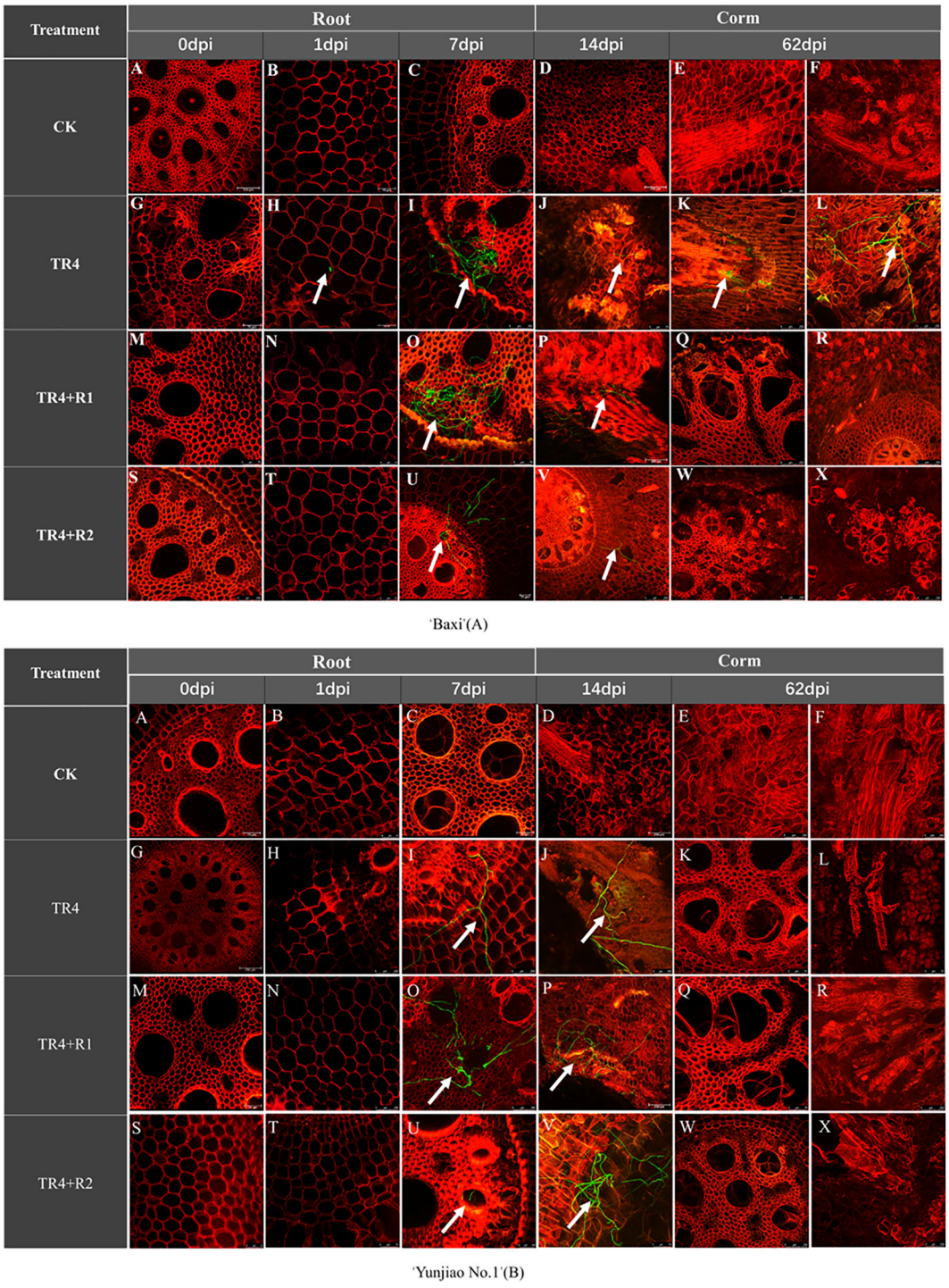

FIGURE 5
Hyphae expansion and infection in the root and corms of banana cultivar 'Baxi' inoculated with green fluorescent protein-tagged TR4 isolates at different time points (0 dpi, 1 dpi, 7 dpi, 14 dpi and 62 dpi). The TR4 hyphae was indicated by white arrows in the plant tissues. Photographs were taken under 488 (GFP) channel and 561 (RFP) channels (A-X). Bar = 250 µm in A, C, D, E, F, I, K, L, P, Q, R, S, U, V, W and X of 'Baxi' **(A)**; Bar = 75 µm in B, G, J, M, N, O and T of 'Baxi' **(A)**;Bar = 250 µm in D, E, G, H, I, N, O, P, R and W of 'Yunjiao No.1' **(B)**; Bar = 75 µm in A, B, C, F, J, K, L, M, Q, S, T, U, V and X of 'Yunjiao No.1' **(B)**.

treatments, while relatively few hyphae were found in the cortical roots of the other treatments. At 62 dpi, massive hyphae were observed in vessels of corms in cultivar 'Baxi' inoculated with TR4 only, while the level of pathogen hyphae in other treatments was relatively rare, so we turned our attention to the central cylinder of corms again. During this period, the central cylinder of the cultivar 'Baxi' inoculated TR4 treatments was ruptured, and many TR4 hyphae were released from the ducts to colonize the central column of the corms, while in other treatments, no hyphae released from the central column were found (Figure 5).

3.4 Effect of isotianil application on TR4 infection between different cultivars and tissues

3.4.1 Cultivar differences in TR4 infection between 'Yunjiao No.1' and 'Baxi'

TR4 hyphae were observed in both roots and corms of 'Baxi' (TR4 inoculated plants) one day after inoculation with TR4, while were not observed in 'Yunjiao No.1'. From 1dpi to 7dpi, the number of hyphae in the root and corms of 'Yunjiao No.1' is lower than that of 'Baxi'. After 14 dpi, quite a lot of hyphae were discovered in the roots of 'Baxi' and 'Yunjiao No.1'. A mass of hyphae was also discovered in the corms in 'Baxi', and almost no hyphae were observed in 'Yunjiao No.1'. These results indicated that the infection difference of TR4 in 'Yunjiao No.1' and 'Baxi' was mainly in the corms, and 'Yunjiao No.1' was more resistant than 'Baxi'. Furthermore, quantity of hyphae in the root is higher than in the corms. These results show that the corms play an important role in blocking the infection of TR4 (Figure 5).

3.4.2 Effect of isotianil application on TR4 infection

One day after inoculated TR4, funguses were only discovered in the roots and corms of 'Baxi', but not in the tissues treated with isotianil. Numerous funguses were discovered in the roots and corms of all plants from 7dpi to 14dpi. However, a mass of funguses was discovered in the roots 62 dpi, while massively hyphae were observed only in the 'Baxi' without isotianil treatment in the corms, while 'Yunjiao No.1' and 'Baxi' treated with isotianil no mycelia were observed (Figure 5) According to the tracking of the TR4 infection process over a period of time and across different plant parts, these results show that isotianil application can trigger the resistance of banana plants and prevent TR4 hyphae from infecting corms.

3.5 Expression of genes related to plant defense in bananas

The relative expression of genes *NPR1, PR1, PR3*, *MYC2*, *ERF1* and *ACC* in corms was analyzed in the control (CK), TR4 inoculated plants (TR4) and isotianil treatment with TR4 inoculated plants (TR4+R1, TR4+R2). In both cultivars, isotianil application significantly induced or repressed the expression of *PR1*, *PR3* and *NPR1* in the pathways regulating salicylic acid at different time point. Compared with TR4 treatment alone, the expression of *PR1* gene was significantly upregulated by routine application in 'Baxi' at different time point. The gene expression levels of TR4 inoculated plants (TR4) and isotianil application with TR4 inoculated plants (TR4+R1, TR4+R2) were as TR4-1 dpi: 4.70 ± 0.88; TR4-7 dpi: 0.36 ± 0.20; TR4-14 dpi: 7.83 ± 1.56; TR4+R1-1 dpi: 32.79 ± 7.50;TR4+R2-1 dpi: 38.87 ± 8.86; TR4+R1-7 dpi: 7.71 ± 2.46; TR4+R2-7 dpi: 5.14 ± 2.20;TR4+R-14 dpi: 19.47 ± 2.23; TR4 +R2-14 dpi: 29.96 ± 9.44, respectively. In 'Yunjiao No.1', the *PR1* gene expression levels of isotianil application with TR4 inoculated plants (TR4+R1, TR4+R2) were as TR4+R1-7 dpi: 8.83 ± 1.37; TR4 +R2-7 dpi: 4.28 ± 0.81; TR4+R1-14 dpi: 3.27 ± 0.66; TR4+R1-62 dpi: 5.34 ± 1.41; TR4+R2-62 dpi: 3.14 ± 1.02, which were significantly upregulated than those of inoculation with TR4 alone (TR4-7 dpi:1.12 ± 0.19; TR4-14 dpi:2.4 ± 0.78; TR4-62 dpi:2.56 ± 0.55), respectively. Additionally, compared with the TR4 treatment alone (TR4), the *NPR1* gene expression of 'Yunjiao No.1' was significantly upregulated by isotianil application (TR4+R1 and TR4+R2), and the expression levels of were as TR4-7 dpi: 0.62 ± 0.09; TR4-14 dpi: 0.37± 0.09; TR4-62 dpi: 0.45 ± 0.14; TR4+R2-7 dpi: 1.22 ± 0.17; TR4+R1-14 dpi:1.28 ± 0.24; TR4+R2-14 dpi: 1.21 ± 0.45; TR4+R1-62 dpi: 1.11 ± 0.39; TR4+R2-62 dpi: 1.34 ± 0.54, respectively. In addition, the genes *ERF1* that ISR-related genes were upregulated and downregulated as a result of the isotianil application in 'Yunjiao No.1' and 'Baxi', respectively. In 'Yunjiao No.1', the *ERF1* gene expression levels of routine application were as TR4+R1-7 dpi: 9.68 ± 1.48; TR4+R2-7 dpi: 36.60 ± 6.65; TR4+R1-14 dpi: 4.45 ± 0.38; TR4+R2-14 dpi: 1.85 ± 0.29; TR4+R1-62 dpi: 76.05 ± 26.04, which were significantly upregulated than those of inoculation with TR4 alone (TR4-7 dpi:2.23 ± 0.16; TR4-14 dpi:0.26 ± 0.11; TR4-62 dpi:3.32 ± 1.06), respectively. Additionally, the *ERF1* gene expression of 'Baxi' were no significantly in TR4 treatment alone (TR4) and isotianil application treatment. Compared with the control treatment (CK), the *ACC and MYC2* genes expression of 'Yunjiao No.1' were significantly upregulated by TR4 (TR4), and the expression levels of *ACC* and *MYC2* were as TR4-7 dpi: 0.76 ± 0.09; TR4-14 dpi: 0.64± 0.13; TR4-62 dpi: 0.45 ± 0.08; TR4-1 dpi: 0.27 ± 0.03; TR4-7 dpi:0.45 ± 0.05; TR4-14 dpi: 0.73 ± 0.31; TR4-62 dpi: 0.25 ± 0.07, respectively, while there was no obvious trend in 'Baxi'. All detailed results were presented in Supplementary Table 3.

3.6 Tyloses accumulation in the vascular bundles of corms

A microscopic observation analysis performed on root and corms samples from banana plants showed that tyloses plays an important role in against the pathogen infection in vascular bundle. At 62 days post inoculation (dpi), numerous tyloses were observed in the cortical root vascular bundle vessels of corms. At the same time, the hyphae were significantly reduced in the tissues with tyloses (Figures 6I–P) whereas a large number of TR4 hyphae were observed in the tissues without tyloses (Figures 6E–M). Isotianil application could induce the formation of tyloses (Figures 6J–P) and the number of tyloses of isotianil treatments (TR4+R1, TR4+R2) were higher than that in TR4 infected plants (TR4). There were also differences between different banana cultivars, with the tyloses in 'Yunjiao No.1' were also being more abundant than in 'Baxi'.

3.7 Determination of starch content and related gene expression levels in corms

In the process of monitoring TR4 infection, there were significantly less TR4 hyphae in cells filled with starch granules

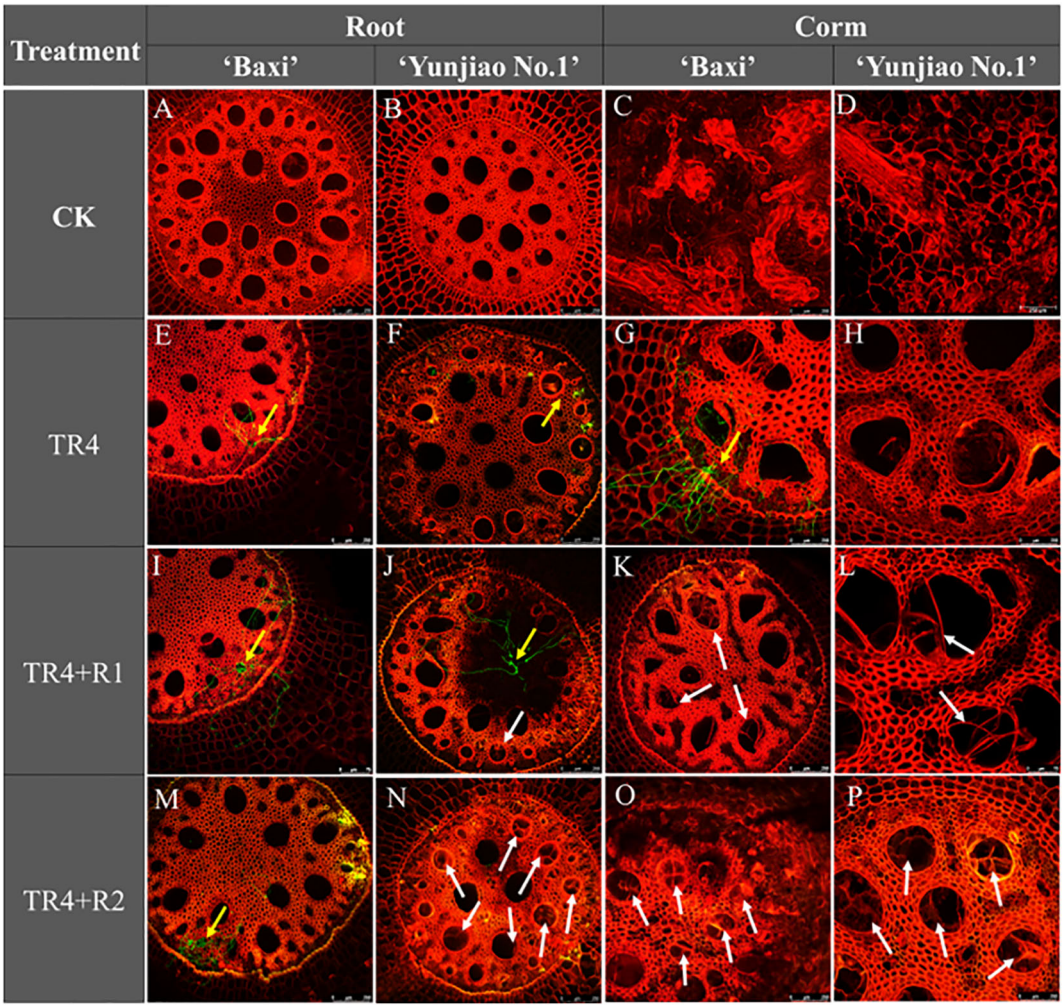

FIGURE 6
Microscopic observation of tyloses in banana corms and root at 62 days post inoculation TR4 (62 dpi) and mock inoculation. The tyloses was indicated by white arrows in the banana plant tissues. The TR4 hyphae was indicated by yellow arrows in the banana plant tissues. Photographs were taken under 488 (GFP) channel and 561 (RFP) channels **(A–P)**. Bar = 250 μm in **(A–H, J, K, M–P)**; Bar = 75 μm in I.

than in tissues with fewer starch granules. In addition, the content of starch granules at 62 dpi was higher than that at 1dpi (Supplementary Figures 1A, B). These results indicated that starch granules in corms may play an important role in preventing TR4 infection (Figure 7). To verify that starch content is related to plant disease resistance, the total starch content in the bulbs was determined (Figure 7A). The results showed that the starch contents in the isotianil applied banana plants (TR4+R1, TR4 +R2) were significantly higher than those in TR4 inoculation alone (TR4) and control (CK) in 'Baxi'. In addition, the starch content in 'Yunjiao No.1' is significantly higher than that in 'Baxi'. The starch content of corm in 'Baxi' and 'Yunjiao No.1' ranged from 15.25 ± 3.18 mg/g to 67.01 ± 4.39 mg/g and 44.70 ± 0.73 mg/g to 123.05 ± 10.89 mg/g, respectively (Figure 7A). These results indicate that isotianil applied plants were induced to produce more starch granules than TR4 inoculated plants (Figure 7). In addition, the key genes (*SBE*, *GBSS*, *AGPase*) related to starch synthesis in the corm were selected for its expression study, and the results showed that the expression of *SBE*, *GBSS* and *AGPase* in two cultivars was significantly different. These genes are significantly more expressed in 'Yunjiao No.1' than 'Baxi' (Figure 7B).

4 Discussion

In the past few decades, the banana production suffered dramatic losses because of the epidemic of FWB, which is a typical soil-borne disease that is difficult to control (Ortiz, 2013; Zhang et al., 2013; Li et al., 2015; Wang et al., 2015; Paz-Ferreiro and Fu, 2016; Zuo et al., 2018; Niwas et al., 2020). According to the historical experience of the first epidemic of FWB, enhancing plant disease resistance is generally considered to be one of the most effective strategies to control FWB. However, the mechanism by which banana plants resist TR4 infection is unclear.

From this study, the results showed that isotianil can significantly reduce the incidence of FWB and alleviate the disease symptoms in both cultivars (Figures 2, 3). Comparing the disease index, the isotianil application treatments (TR4+R1, TR4+R2) was significantly lower than

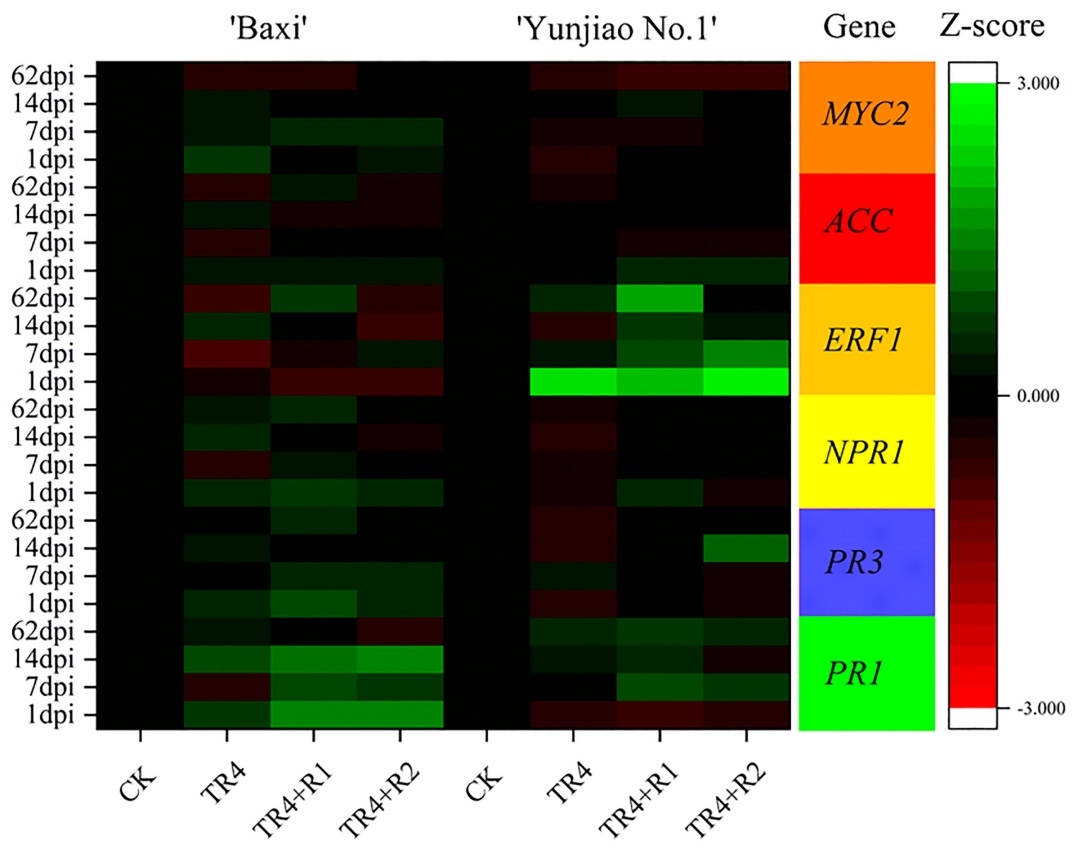

FIGURE 7
Relative expression levels of key genes that induce resistance in corms at 1 dpi to 62 dpi. The heat map illustrates the doubled changes of gene expression (log10 scale) in corms at 1 dpi, 7 dpi, 14 dpi and 62 dpi. Different color means induced or repressed gene expression (Red indicates down-regulation of gene expression; green indicates up-regulation of gene expression; black indicates no effect on gene expression). Three biological replicates and three technical replicates were used in data analysis. MYC2, basic helix-loop-helix (bHLH) transcription factor; ACC, 1-aminocyclopropane-1-carboxylic acid synthase; ERF1, ethylene response factor 1; NPR1, nonexpressorofpathogenesis-relatedgenes1; PR3, pathogenesis-related 3 genes; PR1, pathogenesis-related 1 genes.

that TR4 inoculated alone (TR4). To explain the mechanism of action of isotianil on banana plants, the biomass of TR4 in different banana tissues were measured by qPCR. The results showed that TR4 biomass in corms is lower after isotianil application, and both cultivars have a consistent trend (Figure 4B). The TR4 biomass in the corm was consistent and stable at different growth points. However, no clear tendency of TR4 biomass in roots was found, probably because roots keep growing and only parts of roots were taken for the analysis; sampled roots could be newly grown and uninfected. At the same time, qPCR results showed that at 14 dpi, the content of the pathogenic in corms alone TR4 inoculation gradually decreased, which may be related to the increase of plant resistance during this period. In addition, TR4 biomass in corms was significantly lower than in roots, and these results were consistent with what was observed. Therefore, these results showed that the corm as a physical barrier can play an important role in reducing the harm of FWB by slowing down TR4 infestation. This result is consistent with our previous study (Zhang et al., 2018b). However, the detailed mechanism which prevents TR4 entering the corm is deserved for further study. In the current study, in order to make it easier to monitor the infestation process of TR4 in bananas plants, *GFP*-TR4 was used (Zhang et al., 2018a). The results showed that TR4 hyphae penetrates the root epidermis, invades the xylem vessels, and also invades the vascular bundle vessels from the wound, root hair or intercellular spaces of the cortex. These phenomena are basically consistent with previous research results (Li et al., 2011; Guo et al., 2014; Guo et al., 2015; Li et al., 2017). Compared with other parts during the infection process, we found that TR4 hyphae were more likely to infect from wounds, which is similar to the findings of Dong (Dong et al., 2019). Study of the TR4 infection process in the past was mainly focused on the infection of plant roots or corms (Li et al., 2011; Guo et al., 2015; Li et al., 2017). However, no one observed how TR4 moves from the roots into the corms. In this study, we observed that TR4 hyphae enter the corms from the root through vascular ducts, and the number of TR4 hyphae in the root is much higher than in the corms. Once again, these results confirmed that banana corms play an important role for blocking the infection of pathogens. At the same time, the observation by confocal laser scanning microscope showed that the number of TR4 hyphae after isotianil application corms (TR4+R1, TR4 +R2) was significantly lower than that in TR4 treatment (TR4). When plants were infected by pathogens, the defense response in the xylem vessels would be activated, preventing further spread of the pathogens

(Yadeta and Thomma, 2013; Li et al., 2022). One of the common defense mechanisms produced in xylem vessels is the formation of tyloses (Beckman, 1964; Talboys, 1972; Grimault et al., 1994; Rahman et al., 1999; Fradin and Thomma, 2006; Yadeta and Thomma, 2013). The formation time and number of tyloses in different plants varied greatly, and the contents of tyloses in disease-resistant plants were significantly higher than in disease-susceptible plants (Grimault et al., 1994; Xu et al., 1997; Fradin and Thomma, 2006; Hu et al., 2008).In this study, a mass of tyloses and gums were observed in the cortical roots of banana plants treated with isotianil, while almost no pathogen was observed where the tyloses appeared (Figure 6). Therefore, this result showed that isotianil could induce the formation of tyloses in the root of banana cortex to prevent the further infection of TR4 into corms.

When plants encounter some stress challenges, they can quickly initiate corresponding defense responses to enhance their resistance (Conrath et al., 2002; Acharya et al., 2011; Tanou et al., 2012). Elicitors are a class of substances that can trigger defense responses by mimicking the interaction of corresponding signaling molecules with homologous receptors in plants (Nimchuk et al., 2003). In the study, prior applications of isotianil significantly enhanced the expression of *PR1*, *PR3*, *NPR1* and *ERF1* and further increased the expression in isotianil application plants (TR4+R1 and TR4 +R2) when compared to TR4 inoculated plants. Isotianil pre-application significantly induced the expression of key genes *PR1*, *PR3* and *NPR1* of the SA pathway in banana plants. The result showed that isotianil may initiate the SA pathway to improve banana resistance to Fusarium wilt (Figure 8), this is consistent with previous research results on rice (Toquin et al., 2012). Some studies have shown that ERF1, a key responder downstream of the ET and JA pathway (Zhu et al., 2011; Huang et al., 2016), plays an important role in plant disease resistance (Berrocal-Lobo and Molina, 2004; Meng et al., 2013; Xing et al., 2017). In isotianil application plants, ISR-related genes were also induced to significantly upregulate at some time points, such as *ERF1* (Figure 8), this part of the result is a new discovery. Taken together, these results suggest that isotianil is a potent resistance inducer that can significantly enhance the expression of key genes of SAR and ISR pathways in banana plants (Figure 8).

Another interesting finding was that there was a large accumulation of starch grains in the corm cells (Supplementary Figure 1), and starch granules and TR4 in diseased corms could not coexist in the same time and space (Dong et al., 2019). At the same time, many studies have reported that under abiotic stress, the starch content will decrease or increase (Villadsen et al., 2005; Pressel et al., 2006; Goyal, 2007; Damour et al., 2008). The accumulation of starch granules is not only an important factor for plants to respond to abiotic stress, but also closely related to plant disease response (Takushi et al., 2007; Etxeberria et al., 2009). Combined with the previous observations, the expression of starch synthesis-related genes in corm was measured by qPCR, and the results showed that the expression of key genes in 'Yunjiao No.1'was significantly upregulated, and the upregulation level was higher after isotianil application. (Figure 7B). In addition, the expression of related genes in 'Yunjiao No.1' at some time point was much higher than in 'Baxi'. Further, the starch content in corms of different cultivars was measured, and the results showed that in 'Yunjiao No.1' (disease-resistant cultivar) the level was much higher than that in 'Baxi' (susceptible cultivar). In addition, this result showed that the starch content after the isotianil application was much higher in 'Baxi' (Figure 7A). On the one hand, the accumulation of starch may enhance the density of cell (Kuang et al., 2013) and directly inhibit the diffusion of TR4 in the corm, on the other hand, starch, as an important energy substance, may directly participate in the synthesis of resistance-related substances in cells (Dong et al., 2019). Therefore,

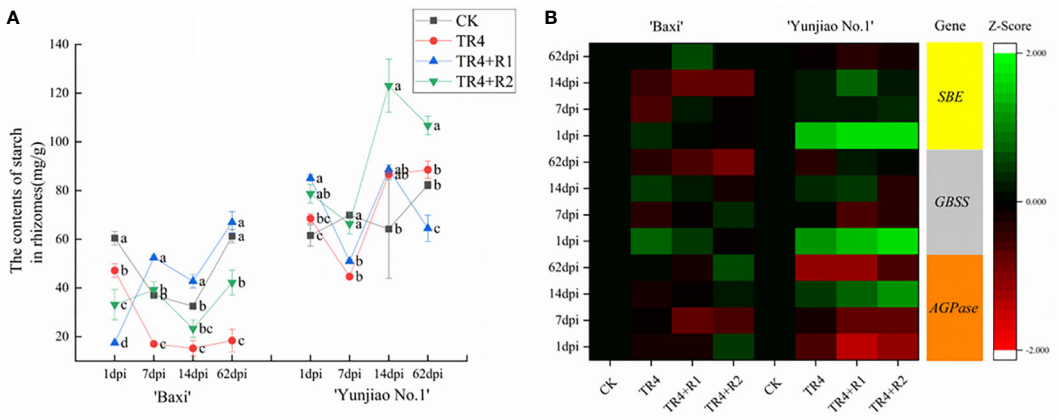

FIGURE 8
(A) Contents of starch in banana corms at different time points past inoculation TR4 (1 dpi, 7 dpi, 14 dpi and 62 dpi). The data represents three independently repeating values. Based on Duncan's multiple range test, a significant difference was determined at $P<0.05$. Lowercase letters a, b, c, d mark significant differences: the same letter means no difference during the same period, while different letters indicate a significant difference. Error bars represent ± standard deviation. **(B)** Relative expression levels of key genes for starch synthesis in corms at 1dpi to 62dpi. The heat map illustrates the double change in the expression(log10) of key genes for starch synthesis in the corm at 1dpi to 62 dpi. Different color means induced or repressed gene expression (Red indicates down-regulation of gene expression; green indicates up-regulation of gene expression; black indicates no effect on gene expression). Three biological replicates and three technical replicates were used in data analysis. AGPase, ADP-glucose pyrophosphorylase; SBE, Starch branching enzyme; GBSS, granule-binding starch synthase.

we speculate that the accumulation of starch grains in the corm cells may be closely related to the disease resistance of plants.

There are differences in the resistance of two cultivars. Our results showed that 'Yunjiao No.1' can significantly reduce the incidence of FWB and alleviate the disease symptoms much more than 'Baxi' cultivar. Comparing 'Baxi' with 'Yunjiao No.1', the disease index of 'Yunjiao No.1' was significantly lower than that of 'Baxi' (Figure 3). In addition, the TR4 biomass in 'Yunjiao No.1' (2058.42 copies/g) is much lower than that of 'Baxi' (10905.69 copies/g) in corms at 62 dpi (Figure 4B). Moreover, the content of tyloses and starch grains in 'Yunjiao No.1' is higher than that of 'Baxi', and there is a consistent trend of disease resistance between 'Yunjiao No.1' and 'Baxi' applicated with isotianil. Furthermore, the expression of resistance (*ERF1*) and starch related (*AGPase*, *SBE* and *GBSS*) genes in 'Yunjiao No.1' are higher than those in 'Baxi'. All the results demonstrated that 'Yunjiao No.1' is more resistant to FWB than 'Baxi', and resistant banana plants may prevent pathogenic fungal infection by inducing the content of tyloses in vascular bundles and starch grains in corms to form a physical barrier and activate immune pathways such as SAR and ISR in the corms.

In the next step of our research, we will continue to explore the defense mechanisms of different resistant banana cultivars in the face of TR4 infestation, especially the relationship between tyloses, starch grain formation and plant resistance.

5 Conclusion

This study has shown that the plant elicitor isotianil can significantly reduce the impact of FWB and protect different banana cultivars. In addition, we further found that corms are important to against further infestation of TR4. The elicitor isotianil is able to induce the formation of tyloses in cortex vascular tissues of corms, preventing pathogen from root entering the corms. It can activate the three major systems of ISR, SAR, and starch granule synthesis in the corms, and inhibit the diffusion of pathogen in the corms, so as to reduce the effect of FWB. In addition, 'Yunjiao No.1' are more resistant than 'Baxi'. In summary, when biological, chemical and agronomic measures are not ideal for the control of FWB, further enhance the resistance of cultivar together with elicitor isotianil application is a promising control strategy for banana growers.

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

Conceptualization, G-DZ, SL and S-JZ. Methodology and software, G-DZ, PH and SL. Validation, G-DZ, PH, SL and S-JZ. Performed the experiment and data analysis, LT, SX, BY, LL, YW, TB and XL. Writing—original draft preparation, G-DZ, PH and SL. Writing—review and editing, SL and S-JZ. Supervision and project administration, SL and S-JZ. Project administration and funding acquisition, S-JZ. 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1145837/full#supplementary-material

SUPPLEMENTARY FIGURE 1

Observation of starch granules in corms cell at different time points (1 dpi, 7 dpi, 14 dpi and 62 dpi) after inoculation TR4. The TR4 hyphae and starch grains was indicated by white arrows and yellow arrows respectively in the banana plant corms. Photographs were taken under GFP channel, GFP channel and through transmitted light (A-P). bar = 250 µm in A, B, C, D, E, G, H, J, K, L, M, O and P of 'Baxi' **(A)**; bar = 75 µm in F, I, and N of 'Baxi' **(A)**; Bar = 250µm in A, B, C, D, E, F, H, K, L, N, O and P of 'Yunjiao No.1' **(B)**; bar = 75 µm in G, I, J and M of 'Yunjiao No.1' **(B)**.

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Comprehensive analysis of the WRKY gene family in *Cucumis metuliferus* and their expression profile in response to an early stage of root knot nematode infection

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Root-knot nematode (RKN) is a major factor that limits the growth and productivity of important *Cucumis* crops, such as cucumber and melon, which lack RKN-resistance genes in their genome. *Cucumis metuliferus* is a wild *Cucumis* species that displays a high degree of RKN-resistance. WRKY transcription factors were involved in plant response to biotic stresses. However, little is known on the function of WRKY genes in response to RKN infection in *Cucumis* crops. In this study, *Cucumis metuliferus* 60 WRKY genes (*CmWRKY*) were identified in the *C. metuliferus* genome, and their conserved domains were classified into three main groups based on multiple sequence alignment and phylogenetic analysis. Synteny analysis indicated that the WRKY genes were highly conserved in *Cucumis* crops. Transcriptome data from of *C. metuliferus* roots inoculated with RKN revealed that 16 *CmWRKY* genes showed differential expression, of which 13 genes were upregulated and three genes were downregulated, indicating that these *CmWRKY* genes are important to *C. metuliferus* response to RKN infection. Two differentially expression *CmWRKY* genes (*CmWRKY10* and *CmWRKY28*) were selected for further functional analysis. Both *CmWRKY* genes were localized in nucleus, indicating they may play roles in transcriptional regulation. This study provides a foundation for further research on the function of *CmWRKY* genes in RKN stress resistance and elucidation of the regulatory mechanism.

KEYWORDS

WRKY family, root knot nematode, cucumis crops, expression patterns, transcriptional regulation

1 Introduction

In plant, WRKY genes belong to one of large plant transcription factors family that is widely involved in various physiological processes (Goyal et al., 2022). WRKY proteins contain one or two highly conserved WRKY domains that contain approximately 60 amino acid residues. The WRKY domain is located in the C-terminal region and includes a highly conserved WRKYGQK motif followed by a zinc finger motif (Kazuhiko et al., 2005). According to the number of WRKY domains and the structure of the zinc finger motif, WRKY proteins were grouped into three groups (1, 2 and 3) (Zhang and Wang, 2005). Group 1 proteins contain an N-terminal and a C-terminal WRKY domains with C2H2 motif. Group 2 proteins contain one WRKY domain but can be classified into five subgroups (2a-2e) according to C2H2 zinc-finger motif structure (Grzechowiak et al., 2022). Group 3 proteins also have one WRKY domain, but the structure of zinc-finger is C2-H-C (Ning et al., 2017). With the release of many plant genomes, whole-genome identification of WRKY genes has been reported in many plant species, and the number of WRKY family members in a species varies from dozens to hundreds (He et al., 2016; Liu et al., 2017; Karkute et al., 2018; Li et al., 2018; Chen et al., 2020; Wei et al., 2022; Zhang et al., 2022; Pan et al., 2023; Ren et al., 2023).

WRKY transcription factors are extensively involved in plant development and growth, and abiotic and biotic stresses responses by binding to the conserved motif in the promoter of their target genes (Duan et al., 2007). One focus of research on WRKY genes is their roles in regulating plant defense against biotic stress (Wani et al., 2021). WRKY genes play important roles in the plant response to diverse pathogens, including bacterial pathogens (Kim et al., 2008; Vo et al., 2018), fungal pathogens (Zheng et al., 2006) and nematodes (Chinnapandi et al., 2017). A function of WRKY genes is to regulate PAMP (pathogen-associated molecular pattern) triggered immunity (PTI) and effector-triggered immunity (ETI). WRKY transcription factors play important roles in the pathways that can activate PTI and ETI (Eulgem, 2006; Jones and Dangl, 2006; Eulgem and Somssich, 2007). In addition, WRKY transcription factors can regulate plant hormone signaling and involved in biotic stress responses. Plant WRKY genes can response to various plant hormones including ethylene, jasmonate, salicylic acid (SA), gibberellin and abscisic acid (Dai et al., 1999; Zhang et al., 2009; Grunewald et al., 2012). WRKY genes can regulate plant biotic defense mechanisms through other pathways. For example, WRKY transcription factors are responsive to regulate biosynthesis of lignin, phosphate acquisition, and other secondary metabolites (Wang et al., 2010; Schluttenhofer and Yuan, 2015; Yang et al., 2016).

Previous studies have shown that WRKY genes play important roles in plant-cyst nematode interactions. Grunewald et al. (2008) reported that knock down WRKY23 expression can reduces infection by the cyst nematode (Grunewald et al., 2008). Yang et al. (2017) reported that overexpression of three WRKY transgenes increases resistance to the cyst nematode (Yang et al., 2017). Huang et al. (2022) conducted soybean RNA-seq transcriptome sequencing of soybean for compatible and incompatible reactions to cyst nematode and observed that interactions between cyst nematode infection and WRKY genes were activated (Huang et al., 2022). An increasing number of studies have shown that WRKY genes also are involved in plant-root knot nematode (RKN) interaction. The tomato WRKY gene *SlWRKY45* is a RKN-responsive gene that enhances susceptibility to RKN (Chinnapandi et al., 2017). Tomato *SlWRKY3* can activate defense-signaling pathways associated with lipids and hormone-mediated pathway and acts as a positive regulator for resistance to *Meloidogyne javanica* (Chinnapandi et al., 2019). Ribeiro et al. (2022) reported that protease inhibitors and nucleotide-binding site leucine-rich repeat WRKY genes are essential for resistance in cowpea (Ribeiro et al., 2022).

Cucumis genus contains two important crops: melon and cucumber. Infections by RKN species cause heavy losses of cucumber and melon. RKN-infections seriously restricts the production and quality of cultivated cucumber and melon, due to the genome lack RKN-resistance genes (Guan et al., 2014). *Cucumis metuliferus* is considered to be a semi-wild or wild relative of melon and cucumber (Weng, 2010). Although cucumber and melon are highly susceptible to RKN infection, several *C. metuliferus* accessions are highly resistant to RKN species, including *Meloidogyne javanica* and *Meloidogyne incognita* (Walters et al., 2006; Ling et al., 2021). However, few genes involved in RKN-resistance in *C. metuliferus* have been reported. Given the lack of RKN-resistance genes in the melon and cucumber genomes, the identification of genes associated with RKN resistance in their wild relative *C. metuliferus* is of great importance. In this study, we identified 60 *CmWRKY* genes and classified them into three groups. Comparative genome analysis indicated that WRKY genes are highly conserved in *C. metuliferus*, cucumber and melon. In addition, the expression profile of *CmWRKY* genes responsive at early stage of RKN infection was investigated. The results will provide a clue for further research on the WRKY genes that regulate RKN-resistance in *Cucumis* crops.

2 Materials and methods

2.1 Genome-wide identification WRKY genes

The genome data of *C. metuliferus* were downloaded from CuGenDB (Zheng et al., 2019). HMMER program was used to search candidate WRKY genes using the WRKY domains (PF03106.7) and the e-valve was set to 1e-10 (http://hmmer.org/). The SMART software (http://smart.embl.de/) was used to prove WRKY domains in the candidate genes.

2.2 Gene structure, conserved motif and phylogenetic analysis

The WRKY domains of *CmWRKY* proteins and seven selected *Arabidopsis* WRKY (*AtWRKY*) proteins were used to create multiple protein sequence alignments using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) with default settings.

The conserved motifs of CmWRKY proteins were identified using MEME software (http://meme-suite.org/) with setting –nmotifs to 10. MEGA11 (Tamura et al., 2021) was used to construct phylogenetic trees for WRKY domains. Bootstrap analysis was performed with 1000 replications to evaluate support rate.

2.3 Chromosomal location, gene duplication, and syntenic analysis of *CmWRKYs*

The locations of the *CmWRKYs* were extracted from the *C. metuliferus* genome gff3 annotation files and a custom Python script was used to display the distribution of *CmWRKYs* on the chromosomes. MCScanX was used to detect the duplication events of *CmWRKY*s. The syntenic analysis among *C. metuliferus*, cucumber and melon were constructed using Bioconda JCVI package (Tang et al., 2008). The WRKY genes of melon and cucumber were obtained from CuGenDB (Zheng et al., 2019).

2.4 Plant materials and RKN (*Meloidogyne incognita*) treatment

The RKN-resistant *C. metuliferus* were planted in greenhouse with temperature of 25°C (day) and 18°C (night). *Meloidogyne incognita* was maintained on susceptible water spinach in a glasshouse at 22–26°C. J2 juveniles of *Meloidogyne incognita* were used as inoculate. J2 juveniles were resuspended in dH2O, and the concentration of *Meloidogyne incognita* used for inoculation was 1,000/mL.

2.5 Transcriptome analysis, differentially expressed genes identification and expression validation by qRT-PCR

For RNA-seq, *Cm3* roots inoculated with *Meloidogyne incognita* were used as treatment and inoculated with dH_2O were used as control. Two time points (3 day after inoculate (3DAI) and 7DAI) were selected to analysis *CmWRKYs* expression profiling at early stage of *Meloidogyne incognita* infection. *Cm3* roots were used to RNA-seq (Hiseq 2500) and performed on three independent biological replicates for each time point. The RNA-seq data processing and DEGs analysis were carried out using salmon pipeline (Patro et al., 2017) and DESeq2 (Love et al., 2014). By comparing the treatment with control at the same time point, the genes were identified as DEGs with adjusted P<0.01 and log2FoldChange >1.

We performed real-time RT-PCR (Qrt-PCR) using cfx96 real time system (Bio-Rad, USA). The PCR program was set to initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55 or 60°C for 30 s and extension at 72°C for 30s. The RT-PCR experiments were carried out at least three biological replicates. Besides of 3DAI and 7DAI samples, other two time points (14 DAI and 28DAI) were added in qRT-PCR experiment, which were late stage of *Mi* infection. The analysis of relative gene expression data were carried out according to Ling et al (Walters et al., 2006).

2.6 Subcellular localization of *CmWRKYs*

The *CmWRKY10* and *CmWRKY28* CDS sequence were cloned into the super1300-GFP vector to generate super1300-*CmWRKY10*-GFP and super1300- *CmWRKY28*-GFP. These vectors were used to transform *Agrobacterium tumefaciens* GV3101. *Nicotiana benthamiana* were grown for 4-week-old and infiltrated with transformed *A. tumefaciens* GV3101 through in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 0.1 mM acetosyringone) to an OD_{600} of 0.5. Leaves were imaged 48h after inoculation; images were acquired by an inverted confocal microscope (Zeiss LSM700, Germany) at 488 nm for GFP.

3 Results

3.1 Genome-wide identification of WRKY genes in *C. metuliferus*

To identify WRKY family genes in the *C. metuliferus* genome, *WRKY* genes of cucumber and melon were used to search against the *C. metuliferus* genome. The homologous proteins were identified as candidate *CmWRKY* genes using HMMER and SMART software. Finally, 60 *CmWRKY* genes in *C. metuliferus* genome were identified. These genes were predicted to contain WRKY domains based on search of the Pfam and SMART databases. All *CmWRKY* genes can be mapped to 12 chromosomes of *C. metuliferus*. Accordingly, we renamed the *CmWRKY* genes from *CmWRKY1* to *CmWRKY60* according to their location order from chromosome 1 to 12 (Figure 1A; Table S1). The distribution of *CmWRKY* genes on the chromosomes was not evenly, with chr06 containing the highest number of *CmWRKY* members (10), followed by chr07 (7) and chr08 (7), whereas chr05 contained only one *CmWRKY* gene. In order to conduct a comparative analysis among *Cucumis* genus, we mapped 60 cucumber WRKY genes (*CsWRKY*) and 58 melon WRKY genes (*MeWRKY*) to their chromosomes, respectively (Tables S2, S3, Figures S1, S2). It was noted that no WRKY gene cluster was detected in their genomes, indicating the lack of tandem replication events of WRKY genes loci in *Cucumis* species.

3.2 Classification and phylogenetic analysis of the *CmWRKY genes*

Multi-sequence alignment analysis of *CmWRKY* proteins was conducted using the WRKY domains. According to the group or subgroup information of *Arabidopsis* WRKY (*AtWRKY*), seven *AtWRKY* domains were randomly selected as group or subgroup representatives for the analysis. As shown in Figure 1B and Figure S3,

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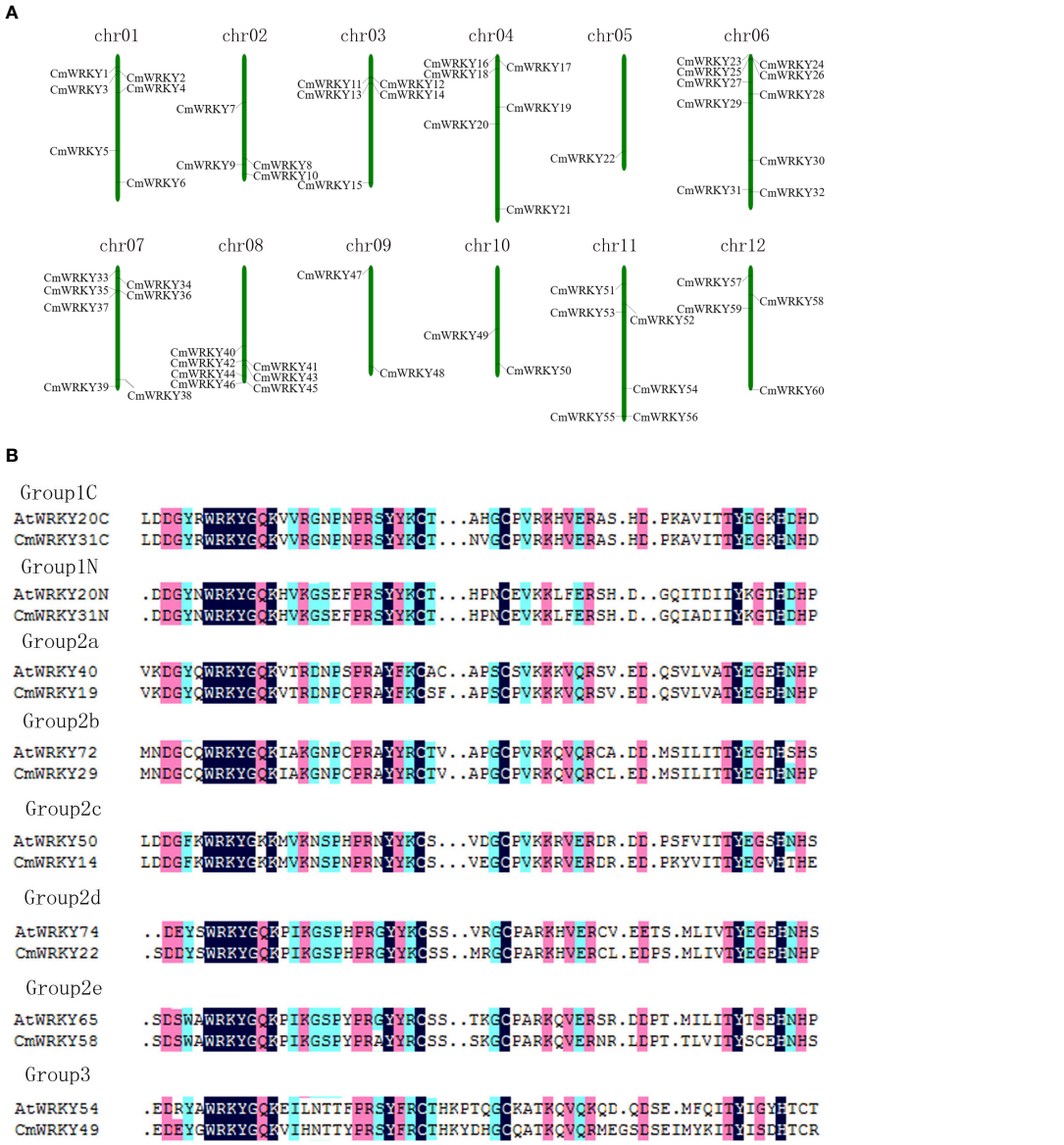

FIGURE 1
Mapping of the WRKY genes to *C. metuliferus* chromosomes **(A)** and alignment of amino acid sequences for selected *CmWRKY* and *AtWRKY* domain **(B)**. **(A)** to simplify the presentation, we renamed the putative WRKY genes from *CmWKRY1* to *CmWRKY60* based on their order on the chromosomes. **(B)** alignment was performed using Clustal W. The suffix 'N' or 'C' indicates the N-terminal WRKY domain or the C-terminal WRKY domain, respectively. The conserved WRKY amino acid signature is highlighted in different colors, and gaps are marked with dots.

the highly conserved amino acid sequence WRKYGQK was detected within 57 *CmWRKY* proteins, whereas the remaining three *CmWRKY* proteins (*CmWRKY14*, *CmWRKY42*, and *CmWRKY55*) had a WRKYGKK motif sequence. To understand the phylogenetic relationships of *CmWRKY* proteins, a phylogenetic tree for *CmWRKY* and *AtWRKY* WRKY domains was constructed. As shown in Figure 2, the *CmWRKY* genes were grouped into three large groups (Groups 1-3). Among the 60 *CmWRKY* proteins, 12 were assigned to group 1, 43 belonged to group 2, and five were assigned to group 3. The phylogenetic tree of the *CmWRKY* domains indicated that the group 2 *CmWRKY* proteins can be further grouped into five distinct subgroups (a-e). In addition, we also constructed a phylogenetic tree using WRKY gene sequences from *C. metuliferus*, cucumber (*CsWRKY*) and melon (*MeWRKY*). The number of WRKY gene family members in the three *Cucumis* crops genomes was almost identical, and the classification of the genes was

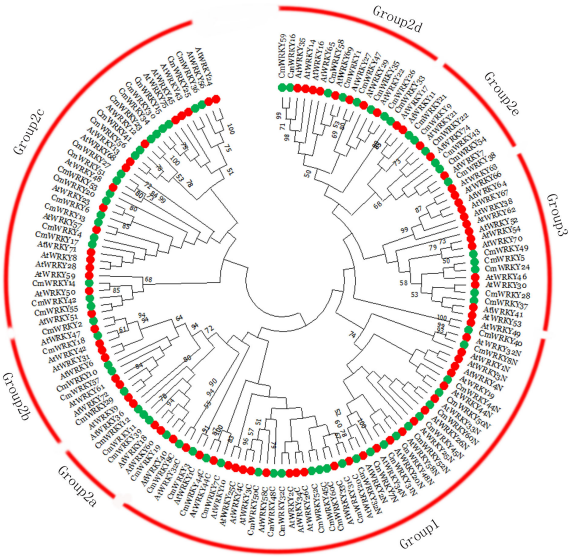

FIGURE 2
Phylogenetic tree representing relationships among WRKY domains of *Cucumis metuliferus* and *Arabidopsis thaliana*. The amino acid sequences of the WRKY domains of all *CmWRKY* and *AtWRKY* proteins were aligned with Clustal W. The phylogenetic tree was constructed using the neighbor-joining method in MEGA. The red arcs indicate different groups (or subgroups) of WRKY domains. Colored circlesrepresent putative orthologs from *C. metuliferus* (green) and Arabidopsis (red).

similar, indicating that WRKY genes are highly conserved in these *Cucumis* crops (Figure S4).

3.3 The *CmWRKY* gene structure and conserved motifs analysis

To detect the *CmWRKY* gene structure variations, the intron and exon structure compositions of *CmWRKY* were analyzed. As shown in Figure 3, among the coding sequences of *CmWRKY* genes, 27 (approximately 45%) had 3 exons, 10 *CmWRKY* genes contained 4 exons, and one WRKY gene (*CmWRKY18*) had the highest number (8) of exons. The average exon number for per *CmWRKY* gene is 3.5, while the average number of *CmWRKY* gene in some groups were significantly higher than the overall average number, with 5.6 for group 2b, 4.9 for group 1 and 4.5 for group2a members.

To evaluate the structural diversification and conservation of *CmWRKYs*, the different motifs of all *CmWRKY* proteins were identified using MEME. As shown in Figure 3, motif1, motif2 and motif3 were the most conserved motifs, and motif1 is shared by all *CmWRKYs*, while motif9 is the rarest which is shared by only 5 members(*CmWRKY2,10,18,29* and *57*) of group 2b (Table S4). Each member of *CmWRKYs* family contains 3.6 motifs on average, while group 2b members contain the largest number of motifs (6), followed by group 1 *CmWRKYs*(4.4) and group 2e (4). It is noted that, in the same phylogenetic clade, the motifs of *CmWRKY* proteins exhibited similar compositions and distributions, suggesting that *CmWRKY* genes of the same clade may be genes with similar functions.

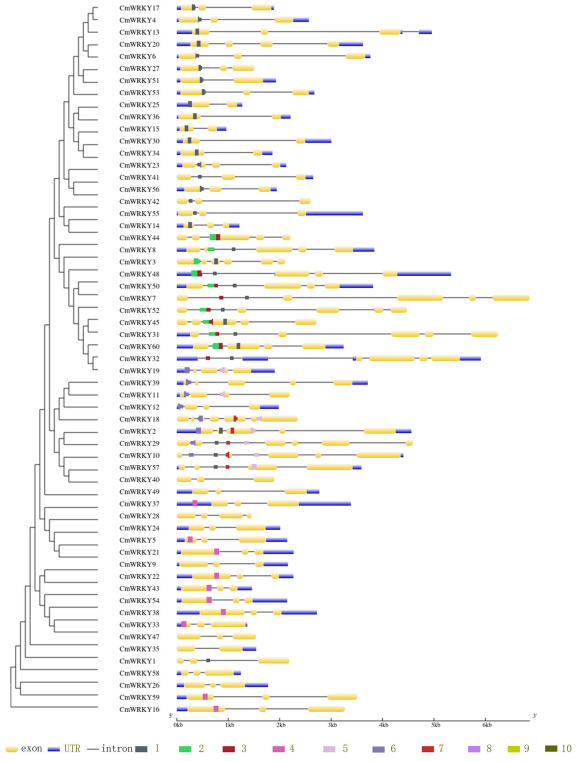

FIGURE 3
The gene structure and conserved motifs of CmWRKY genes. Left panel: the phylogenetic tree of CmWRKY domain. Right panel: the gene structure and conserved motifs. The exons, UTR and introns are indicated by yellow boxes, black lines and blue boxs, respectively.The motifs are represented by different colored boxes from number 1-10.

3.4 Synteny analysis of *CmWRKY genes*

To further explore the expansion and evolution of *CmWRKY* genes, the gene duplication events within the *CmWRKY* gene family were analyzed by conducting a genome synteny analysis. Thirteen recent duplication events involving 23 *CmWRKY* genes were detected (Figure 4A). In contrast, tandem duplication events were not observed among *CmWRKY* genes. These results indicated that some *CmWRKY* genes were possibly derived from genome segmental duplication events and segmental duplication events play an important role in the evolution of *CmWRKY* genes.

To examine the potential evolutionary relationships among *CmWRKY* genes, *CmWRKY*, *CsWRKY* and *MeWRKY* genes were used to conduct comparative syntenic analysis. Fifty-three *CmWRKY* genes and 52 melon WRKY genes showed syntenic relationships with CsWRKY genes, respectively (Figure 4B). A total of 48 syntenic relationships were detected among *C. metuliferus*, cucumber and melon WRKY genes, suggesting that most WRKY genes in the three genomes were conserved. Combing syntenic and phylogenetic analyses, all WRKY genes in these three *Cucumis* genomes displayed orthologous or paralogous gene relationships. No species-specific WRKY genes were detected, indicating that no new WRKY genes had evolved among *Cucumis* crops after their divergence from *Citrullus* genus.

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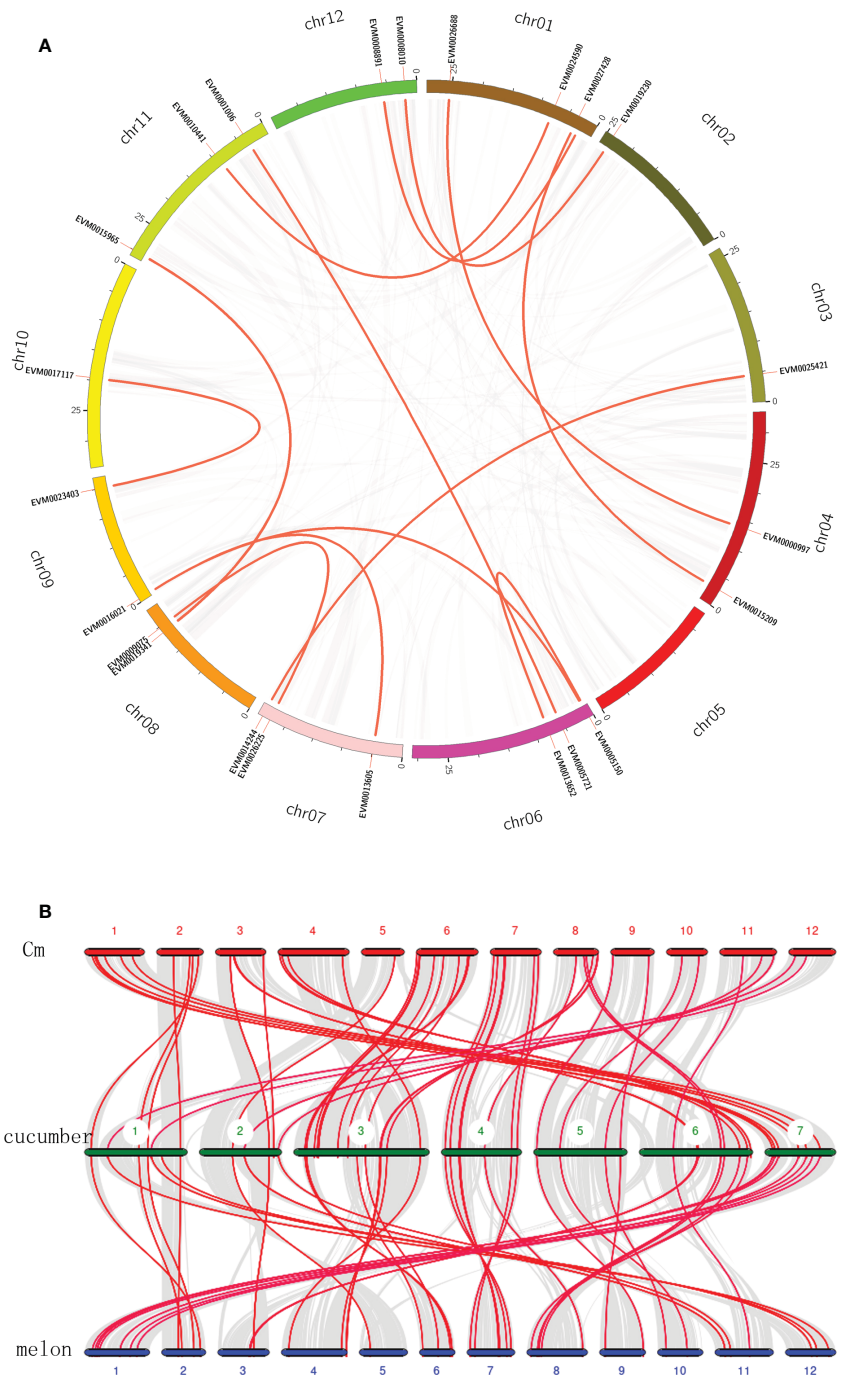

FIGURE 4
(A) Schematic representations of inter-chromosomal relationships of *CmWRKY* genes. Gray lines indicate synteny blocks in the *Cucumis metuliferus* genome, duplicated WRKY gene pairs are connected with red lines. **(B)** Synteny analysis of WRKY genes among *Cucumis metuliferus*, cucumber and melon. Gray lines in the background indicate the collinear blocks within these three *Cucumis* crops, and the red lines highlight the syntenic WRKY gene pairs. Cm represents *C. metuliferus*.

3.5 Expression patterns of *CmWRKY* genes at an early stage of RKN infection

Transcriptome data from *C. metuliferus* roots inoculated with *M. incognita* at an early stage of infection (3 and 7 days after infection (DAI) were used to detect the expression profile of *CmWRKY* genes in response to RKN infection. A total of 16 *CmWRKY* genes displayed a differential expression pattern at corresponding time points (3 or 7DAI), of which 13 *CmWRKY* genes were significantly upregulated, and three *CmWRKY* (*CmWRKY10, 52* and *59*) genes were significantly downregulated ($p<0.01$) (Figure 5). The expression of these differentially expressed genes (DEGs) were further verified by real-time PCR (RT-PCR). The results of RT-PCR were basically consistent with the RNA-

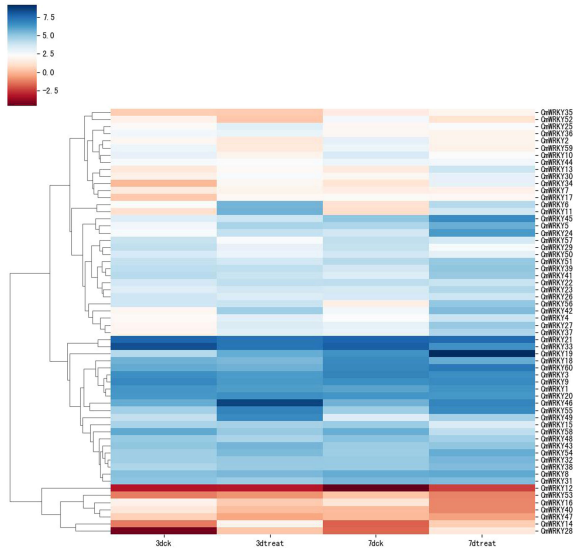

FIGURE 5
Expression pattern of *CmWRKY* genes in roots of *Cucumis metuliferus* in response to *Meloidogyne incognita* inoculation. The transcript levels of *CmWRKY* genes at 3 and 7DAI were investigated based on transcriptome data. The expression level of *CmWRKY* genes is shown as a heatmap using TPM value (log2). 3D-CK and 7D-CK represent the control of at 3DAI and 7DAI, respectively. The color scale shows increasing expression levels from red to blue.

sequencing (RNA-seq) (Figure 6; Table S5). However, in some instances, the differential expression in the RNA-seq data was significantly higher than that observed by RT-PCR. For example, at 3 DAI, the RNA-seq data showed indicated that *CmWRKY28* expression was 24 times higher compared with that of the control, whereas RT-PCR indicated that expression increased only eight times. It was noted that all DEGs did not show differential expression at 14 and 28 DAI, indicating that these WRKY DEGs only respond to RKN infection at an early stage of infection.

3.6 Subcellular localization of *CmWRKY* proteins

In this study, we selected the downregulated *CmWRKY10* and the upregulated *CmWRKY28* to investigate WRKY gene function in response to RKN infection (Figure 7A). *CmWRKY10* and *CmWRKY28* proteins were predicted to be localized nucleus, using protein PredicProtein software. To verify prediction for two *CmWRKY* proteins, the fusion GFP proteins vectors super1300:-*CmWRKY10*-GFP and super1300:-*CmWRKY28*-GFP were transiently expressed in *N. benthamiana* leaves. The signal from the green fluorescent protein (GFP) signal was observed with a laser-scanning confocal microscopy. The epidermal cells from leaves expressing the respective *CmWRKY* fusion protein were only detected in the nucleus. In contrast, super1300: GFP was detected in the cytoplasm and the nucleus (Figure 7B). These data suggested that the *CmWRKY10* and *CmWRKY28* proteins were nuclear localized proteins, which was consistent with the forementioned predictions. The two *CmWRKY* transcription factors may play roles in the response of *C. metuliferus* to RKN infection.

4 Discussion

There are several economically important crops in the *Cucumis* genus, such as melon and cucumber. Owing to the lack of RKN-resistance genes in the cucumber and melon genomes, RKN seriously restricts the yield and quality of cultivated melon and cucumber (Weng, 2010). *C. metuliferus* is a wild relative of cucumber and melon that shows a high degree of resistance to RKN (Walters et al., 2006). The resistance of *C. metuliferus* to RKN is attained through inhibition of the development and growth of RKN at the J2 stage (Ling et al., 2021). However, the resistance mechanism of *C. metuliferus* remains unclear, and the genes involved in the interactions between *C. metuliferus* and RKN have not been identified. With publication of the *C. metuliferus* genome (Ling et al., 2017), it is feasible to identify important pathogen-resistance gene using the *C. metuliferus* genomic data. WRKY genes are important transcription factors family that are extensively involved in plant response to biological stress. In this study, we identified 60 *CmWRKY* genes in the *C. metuliferus* genomes. The number of *CmWRKY* genes is similar to that in the genomes of cucumber (60) and melon (58). It was noted that the member number of WRKY genes in *C. metuliferus*, cucumber, and melon is significantly lower than that of *Arabidopsis* (72) and rice (101) (Abdullah-Zawawi et al., 2021). No genome-wide duplication events were detected in the reported genomes of *C. metuliferus*, cucumber and melon. This can be attributed to lack of segmental duplication and tandem duplication events in the genomes of *Cucumis* crops genome (Ling et al., 2011; Chen et al., 2020). Genome duplication is a source of new genes that assume novel functions during evolution (Lawton-Rauh, 2003). Given the lack of genome duplication events, some new WRKY genes with new functions may be absent in *Cucumis* crops compared with other plants, such as *Arabidopsis* and rice. In the present study, we observed that WRKY genes were highly conserved in *C. metuliferus*, cucumber and melon. Most of the WRKY genes (48) displayed a syntenic relationship and no species-specific WRKY genes were detected in any of genomes. Taken together, these results indicated that the WRKY genes in *Cucumis* might retain the functions of the ancestral WRKY genes in plants.

Ling reported that NBS-LRR genes, which are an important pathogen-resistance genes, comprise more members (104) in *C. metuliferus* compared with those in cucumber (66) and melon (67) (Ling et al., 2017). Insertion/deletion (indel) events in the *Cucumis* genome have deleted many NBS-LRR genes in cucumber and melon. However, we did not detect indel events in the WRKY gene loci in the three genomes. Unlike NBS-LRR genes, WRKY genes not only function in disease and pathogen resistance, but also participate widely in various important physiological activities of plants. Thus, the deletion of WRKY genes may severely affect the physiology of plants. The number of WRKY gene members in *C. metuliferus*, cucumber and melon remained essentially unchanged.

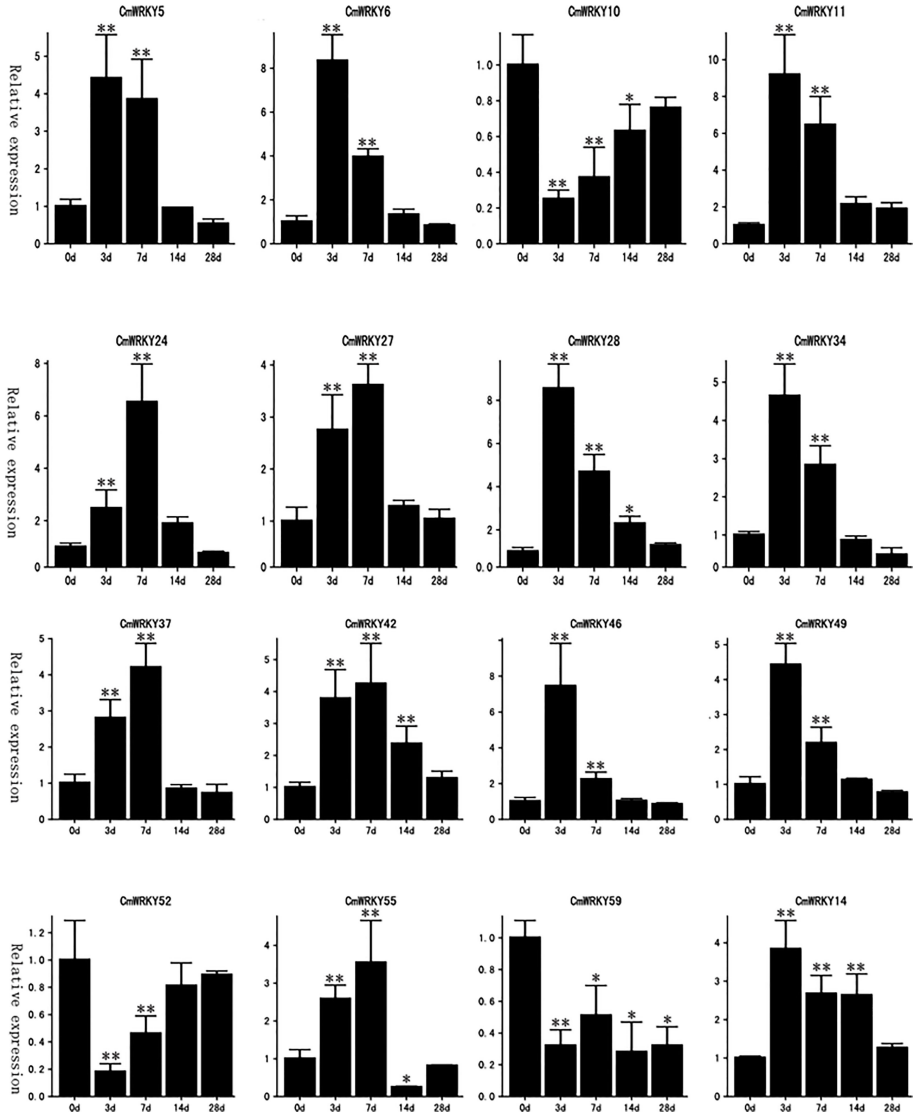

FIGURE 6
Real-time PCR detection of the expression patterns of 16 *CmWRKY* genes in roots of *Cucumis metuliferus* response to *Meloidogyne incognita* inoculation. The relative expression (y-axis) was calculated in accordance with the description in the Methods. The time points 0d, 3d, 7d, 14d, and 28d (x-axis) indicate the time(days) after inoculation. The error bars were calculated based on three replicates. Stars indicate a significant differences compared with the control (0d) (*p<0.05, **p<0.01).

Recent studies have revealed that WRKY genes are involved in plant-nematode interactions (Chinnapandi et al., 2019; Ribeiro et al., 2022). Most of this research has focused on tomato and soybean. The response of WRKY genes to nematode infection in cucurbitaceous crops has not been reported previously. A previous transcriptomic study of *C. metuliferus* roots infected by RKN has showed that plant hormone-related genes are involved in the response to RKN infection and that cytoskeleton-related genes are crucial regulators of *C. metuliferus* resistance to RKN (Ling et al., 2011). However, this study did not report on whether *CmWRKY* genes responded to nematode infection. In the present study, we analyzed the response of WRKY genes at an early stage of RKN infection, and identified 16 differentially expressed WRKY genes, of which 13 were upregulated and three were downregulated. The present expression analysis showed that *CmWRKY* genes are involved in the early response of the plant to nematode infection. We identified the orthologous genes of *CmWRKY10* and *CmWRKY28* in cucumber, which corresponds to Csav3_3g021980 and CsaV3_1G002180, respectively. The expression profiles of the two *CsWRKY* genes under RKN treatment were investigate, and no significantly differential expression can be detected for the two genes under RKN treatment in 3DAI and 7DAI (Figure S5). The results indicated that the differential expression of *CmWRKY* genes was related to nematode resistance. This is the first report on the response of WRKY genes to nematode in a cucurbitaceous crop. Therefore, this research enhances our understanding of WRKY gene regulation of plant resistance to RKN. However, the downstream target genes regulated by WRKY genes need to be identified to further clarify the anti-nematode function of WRKY genes.

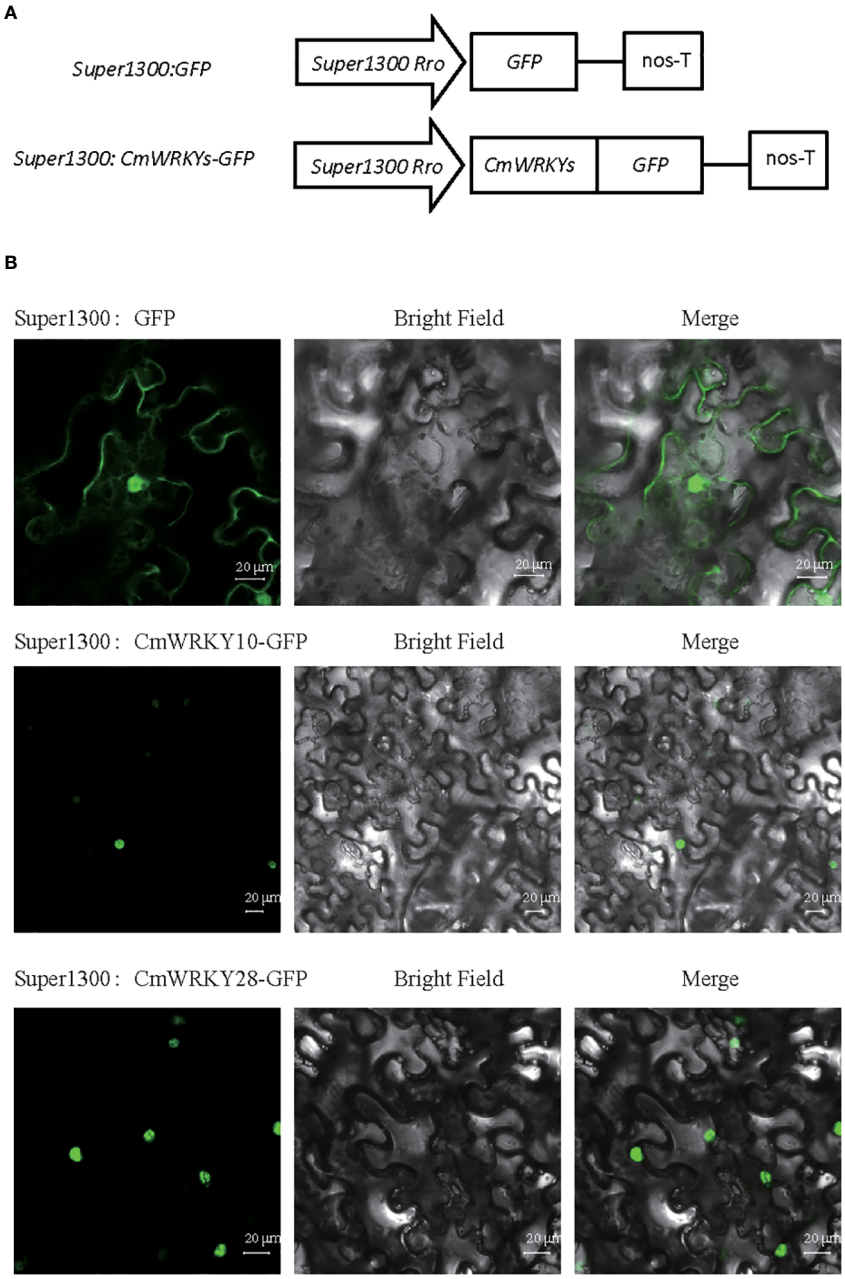

FIGURE 7
Subcellular localization analysis of *CmWRKY10* and *CmWRKY28* genes in tobacco leaf cells. **(A)** Schematic diagram of the control (super1300:GFP) and super1300:*CmWRKYs*-GFP fusion protein. **(B)** Transient expression of super1300: GFP and super1300:*CmWRKYs*-GFP in the tobacco leaf. A 48 h of after inoculation, green fluorescence signal was observed by laser confocal fluorescence microscopy.

5 Conclusion

Due to lack of root knot nematode (RKN) resistant genes in their genome, two important *Cucumis* crops melon and cucumber are suffered from serious RKN disease. *Cucumis metuliferus* is a wild *Cucumis* species, which displays a high level of RKN-resistance. WRKY transcription factors were involved in plant response to biotic stresses. In this study, we identified a total of 60 WRKY genes in *Cucumis metuliferus* genome. Syntonic analysis indicated that WRKY genes are highly conserved in *Cucumis metuliferus*, cucumber and melon. The expression patterns of *CmWRKY* genes under RKN stress treatment were also investigated, and 16 *CmWRKY* genes displayed differential expression at early stage of RKN infection were identified, in which two selected *CmWRKY* genes were located on nuclear by subcellular localization analysis. This is first report on WRKY genes expression profile under RKN stress in Cucumis crops. This study provided clues for further research on *CmWRKY* function in RKN-resist mechanism. Analysis of RKN-responding gene in RKN-resist Cucumis metuliferus can also provide clues to explore resistant genes in RKN-sensitive cucumber and melon.

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://ngdc.cncb.ac.cn/, PRJNA330972.

Author contributions

JL, JZ and ZM conceived the project, XP and YH contributed to WRKY gene family analysis, RL and QY contributed to functional experiment, YY, XL and BX contributed to experiment design. 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/fpls.2023.1143171/full#supplementary-material

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Molecular interaction between plants and *Trichoderma* species against soil-borne plant pathogens

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Trichoderma spp. (Hypocreales) are used worldwide as a lucrative biocontrol agent. The interactions of *Trichoderma* spp. with host plants and pathogens at a molecular level are important in understanding the various mechanisms adopted by the fungus to attain a close relationship with their plant host through superior antifungal/antimicrobial activity. When working in synchrony, mycoparasitism, antibiosis, competition, and the induction of a systemic acquired resistance (SAR)-like response are considered key factors in deciding the biocontrol potential of *Trichoderma*. Sucrose-rich root exudates of the host plant attract *Trichoderma*. The soluble secretome of *Trichoderma* plays a significant role in attachment to and penetration and colonization of plant roots, as well as modulating the mycoparasitic and antibiosis activity of *Trichoderma*. This review aims to gather information on how *Trichoderma* interacts with host plants and its role as a biocontrol agent of soil-borne phytopathogens, and to give a comprehensive account of the diverse molecular aspects of this interaction.

KEYWORDS

soil-borne phytopathogen, *Trichoderma*, molecular interaction, disease management, host plant

1 Introduction

Agriculture is an economic activity that deals with the scientific production of crops to address world hunger. Food is a fundamental right of humans; therefore, it is the utmost concern of all countries to increase their agricultural production, as the global population is expected to reach nearly 10 billion by 2050 (Gill and Garg, 2014; Dutta et al., 2022a). However, one of the major challenges encountered by agriculture today is the sustainable production of high-quality food in a sufficient quantity to meet the needs of the producer and consumer. Among the various biotic and abiotic factors contributing to the economic yield loss of crops, destruction due to diseases caused by filamentous fungi is of foremost importance (Singh, 2014). Soil-borne plant pathogens lead to a significant reduction in crop yield by causing diseases such as die-back, wilting, and root rot. They usually target the roots to enter into the plant system and directly influence water and nutrient uptake capacity. Soil-borne diseases therefore have a direct negative impact on plant growth and development (Dignam et al., 2022). The management of soil-borne plant diseases is a cumbersome task. Large amounts of chemical pesticides are administered early in the farming process to counteract these phytopathogens. The use of chemical pesticides, however, has a negative impact on the environment, such as residual toxicity and soil pollution. Therefore, the biological management of plant diseases with different bacterial and fungal biocontrol agents is considered a safer option. *Trichoderma*, a soil-inhabiting ascomycete fungus, is widely used for its versatile plant growth-promoting (PGP) and biocontrol activity. First described by Persoon (1794), the genus *Trichoderma* outraces phytopathogens in the competition for space, nutrients, antibiosis, and mycoparasitism (Mukherjee et al., 2013). Furthermore, *Trichoderma* is also known to colonize plant roots, to enhance plants' systemic defenses, viz., systemic acquired resistance (SAR) and induced systemic resistance (ISR), and to promote plant growth by modulating the phytohormonal blend. To do so, *Trichoderma* needs to interact and establish a good relationship with the host plant. Proteins or peptides are the communicating molecule in any plant–fungus interaction. Plants and fungi communicate and perceive their surroundings *via* the secretion and perception of different peptides. Understanding the soluble secretome of *Trichoderma* will shed light on the mechanisms of molecular crosstalk between plant roots and *Trichoderma*, and will explain the mechanism behind PGP and biocontrol activities.

2 *Trichoderma*: role as a biocontrol agent

In the early 1930s, the biological control potential of *Trichoderma* was realized. Weindling, while working with *T. lignorum* and *Rhizoctonia solani*, observed that the mycelial growth of *R. solani* was inhibited by the profuse mycelial growth of *Trichoderma*. Microscopic observation led to the discovery of a new phenomenon, whereby the hyphae of *T. lignorum* coil around the hyphae of the phytopathogen and penetrate them, which subsequently leads to complete dissolution of the host cytoplasm. The mechanism of parasitization of another fungus by *Trichoderma* was named mycoparasitism by Weindling (1932). The discovery of the mycoparasitic nature of *Trichoderma* led a great volume of work on the subject by many researchers. The studies carried out led to the discovery of different biocontrol mechanisms exhibited by fungi from the genus *Trichoderma*. Genome profiling of three *Trichoderma* species, viz., *T. virens*, *T. atroviride*, and *T. reesei*, by Mukherjee et al. (2012) has opened avenues for understanding the molecular mechanism behind their advantageous biocontrol activities. Mycoparasitism, competition with other soil inhabitants/invaders, and antibiosis are the major modes of action for the biocontrol activity of *Trichoderma* (Zhang et al., 2021). Synchronization between mycoparasitism and antibiosis is necessary for the proper functioning of this biocontrol agent (BCA) (Keswani et al., 2014). Moreover, the host defense activation triggered by *Trichoderma* is a key part of its ability to protect plants against several phytopathogens. Therefore, it can be said that a combination of competitive exclusion, antibiosis, mycoparasitism, and induced systemic resistance (Figure 1) is crucial for *Trichoderma*-mediated disease suppression/management (Sharma et al., 2017). *Trichoderma* is a very fast-growing BCA that rapidly colonizes the spermosphere and/or rhizosphere, thereby providing protection to germinating seeds against major soil-borne, seed-borne, and air-borne plant diseases (Mukherjee et al., 2022).

3 Major biological control strategies of *Trichoderma*

3.1 Mycoparasitism: *Trichoderma*'s deadly weapon for the management of phytopathogens

The phenomenon of mycoparasitization by *Trichoderma*, as first reported by Weindling (1932), is a complex process involving sequential events. Direct confrontation with the fungal pathogen

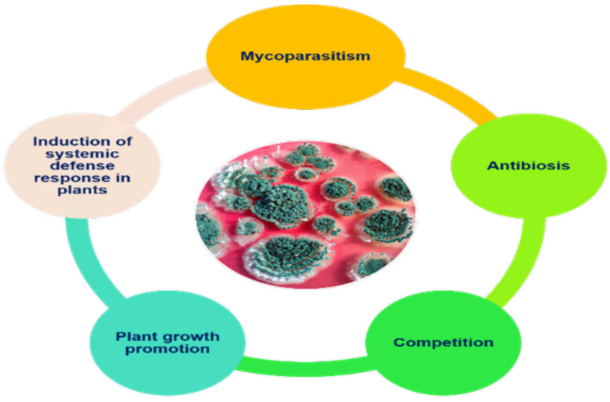

FIGURE 1
Key components of *Trichoderma*-mediated disease suppression.

and the secretion of cell wall-degrading enzymes (CWDEs) is followed by the penetration and subsequent killing of the fungal phytopathogen (Woo and Lorito, 2007). The primary identification and attachment between *Trichoderma* and the prey fungi is mediated by the binding of the cell wall carbohydrates of *Trichoderma* to the lectin of the target fungi, which is followed by hyphal coiling. The adhesion of *Trichoderma* to the mycelium of the host fungi is facilitated by hydrophobins, which is evident from the expression of the *Vel1* gene of *T. virens* encoding hydrophobins (Viterbo and Chet, 2006). Penetration into the target hyphae occurs *via* the development of appressoria containing a high concentration of osmotic solutes such as glycerol, which is necessary for exerting mechanical pressure to invade the hyphal wall. The use of fungitoxic CWDEs, such as chitinases, glucanases, and proteases, by *Trichoderma* combined with the mechanical strength exerted by the appressorium is crucial to the successful penetration of the host hyphae. Following penetration into the lumen of the target hyphae, the cumulative effect of the CWDEs dissolves the host cell wall, which ultimately results in parasitization and facilitates the assimilation of cell wall content, leading to the subsequent killing of the target fungus (Howell, 2003; Harman et al., 2004; Sood et al., 2020). In addition, *Trichoderma* disarms the target fungi by deactivating the enzymes necessary for pathogenic fungi to colonize and penetrate the plant tissue (Harman et al., 2004). There are approximately 75 species of *Trichoderma* reported to exhibit mycoparasitic activity against a wide range of phytopathogens (Verena et al., 2011; Harwoko et al., 2021). Different studies have revealed the significant effect of several strains/species of *Trichoderma* in the management of phytopathogens such as *Fusarium oxysporum*, *F. culmorum*, *Gaeumannomyces graminis* var. *tritici*, *Pythium aphanidermatum*, *R. solani*, and *Sclerotium rolfsii* in both greenhouse and field conditions (Das et al., 2006; Dutta et al., 2008; Gajera et al., 2013). The hyperparasitization of *F. graminearum* by *Trichoderma* takes place by the *Trichoderma* clutching and coiling around the target mycelium, interpenetration, and other mechanisms, resulting in deformed mycelium of *F. graminearum* that eventually disappear (Tian et al., 2018). Chitinase secreted by *T. hamatum* plays an important role in promoting disintegration of fungal cell wall, chitin assimilation, mycelial autolysis, mycoparasitism, and impeding mycelial growth, spore germination, and spore formation (Saravanakumar et al., 2017). Similarly, *T. koningiopsis* exhibits mycoparasitic activity against *S. sclerotiorum* by invading the host hyphae, which it achieves by attaching to and wrapping around the targeted hyphae and then breaking them into small fragments until it completely disintegrates (Shaw et al., 2016). *Trichoderma* perceives the presence of target fungi in its surroundings *via* seven transmembrane G protein-coupled receptors, e.g., Grp1 (Omann et al., 2012). When pathogen ligands bind to the receptor, it causes a downstream signaling cascade by stimulating G proteins and mitogen-activated protein kinases (MAPKs). There are three MAPKs known in different *Trichoderma* species, viz., MAPKKK, MAPKK, and MAPK. Signal transduction *via* these pathways may have an important role in the mycoparasitization and biocontrol of phytopathogens. Furthermore, the synthesis and secretion of pathogenesis-related enzymes, viz., CWDE and fungitoxic secondary metabolites such as peptaibols, is an extremely useful chemical resource used by *Trichoderma* to eradicate pathogens (Figure 2) (Omann et al., 2012; Gajera et al., 2013; Dutta et al., 2022b). Although *Trichoderma* spp. are traditionally known as necrotrophic mycoparasites, an extensive scientific study (Mukherjee et al., 2022) has also revealed hemibiotrophic nature. Hemibiotrophic *Trichoderma* causes minor damage to the cell wall of the host fungi and is reported to exist intracellularly for a notable period of time.

3.1.1 Evolution of mycoparasitism from a genomic perspective

The ability of the fungi to grow indefinitely as hyphae with great metabolic diversity and their ability to interact with other living components of the ecosystem account for their evolutionary success (Naranjo-Ortiz and Gabaldon, 2019). Mycoparasitic associations were found in the oldest fungal fossil, aged 410 million years (Hass et al., 1994). Comparison of the genome sequences of *T. atroviride*, *T. reesei*, and *T. virens* revealed information about the common ancestral mycotrophic lifestyle of these species (Kubicek et al., 2011; Schmoll et al., 2016; Karlsson et al., 2017). Their mycotrophic lifestyle further evolved to slowly colonize dead wood, plants, animals, and immunocompromised humans, providing new ecological niches for their growth and development (Druzhinina et al., 2018). It is speculated that the ancestor of *Trichoderma* had limited cellulolytic capacity and fed on either fungi or arthropods, and lateral gene transfer (LGT) is to some extent considered responsible for the formation of this genus. Nearly half the genes for plant CWDEs [belonging to the group called carbohydrate active enzymes (CAZymes)] found in genome profiling of *Trichoderma* were from plant-associated Ascomycota, indicating a competitive advantage for the mycoparasite in colonizing and feeding on these fungi. However, LGT is not reported in *Trichoderma* for the mycoparasitism of unrelated fungi such as basidiomycetes and oomycetes (Druzhinina et al., 2018; Mukherjee et al., 2022). A comparative analysis of the presence in 12 *Trichoderma* spp. of peculiar gene families that are not shared by other fungi might be useful in explaining their ability to deconstruct host/prey cells. An extremely high number of genes gained by

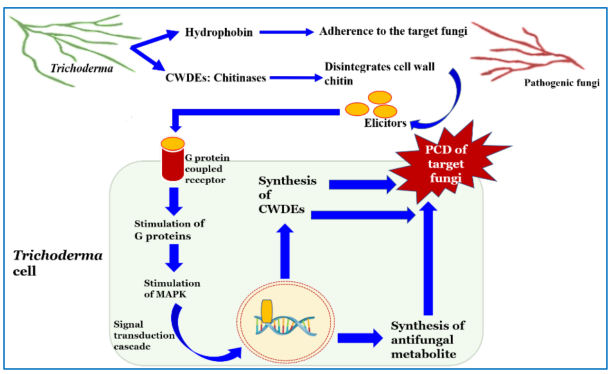

FIGURE 2
Mode of action of *Trichoderma* against phytopathogens.

Trichoderma belong to different domains, viz., heterokaryon incompatibility (HET), ankyrin repeat, and the major facilitator superfamily (MFS) transporter families. In comparison with other fungi, *Trichoderma* genomes have been found to possess a higher number of gene families encoding CAZymes, secondary metabolism-related genes, and transcription factors. Moreover, as well as heterokaryon incompatibility, the HET genes may play a important role in sensing the prey fungus (Kubicek et al., 2019).

The study of the transcriptomics of *Trichoderma*'s interactions with host fungi revealed that the strategies taken up against prey fungi differ among different *Trichoderma* species. Atanasova et al. (2013) conducted a transcriptomic study on the specific interactions of three *Trichoderma* spp., viz., *T. atroviride*, *T. virens*, and *T. reesei*, with *R. solani*, which revealed the different strategies adopted by these BCAs. Th results revealed that *T. atroviride* uses diverse strategies that include the up-regulation of the biosynthesis of secondary metabolites and enzymes such as GH16 β-glucanases, proteases, and small secreted proteins. *T. reesei* was found to increase the expression of cellulases, hemicellulases, and transporter-encoding genes, whereas *T. virens* relied strongly on its toxic secondary metabolite, expressing primarily the genes responsible for gliotoxin biosynthesis. Expression of genes encoding β-1,3- and β-1,6-endoglucanase were observed in the transcriptome of *T. harzianum* during co-culture with host fungi. Similar results were obtained for *T. asperellum* during co-culture with mycophytopathogens (Mukherjee et al., 2022). Further research on transcriptomics revealed the up-regulation of genes encoding proteases in *Trichoderma* transcriptomes, indicating that proteolysis is a prominent process in this BCA's mycoparasitism (Morán-Diez et al., 2019). Zapparata et al. (2021) studied the interactions of *T. gamsii* with *F. graminearum*, with a special focus on transcriptomic changes in both organisms during the sensing phase. The findings revealed an increase in the expression of genes for ferric reductase in *T. gamsii*, which are essential for iron competition among both fungi; similarly, the expression of defensive genes such as genes encoding killer toxin and transporters was upregulated by *F. graminearum*.

3.2 Antibiosis and role of secondary metabolites of *Trichoderma*

Study of the mycoparasitic activity of *T. lignorum* on *R. solani* (Weindling, 1932) *via* coiling of hyphae, coagulation of protoplasts, and loss of vacuolated structures led to the discovery of a lethal principle with the ability to suppress *R. solani* in pot cultures (Weindling and Emerson, 1936; Weindling and Fawcett, 1936). The lethal principle was later identified as gliotoxin—a highly antimicrobial secondary metabolite of *Trichoderma* (Weindling, 1934). *Trichoderma* secretes a wide range of chemically divergent secondary metabolites with broad-spectrum antimicrobial activity in the vicinity of their niche, which in turn inhibit the growth or spore germination of mycopathogens, a process known as antibiosis (Keswani et al., 2014). Owing to their biochemical nature, the secondary metabolites of *Trichoderma* perform antibiosis by acting as the metabolic inhibitor of translational pathways, thereby blocking protein synthesis and promoting mycoparasitism by helping in the penetration of the hyphae of prey fungus, as well as inhibiting cell wall synthesis, growth, reproduction, sporulation, nutrient uptake, and metabolite production by target pathogens. Moreover, the antibiosis mediated by the secondary metabolites of *Trichoderma* is greatly influenced by the species and strain of the fungal agent (Khan et al., 2020). A pot assay targeting *R. solani* and *P. debaryanum* in cucumber and pea revealed that the suppression of targeted pathogens was attributed to the antibiosis mechanism of *T. virens* (Allen and Haenseler, 1935). Diverse studies conducted since then have revealed considerable antifungal/antimicrobial activities exhibited by several secondary metabolites of *Trichoderma* against a wide range of phytopathogens (Table 1). Viridiol is another antifungal compound released by *Trichoderma* spp. that also inhibits the enzyme activity of 5′-hydroxyaverantin dehydrogenase, which is necessary for aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus*, and, therefore, affects aflatoxin biosynthesis (Sakuno et al., 2000; Wipf and Kerekes, 2003). In addition to antibiosis, these metabolites could also play a significant role in competition, mycoparasitism, and stimulating the plant's immune system (Zeilinger et al., 2016). It is, therefore, very difficult to study mycoparasitism in isolation. A comparative assessment of sclerotial parasitism, hyphal parasitism, and antibiosis exhibited by *T. virens* (P strain), led Mukherjee et al. (1995), concluded that sclerotial parasitism is the major mechanism used in controlling *S. rolfsii* and *R. solani* in soil (Dutta et al., 2013; Dutta, 2018). In a mutant of *T. virens* (developed using gamma ray-induced mutagenesis) with upregulated genes for plant interaction, the production of secondary metabolites was found to provide excellent protection against collar rot in lentil and chickpea in both greenhouse and on-farm trials (Mukherjee et al., 2019). A UV-induced mutant of *T. virens* deficient in genes for mycoparasitism was observed to be an equally efficient biocontrol agent against *R. solani* as its parental type, and gliotoxin-deficient mutants exhibited a similar result (Howell, 1987; Howell, 2003); however, sclerotial parasitism remained unexamined by these experiments. Therefore, this case raised the question of the mechanism used by *Trichoderma* for efficient control of phytopathogens, and the probable role of induced systemic resistance in host plant was emphasized. Tu (1980) observed that the sclerotia of *Sclerotinia sclerotiorum* were readily parasitized by *T. virens*, with extensive hyphal growth of the mycoparasite inside the colonized sclerotia; however, they could not find any conidia inside. A study conducted by Liu et al. (2009) revealed that, during symbiotic colonization of plant roots by *Trichoderma*, *Trichoderma* secretes a higher number of secondary metabolites, such as pachybasin and chrysophanol, with a lower degree of oxidation and less antimicrobial activity. Interestingly, when the host plant is encountered by any phytopathogens, the reactive oxygen species (ROS) released by the plant convert these weakly antimicrobial metabolites to highly antimicrobial oxidized secondary metabolites, viz., 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and emodine. They perform dual beneficial roles by both acting as a powerful antifungal agent that helps in promoting the competitive efficiency of *Trichoderma* and

TABLE 1 Diverse secondary metabolites secreted by *Trichoderma* spp. and their functions in plant disease management.

Class of secondary metabolite	Name of the compound	Activity performed	Reference
i) Against bacterial phytopathogens			
Peptaibols	Trichokonin VI, VII, and AVIII	Highly effective against the Gram-positive bacterial phytopathogen *Clavibacter michiganensis* subsp. *michiganensis*, causing bacterial wilt and canker in tomato, potato, and maize	Song et al. (2006)
Pyridone	Trichodin A	Antibiotic activity against Gram-positive bacteria	Wu et al. (2014)
Viridiofungin	Viridiofungin A	Effective against *Erwinia amylovora* and *C. michiganensis*	El-Hasan et al. (2009)
–	Secondary metabolites obtained from *T. pseudoharzianum* (T113) and *T. viridae*	Effective against bacterial phytopathogens, viz., *Ralstonia solanacearum* and *Xanthomonas campestris*	Khan et al. (2020)
ii) Against fungal phytopathogens			
Pyrones	6-Pentyl-2H-pyran-2-one	Antifungal activity against *Rhizoctonia solani* and *Fusarium oxysporum* Effective in reducing *Botrytis* fruit rot of kiwi fruits	Scarselletti and Faull (1994); Poole et al. (1998)
	Viridepyronone	Exhibits 90% antagonistic activity against *Sclerotium rolfsii* at MIC 196 mg/ml	Hill et al. (1995); Evidente et al. (2003); Kishimoto et al. (2005)
	Massoilactone and δ-decanolactone	Effective against *Phytophthora* and *Botrytis*	Hill et al. (1995)
Koninginins	Koninginins A, B, D, E, and G	Antifungal activity against *Gaeumannomyces graminis* var. *tritici*	Almassi et al. (1991); Ghisalberti and Rowland (1993)
	Koninginins A, B, and D	Broad antifungal activity against several fungal phytopathogens, viz., *F. oxysporum*, *Bipolaris sorokiniana*, *Phytophthora cinnamomi*, and *Pythium middletonii*	Dunlop et al. (1989); Chen et al. (2015)
Steroids	Stigmasterol	Antifungal activity against *R. solani*, *S. rolfsii*, *Macrophomina phaseolina*, and *F. oxysporum*	Ahluwalia et al. (2014); Ahluwalia et al. (2015)
	Ergosterol, 3,5,9-trihydroxyergosta-7,22-dien-6-one	Effective against *Pyricularia oryzae*, *Aspergillus niger*, and *Alternaria alternata* at MIC 32 µg/mL	Xuan et al. (2014)
Furanosteroids	Viridin	Broad spectrum antifungal activity against *A. niger*, *Botrytis allii*, *Colletotrichum lini*, *F. caeruleum*, *Stachybotrys atra* and *P. expansum*	Brian and McGowan (1945)
Pyridone	Harzianopyridone	Antagonists to *Botrytis cinerea*, *G. graminis* var. *tritici*, *R. solani*, *Phytophthora* spp., *Leptosphaeria maculans*, *S. rolfsii* and *F. oxysporum*	Dickinson et al. (1989); Vinale et al. (2006); Vinale et al. (2009); Ahluwalia et al. (2015)
	Harzianic acid	Highly antifungal activity against soil-borne plant pathogens such as *Pythium irregulare*, *Sclerotinia sclerotiorum*, and *R. solani*	Vinale et al. (2009)
Epipolythiodioxopiperazines	Gliotoxin	Inhibitory against *Rhizoctonia bataticola*, *M. phaseolina*, *Pythium debaryanum*, *Pythium aphanidermatum*, *S. rolfsii* and *R. solani*,	Jones and Pettit (1987); Singh et al. (2005)
	Gliovirin	Antagonistic activity against *Pythium ultimum* and *R. solani*	Howell and Stipanovic (1983); Nakano et al. (1990)
Peptaibols	Trichokonins VI, VII, and VIII	Highly antagonistic to soil-borne phytopathogens such as *R. solani*, *F. oxysporum*, *Verticillium dahliae*, and *B. cinerea*, and *Phytophthora parasitica*	Song et al. (2006); Shi et al. (2012); Zhao et al. (2018)
	Trichokonin	Induces ROS production, synthesis, and accumulation of phenolic compounds at the application site, and activation of multiple defense signaling pathways in plants	Luo et al. (2010)
	Trichorzianines A1 and B1	Exhibit antifungal activity by acting as an inhibitor of spore germination and hyphal elongation of phytopathogenic fungi	Goulard et al. (1995); Lee et al. (1999)
	A-aminoisobutyric acid and isovaline		Dutta et al., (2022b)

(Continued)

TABLE 1 Continued

Class of secondary metabolite	Name of the compound	Activity performed	Reference
		Highly effective against oomycetes fungi, act as an inhibitor of β-glucan synthase	
	Trichostromaticins A–E	Antagonistic activity against *Moniliophthora perniciosa*, a causal agent of witches' broom disease in cocoa	Degenkolb et al. (2008)
	Trichogin GA IV and its derivatives	Preventive efficacy against *B. cinerea* infection in tomato plants	Baccelli et al. (2022)
Butenolides	Harzianolide	Antagonistic to pathogens that cause take-all in wheat, viz., *G. graminis* var. *tritici*, *P. ultimum*, *R. solani*, and *B. cinerea*	Almassi et al. (1991); Vinale et al. (2006); Vinale et al. (2009)
	T39butenolide	Highly antagonistic to *G. graminis* var. *tritici*, inhibitory to *P. ultimum*, *R. solani*, and *B. cinerea*	Almassi et al. (1991); Vinale et al. (2006); Vinale et al. (2009)
	5-Hydroxyvertinolide	Antifungal activity against *Mycena citricolor*, the causal organism of American leaf spot disease in coffee	Andrade et al. (1992)
Azaphilones	T22azaphilone, harziphilone, fleephilone,	Antagonistic activity against *P. ultimum*, *G. graminis* var. *tritici*, *R. solani*, *B. cinerea*, *P. cinnamomi*, and *L. maculans*. Provides self-protection to ROS liberated during mycoparasitic interaction with *F. oxysporum* f. sp. *Cubanse* race 4	Vinale et al. (2006); Vinale et al. (2008); Vinale et al. (2009); Pang et al. (2020)
Koninginins	Koninginins A-E	Inhibitory to *G. graminis* var. *tritici;* antifungal activity against *Alternaria panax*, *B. sorokiniana*, *F. oxysporum*, *F. solani*, *P. cinnamomi*, and *P. middletonii*	Almassi et al. (1991); Dunlop et al. (1989); Ghisalberti and Rowland (1993); Chen et al. (2015)
Anthraquinones	1,8-Dihydroxy-3-methylanthraquinone, 1-hydroxy-3-methylanthraquinone	Exhibit antifungal activity against *G. graminis* var. *tritici* and *P. ultimum*	Vinale et al. (2006)
	Chrysophanol, pachybasin	Reduced antimicrobial activity; released in symbiotic interaction with plant roots	Liu et al. (2009)
	1,5-Dihydroxy-3-hydroxymethyl-9,10-anthraquinone; emodin; 1,7dihydroxy-3-hydroxymethyl-9,10-anthraquinone	Powerful antimicrobial agent, effective against *R. solani* and *B. cinerea* Escalates host plant's defense response against phytopathogen	Liu et al. (2009)
Lactone	Cremenolide	Antagonistic activity against *R. solani*, *B. cinerea*, *and F. oxysporum;* exhibit PGP activity	Vinale et al. (2016)
	Aspinolide C	Exhibit antibiotic activity against *B. cinerea* and *Fusarium sporotrichioides;* activates host plant defense against phytopathogenic fungal invasion	Malmierca et al. (2015)
	Cerinolactone	Strong antifungal activity against *Rosellinia necatrix*—the causal agent of white root rot in apple, pear, apricot, strawberry, etc.	Vinale et al. (2012); Arjona-Girona et al. (2014)
	Nafuredin C, nafuredin A	Exhibit moderate antifungal activity	Zhao et al. (2020)
Trichothecenes	Trichodermin	Broad antifungal activity against several phytopathogenic fungi, such as *B. cinerea*, *Colletotrichum lindemuthianum*, *Colletotrichum gloeosporioides*, *Cochliobolus miyabeanus*, *F. oxysporum*, *R. solani*, and *Thanatephorus cucumeris*	Shi et al. (2009); Sha et al. (2013); Shentu et al. (2014)
	Trichodermarins G–N, trichodermol, trichodermin, trichoderminol, trichodermarins A and B, and 2,4,12-trihydroxyapotrichothecene	Exhibit antifungal and antimicrobial activity; highly effective against *B. cinerea*, *C. miyabeanus*, *F. oxysporum* f. sp. *cucumerium*, *F. oxysporum* f. sp. *niveum*, and *Phomopsis asparagi*	Shi et al. (2020)
	Trichobreols A–E	Exhibit broad antifungal activity	Yamazaki et al. (Yamazaki et al., 2020a and Yamazaki et al., 2020b)
	Trichothecinol A, 8-deoxy-trichothecin, trichothecinol B, and trichodermene A	Antagonistic activity against a broad range of soil-borne phytopathogens	Du et al. (2020)

(Continued)

TABLE 1 Continued

Class of secondary metabolite	Name of the compound	Activity performed	Reference
Isocyanide	Dermadin	Antibiotic activity against *Phytophthora* spp.	Dutta et al., (2022b)
Polyketides	Trichoharzianol	Antifungal activity against *Colletotrichum gloeosporioides*	Jeerapong et al. (2015)
Peptide	Alamethicin	Activation of plant defense, viz., ISR and SAR in lima bean	Engelberth et al. (2001)

MIC, minimum inhibitory concentration; PGP, plant-growth promoting.

escalating the immune response of the host plant to other phytopathogens. This finding indicates that, in a tripartite interaction of plant–*Trichoderma*–pathogen, the plant has been previously well equipped with a reservoir of anthraquinone secondary metabolites of *Trichoderma* that engage in antagonistic activity only when the plant encounters a pathogenic invasion. A study of the genomics of *T. virens* revealed that *NRPS Tex2* (non-ribosomal peptide synthetase-encoding gene Tex2) is responsible for the assemblage of 11- and 14-module peptaibols (Mukherjee et al., 2011), which elicit strong antimicrobial effects. Trichokonin VI, a peptaibol isolated from *T. pseudokoningii*, is reported to cause programmed cell death in *F. oxysporum via* formation of voltage-gated channels in the pathogen membrane. Similarly, *SMF2*-derived trichokonin VI in *T. pseudokoningii* was reported to exhibit antimicrobial activity against wide range of fungal phytopathogens by stimulating wide-ranging apoptotic programmed cell death (PCD) (Tijerino et al., 2011; Shi et al., 2012; Sood et al., 2020). Gliotoxin and gliovirin are the polyketides synthesized by the P and Q group strains of *Trichoderma* and have a significant role in managing deadly soil-borne phytopathogens. Interestingly, the *T. virens* P group strain is highly antagonistic to *P. ultimum*, but not to *R. solani*. Similarly, the Q group strain adversely affects *R. solani* (Howell et al., 2000). Further research into the genomic perspective of the secondary metabolism of *Trichoderma* revealed that the *T. virens* gene *veA* ortholog *vel1* encoded the VELVET protein, which is responsible for regulation of both the biosynthesis and biocontrol activity of gliotoxin, and also adjusts the expression of other genes involved in the secondary metabolism (Mukherjee et al., 2012).

3.3 Competitive exclusion of phytopathogens by *Trichoderma*

Trichoderma is known as an aggressive colonizer of plant roots that competes for space, nutrients, water, or oxygen by mobilizing immobile soil nutrients, thereby eliminating other micro-organisms that inhabit their niche (Elad et al., 2000; Dutta et al., 2022b due to the diversified composition of root exudates secreted by plants. Competition among micro-organisms is a strategy to utilize the nutrient hotspots present in the rhizosphere by eliminating other competitors (Guzmán-Guzmán et al., 2023). Therefore, to be an effective colonizer of plant roots, those organisms must have metabolic versatility and the competitive capacity to occupy the nutrient hotspots. In this regard, *Trichoderma* can be considered as an aggressive competitor because it has the capacity to secrete a plethora of chemically diverse secondary metabolites that have an antagonistic effect on other micro-organisms (i.e., competitive capacities) and it also exhibits rapid growth and colonization strategies (indicating metabolic versatility) that enable it to occupy space in rhizosphere, enhance plant growth, and restrict further growth of potentially pathogenic micro-organisms (Saravanakumar et al., 2017). The presence of ATP-binding cassettes transporters (ABC transporters) in *Trichoderma* ensures enhanced competitive ability by conferring resistance to toxic metabolites secreted by other micro-organisms (Harman et al., 2004). Moreover, *Trichoderma* is compatible with sublethal doses of chemical fertilizers such as urea and muriate of potash, and many chemical pesticides such as thiamethoxam, methomyl, imidacloprid, and methyl bromide, which is attributed to the presence of ABC transporters in *Trichoderma* (Chet et al., 1997; Gajera et al., 2013; Dutta et al., 2017). *Trichoderma* releases certain iron chelators, i.e., siderophores, which become bound to iron present in soil. Iron is a key micronutrient for the viability of fungi, and therefore the release of iron-chelating siderophores by *Trichoderma* is detrimental to the growth of other fungi. This is one of the main reasons for the biocontrol potential of *Trichoderma* against soil-borne phytopathogens such as *Pythium*, *Fusarium*, and *Botrytis*, which is inversely proportional to the concentration of nutrients in soil (Tjamos et al., 1992). The discovery of Gtt1 (high-affinity glucose transporter) in *T. harzianum* CECT 2413 raised questions about the probable role of glucose transporters during competition by *Trichoderma*. Delgado-Jarana et al. (2003) observed that *Gtt* gene expression is upregulated when *T. harzianum* CECT 2413 is subjected to growth in nutrient-deficient media. Moreover, a mutant of *Trichoderma* with an additional copy of the glucose transporter gene performed strongly, with a two- to threefold increase in glucose uptake. Glucose metabolism is essential in the assimilation of enzymes and permeases, as well as proteins involved in membrane and cell wall modifications (Delgado-Jarana et al., 2003).

3.4 Impact of *Trichoderma* colonization on plant defense and growth promotion

The sessile lifestyle of plants depends on their ability to adapt to the challenges presented by the outside environment in terms of pathogen attack, nutrient starvation, and exposure to toxins and contaminants which are detrimental to its growth. Owing to the abiotic and biotic stresses faced by plants, growth–defense trade-offs take place, which prioritize the acquisition and use of resources

(Hacquard et al., 2016). The production of ROS under such conditions determines important developmental processes and cross-kingdom relationships (Segal and Wilson, 2018).

Plant immunity consists of a robust three-layered protection (Figure 3). The first layer of defense safeguards them from foreign invasion and takes the form of physical barriers such as wax, a cuticle layer, stomata, and the cell wall (Lawry, 2016). The second and third layers are based on molecular pattern recognition. All micro-organisms, irrespective of whether they are pathogenic or beneficial, possess unique molecular patterns known as microbe-associated molecular patterns (MAMPs). The unique molecular patterns present in pathogenic microbes are known as pathogen-associated molecular patterns (PAMPs). The second layer of plant immunity consists of different enzymes and pattern recognition receptors (PRRs) that recognize the MAMPs/PAMPs, which leads to the activation of active immune responses. Another possible element of this layer of plant immunity is damage-associated molecular pattern (DAMP) recognition. DAMP recognition receptors help the plant in recognizing any damage caused to the plant system due to invasion by a micro-organism. Therefore, the innate or basal plant immunity comprises three components, viz., MAMP, PAMP, and DAMP recognition receptors, and together this layer of plant immunity is known as molecular pattern-triggered plant immunity (MTI) (Jones and Dangl, 2006; Benedetti et al., 2015). The third layer is based on effector recognition and is known as effector-triggered immunity (ETI). Effectors (previously known as avirulence or Avr proteins) are the molecules released by pathogens/micro-organisms to escape the MTI of plants. Effectors released by micro-organisms help them to counter MAMP-triggered immunity by, for example, scavenging MAMPs, degrading proteases released by plant, and/or deregulating the primary and secondary signaling pathways of plant host. Containing a nucleotide-binding site and leucine-rich repeats (NBS-LRR), the resistance protein (R protein) present in the plant responds to the effectors and triggers a systemic resistance response (i.e., SAR) due to the accumulation of salicylic acid. However, the effector-triggered interaction is always under a tremendous selection pressure that would enable the pathogen/micro-organism to overcome plant immunity and the plant host to retain its immunity (Lawry, 2016). These two layers of plant immunity (viz., MTI and ETI) greatly influence the plant's response to invading microbes and trigger strong systemic resistance reactions in the plant. Therefore, in order to successfully enter the plant roots and colonize them, *Trichoderma* need to breach

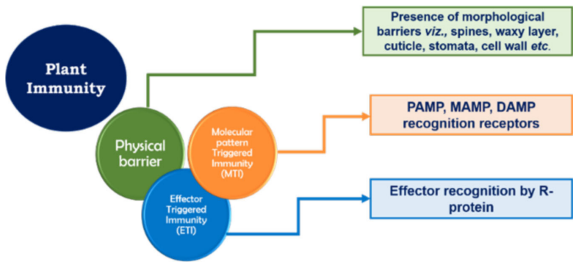

FIGURE 3
Layers of plant immunity.

these layers of plant immunity by establishing molecular dialogs with the host plant.

The soil-inhabiting fungi *Trichoderma* are mainly found to be root colonizers. They can establish themselves in the plant as an endophyte after a process of molecular crosstalk that brings a plethora of positive changes for the host. The sucrose-rich plant root exudates act as an attractant for *Trichoderma*, causing root colonization by *Trichoderma*, which activates the plant defense responses and enhances leaf photosynthesis (Vargas et al., 2009). The colonization of plant roots involves *Trichoderma*'s ability to recognize and adhere to roots, penetrate them, and withstand the toxic metabolites produced by the plant in response to invasion. Activation of plant defense takes place through MTI and ETI, which leads to the production of ROS such as H_2O_2, O_2^-, and hydroxyl radical. The ROS further act as a signaling molecule in signal transduction *via* mitogen-activated protein kinase (MAPK), thereby stimulating different pathways of plant defense, such as activation of the phenylalanine ammonia lyase (PAL) enzyme, which is essential in phytoalexin production and synthesis and the accumulation of pathogenesis-related (PR) proteins, and activates the host's defense responses (Mendoza-Mendoza et al., 2017; Figure 4). NADPH oxidase (Nox) is the key enzyme that regulates the production of ROS. Studies have revealed that Nox proteins, particularly NoxR and Nox1, greatly influence the molecular dialog between plant roots and *Trichoderma* during their interaction (Villalobos-Escobedo et al., 2020). The defense responses exhibited by plants to any microbial invasion are energy consuming, and are expressed at the cost of the plants' own growth and development. Therefore, *Trichoderma* elicits plant growth and development alongside the induction of a strong immune response in plants (Hermosa et al., 2013). In this context, the Nox protein plays a significant role. In a study conducted with a *Trichoderma atroviride* mutant expressing the NoxR protein, co-culture with *Arabidopsis* produced a decrease in feeder root proliferation and phytostimulation when compared with the wild-type strain. However, this also caused an exacerbated response of jasmonic acid-mediated systemic resistance response in the plant when compared with treatment with wild-type *T. atroviride* (Villalobos-Escobedo et al., 2020). Reduction in lateral growth and development in plants when cultured with a *Trichoderma* NoxR mutant may be due to the overactivation of jasmonic acid-mediated responses, leading to a shortage of carbon/other energy resources in the plant that are required for the development of lateral root primordia (Guo et al., 2018).

Invasion and colonization by *Trichoderma* lead to the synthesis and accumulation of different phytohormones, viz., salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). Due to their ability to modulate plant immune responses, these phytohormones are known as the central players of plant defense. The timing, composition, and quantity of the phytohormonal blend produced by plants in response to microbial invasion is greatly influenced by the strain, time, and/or inoculum concentration of the microbe (Pieterse et al., 2009). A study conducted with cucumber and *Trichoderma* in a hydroponic system by Segarra et al. (2007) revealed that, 4 h post inoculation of cucumber roots with *Trichoderma*, the plants exhibited a SAR-like response *via* up-regulated activity of peroxidase and SA. Furthermore, application of a higher inoculum density of

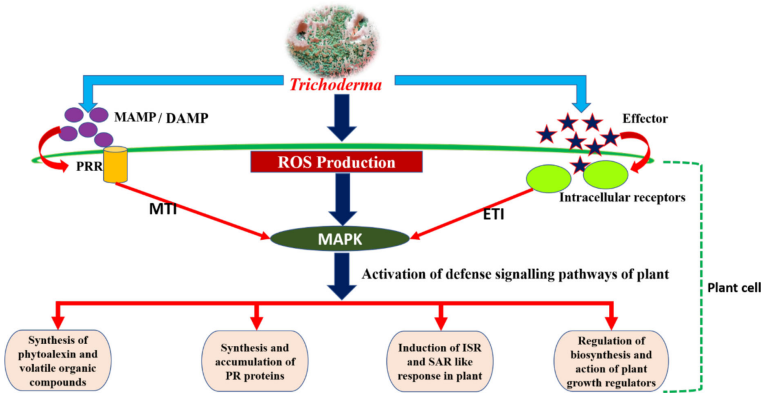

FIGURE 4
Pictorial representation of plant–*Trichoderma* interaction.

Trichoderma induced a systemic increase in SA and JA levels within the plant system. This may be due to the expression of ETI causing oxidative bursts in the plant cells, leading to a hypersensitive response in the plant system that ultimately results in the activation of SAR *via* an SA-mediated pathway. These defense responses of plants due to the activation of SA-dependent pathways can be overcome by *Trichoderma* by increasing the JA/ET and auxin responses in plant, which can act as antagonists to SA (Hermosa et al., 2012). When *Trichoderma* was inoculated into the roots of the model plant *Arabidopsis* by Morán-Diez et al. (2012), a decrease in plant defense mediated by SA and JA was observed after 24 h; however, over a longer period, the level of plant defense increased both locally and systematically. They suggested that the lower level of plant defense during the initial 24 h of *Trichoderma* inoculation could be because the plant did not consider the *Trichoderma* as hostile at that point in time. Over time, *Trichoderma* colonizes the epidermal and cortical cells of roots, which plants perceive as a threat, and subsequently the plants try to limit *Trichoderma* from entering into their vascular system by activating plant defenses both locally and systematically *via* upregulation of genes mediating ISR and SAR. Therefore, it can be said that the SA-mediated response of plants is essential in limiting the root colonization by *Trichoderma* to the first two layers of root cortical cells, preventing further invasion into the vascular system (Segarra et al., 2007). Moreover, the ability of *Trichoderma* strains to withstand the highly oxidizing, toxic environment created within the plant system is also a determinant of the degree to which they are effective in colonizing the plant roots (Chen et al., 2011).

The phytohormonal blend produced by plants in response to root colonization by *Trichoderma* also plays an important role in determining plant growth and development. A balanced trade-off between growth and defense in plants upon colonization by *Trichoderma* can be explained in terms of cross-communication among phytohormones, viz., ET, SA, and JA (the central players of defense); abscisic acid (ABA), which is related to abiotic stress and plant growth; indole acetic acid (IAA), which is commonly associated with plant growth and lateral root growth of plants; and gibberellins, which modulate plant growth and defense responses *via* degradation of the DELLA protein (D, aspartic acid; E, glutamic acid; L, leucine; L, leucine; A, alanine) (Hermosa et al., 2012). During *Trichoderma*–plant interactions, the 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity of *Trichoderma* reduces ET production by lowering the availability of the substrate 1-aminocyclopropane 1-carboxylic acid (ACC), which is necessary for ET biosynthesis. As a result, ABA biosynthesis decreases and the activation of gibberellin signaling takes place *via* degradation of the DELLA protein, which results in an increase in PGP activities. Moreover, JA- and SA-mediated defense responses in plants are also modulated by gibberellin through regulation of DELLA protein degradation. Furthermore, IAA and ET can reciprocally regulate biosynthesis of each other (Stepanova et al., 2007) and, according to this finding, ABA biosynthesis is regulated by exogenous auxin-stimulated ET biosynthesis *via* ACC synthase (Hermosa et al., 2012). A decrease in ABA biosynthesis is inversely proportional to stomatal conductance, thereby ensuring a higher rate of photosynthesis, and *vice versa*. Tucci et al. (2011) observed an increase in PGP activity in tomato plants subjected to treatments containing *T. atroviride* and *T. harzianum* (Dutta and Das, 1999; Dutta and Das, 2002). The probable reasons for a reduction in ET production were suggested to be either a decrease in the precursor ACC through microbial degradation of IAA in the rhizosphere or the presence of ACCD activity in *Trichoderma* (Tucci et al., 2011). *T. asperellum* mutants with RNA interference (RNAi) silencing of the ACCD gene showed an inability to promote root elongation in treated canola seedlings, suggesting the important role of ACCD in root elongation and development (Viterbo et al., 2010; Kubicek et al., 2011). Exogenous production of IAA by *Trichoderma* stimulates ET biosynthesis through ACC synthase (Figure 5). Liu et al. (2021) conducted an experiment to identify the growth-promoting effect of the *T. guizhouense* NJAU 4742 strain on cucumber seedlings in a hydroponic study. They observed a significant increase in plant biomass and the modification of lateral root architecture, with a 64.7% increase in lateral root tips of treated plants compared with control. Further study on *in situ* biosynthesis of auxin by *T. guizhouense* during interaction with cucumber roots revealed a gradual increase of auxin in the growing media, which was 1.15 and 0.5 times more than the control and IAA-containing treatments (external source) at 30 days post inoculation. These findings indicate that, after interaction with host roots, the exogenous production of IAA by *Trichoderma* increased considerably, which

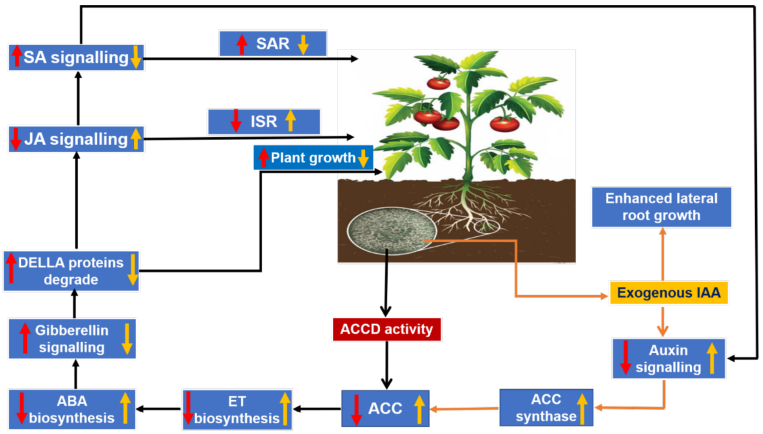

FIGURE 5
Trichoderma–plant cross-communication model *via* regulation of the phytohormonal blend: red arrows indicate the effects on the plant due to 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity in *Trichoderma*; and yellow arrows indicate the modulation in phytohormonal concentration due to exogenous production of indole acetic acid (IAA) by *Trichoderma*.

could be the underlying reason behind plant growth promotion. Similarly, Dutta et al. (2021) observed that groundnut plants treated with a *T. harzianum*-based bioformulation, made from a native isolate of Meghalaya, were not only protected from tikka disease but also exhibited enhanced plant growth parameters along with increased lateral root growth and root nodulation. Thus, the role of the phytohormonal blend resulting from *Trichoderma* colonization in determining plant growth promotion and immune response cannot be denied. Therefore, it can be said that plant root colonization by *Trichoderma* and the existence of *Trichoderma* within the plant as an endophyte (Zheng et al., 2021), which stimulate the plant's immunity responses, constitute a complex yet profitable relationship that enables the plant to withstand subsequent biotic and abiotic stresses.

4 Soluble secretome of *Trichoderma*: role in host–plant interaction and biological control of plant diseases

The emergence of the era of molecular science in the 1940s and 1950s, and its subsequent progress, with the development of different biotechnology tools, made it possible for scientists to isolate, study, and determine the chemical composition of individual genes present in any organism, and ultimately paved the way for whole-genome sequencing. The ability to map and study genes present in a genome made it easier for scientists to understand how genes are assembled in a genome and how they perform their function. In this context, the development of evolutionary trees was also fine-tuned by the detailed knowledge obtained from the understanding of genomics (Merrill and Mazza, 2006). The comprehensive study or global assessment of a set of molecules is referred to as "omics". Next-generation sequencing of genetic materials and the development of other high-throughput technologies has led to availability of omics data worldwide. The first omics to appear was genomics, which deals with the study of the whole genome of an organism (Hasin et al., 2017). Different omics, viz., genomics, transcriptomics, proteomics, and metabolomics, contribute to the wealth of omics data available publicly across the globe (Figure 6). *Trichoderma reesei* was the first species of *Trichoderma* to have its whole genome sequenced (Martinez et al., 2008). Subsequently, the complete genome sequencing of many species of *Trichoderma* was carried out, and the genomic information is available from the NCBI (National Center for Biotechnology Information) GenBank database (Table 2).

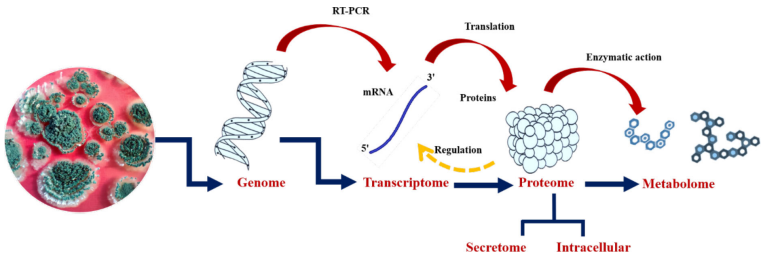

FIGURE 6
Omics strategies for a better understanding of molecular dialogues in *Trichoderma*–plant/phytopathogen interaction.

Sanger expressed sequence tag (EST) projects have made transcriptomics studies of *Trichoderma*–plant interactions easier (Steindorff et al., 2012; Silva et al., 2019). In addition, a transcriptomic study of *Trichoderma* genes present in the fungal cell wall can be obtained using high-throughput techniques such as suppression subtractive hybridization (SSH) (Vieira et al., 2013). Furthermore, a combined omics strategy can be adopted for an in-depth study of *Trichoderma*–plant/pathogen interactions (Figure 6).

5 Omics in determining the adaptation behavior of *Trichoderma* to host root colonization

Trichoderma, being a beneficial microbe, has attracted the attention of researchers, who have studied how it adapts to plant root colonization. The upregulation of genes responsible for the formation of infection structures was observed in an early transcriptomic study of *Trichoderma* colonizing tomato roots in a hydroponic system (Samolski et al., 2009). Similarly, Lawry (2016) observed the presence of an appressorium-like structure in a *Trichoderma virens*–maize hydroponic system that helped the biocontrol agent penetrate the plant cell wall and form an intercellular infection peg. The infection pegs possess structures similar to a haustorium, thereby indicating intracellular colonization of maize roots by *T. virens*. Moreover, forced penetration, by pushing away the outer cell layer of the epidermis, was observed in maize grown in *T. virens*-inoculated soil, and may be considered as another root colonization mechanism of *Trichoderma*. Rubio et al. (2014) reported a decrease in nutrient and carbohydrate metabolism activity in plants in response to hyphal attachment of *Trichoderma*. After 20 h of interaction between *Trichoderma* and tomato grown in a hydroponic system, Rubio et al. (2012) observed the differential regulation of genes in *Trichoderma* as a response to the host's fluctuating behavior. It revealed an upregulation of *Trichoderma* genes involved in carbohydrate metabolism, nutrient exchange with the plant, the generation of building blocks, and cell wall synthesis, and a downregulation of genes that indicate a sufficient availability of nitrogen. Furthermore, the upregulation or downregulation of genes in *Trichoderma* during root colonization of either tomato or maize plants is greatly regulated by the host itself. In a *T. virens*–maize co-culture system, the genes that are mostly upregulated belong to classes such as the glycosyl hydrolases (GHs), oxidoreductases, and small secreted proteins, and the symbiosis-related invertase TvInv (Vargas et al., 2009). The gene most preferentially expressed in *T. virens* during its interaction with tomato was revealed to be a secreted quino-protein, glucose dehydrogenase. Several studies on the secretome of different species of *Trichoderma* co-cultured with plants have reported an increased expression of genes encoding glycosidases and peptidases during the initial phase of their interaction (Lamdan et al., 2015; González-López et al., 2021). Reduction in tomato root colonization by a *T. harzianum* mutant with a silenced *thpg1* gene encoding endopolygalactouronase indicates the probable role of fungal glycosidases and peptidases in plant–*Trichoderma* interactions. Therefore, it can be said that the lytic enzymes released by *Trichoderma* during the initial phase of their interaction with plants is essential for disintegrating cell wall components to aid in the successful colonization of plant roots (González-López et al., 2021). Furthermore, during the first 24 h of interaction, the genes encoding a group of antioxidant enzymes were found to be exclusively expressed, which is necessary for ROS detoxification, and therefore demonstrates *Trichoderma*'s strategy of adaptation to the highly reactive environment of plants (González-López et al.,

TABLE 2 Genomic information for different *Trichoderma* species available from the NCBI GenBank database.

Species name	Clade under Hypocreaceae	Genome size (Mb)	Total number of genes	Reference
T. atroviride	Clade Viridae	36.1	11,863	Kubicek et al. (2011)
T. atrobruneum	Clade Harzianum	39.15	8,649	Fanelli et al. (2018)
T. arundinaceum	Clade Brevicompactum	36.87	10,473	Proctor et al. (2018)
T. asperellum	Clade Viridae	37.66	12,586	Druzhinina et al. (2018)
T. citrinoviride	Clade Longibrachiatum	33.2	9,737	Druzhinina et al. (2018)
T. gamsii	Clade Viridae	37.9	10,709	Baroncelli et al. (2015)
T. guizhouense	Clade Harzianum	38.8	11,297	Druzhinina et al. (2018)
T. hamatum	Clade Viridae	38.2	12,391	Studholme et al. (2013)
T. harzianum	Clade Harzianum	40.9	14,095	Druzhinina et al. (2018)
T. koningiopsis	Clade Viridae	36.58	12,661	Castrillo et al. (2017)
T. longibrachiatum	Clade Longibrachiatum	31.7	9,409	Xie et al. (2014)
T. parareesei	Clade Longibrachiatum	32.07	9,292	Yang et al. (2015)
T. reesei	Clade Longibrachiatum	34.1	9,129	Martinez et al. (2008)
T. virens	Clade Virens	39.0	12,427	Kubicek et al. (2011)

2021). However, there is still a need to perform more function-oriented experiments to obtain a clear understanding of the biochemical significance of host specificity at a transcriptome level. Overall, it can be summarized that the host and *Trichoderma* appear to coordinate their counterattacks, and *Trichoderma* has several genes for secreted proteins that apparently play a key role in determining its ability to survive in the complex rhizosphere ecosystem.

Proteomic study of the secretome of *Trichoderma* revealed that 3.5%–6.0% of total proteins are released *via* the type II secretion system into the apoplastic space of the plant cell, which is also known as the soluble secretome of *Trichoderma*. Gene ontology studies of the soluble secreted proteins of *Trichoderma* have revealed the range of different functions exhibited by these proteins, which are primarily CWDEs, cell wall adherence proteins (such as adhesins, hydrophobins, and tandem repeats), effector-like proteins, proteins determining host surface attachment and recognition, and proteins involved in secondary metabolisms (Mendoza-Mendoza et al., 2017). An effort is made here to briefly discuss the literature available on the soluble secretome of *Trichoderma* with regard to plant root colonization and its role in the biological control of plant diseases.

6 Cell wall-degrading enzymes

In every plant–microbe interaction, the host cell wall is at the forefront and so is the basal defense. Therefore, it is essential that the micro-organisms digest cell walls by releasing CWDEs in order to break through to the host system. Plant CWDEs are considered a major source of communication molecules and belong to CAZymes groups. GHs essentially digest plant cell walls and facilitate fungal entry into the host tissue. A study conducted with different species of *Trichoderma* revealed that *T. harzianum* and *T. guizhouense* secrete a higher number of CAZyme modules than other *Trichoderma* species. This may be indicative of their larger genome size and/or their particular behavior in the competitive environment of soil and when interacting with plants and pathogens (Li et al., 2013). In soil, *Trichoderma* releases a lignocellulolytic enzyme that helps it to live a saprophytic life. However, in the presence of host root exudates they respond differently, synthesizing and secreting CWDEs (Cragg et al., 2015). Harman et al. (2004) reported two glucosyltransferases (GTs) from the soluble secretome of mycoparasitic fungi *T. virens* and *T. atroviride*.

The soluble secretome of *Trichoderma* contains a significant amount of CWDEs. They are responsible for alerting the plant immune system to the presence of an invader. As reported by Avni et al. (1994), inactive cellulase and xylanase were the first MAMPs obtained from *Trichoderma*. Furthermore, CWDEs cause damage to the plant cell wall, thereby generating DAMP signals. A study of *T. harzianum*–root interaction in *Arabidopsis* and tomato led to the discovery of the first DAMP, which corresponds to the oligogalactouronides produced by the enzymatic activity of CWDE endopolygalacturonase ThPG1 in *T. harzianum*, which was found to be capable of inducing systemic defense in the plants (Moran-Diez et al., 2009). In a study conducted by Baroncelli et al. (2016), the expression of two endo-polygalcturonase genes (viz., *TvPg1* and *TvPg2*) from *T. virens* I10 was examined during the interaction with tomato roots. The results revealed that, while interacting with, in particular, host roots or pectin, expression of *TvPg1* was induced, whereas *TvPg2* was later expressed constitutively. According to Sarrocco et al. (2017), this constitutively produced endopolygalacturonase was responsible for eliciting ISR in the host. Similarly, the direct activity of plant chitinases or the mycotrophic nature of *Trichoderma* against rhizospheric fungi yields chito-oligosaccharides, which can also function as DAMPs in the activation of systemic plant immunity (Woo et al., 2006). Apart from inducing plant immunity by generating MAMP and DAMP signals, CWDEs also play an important role in determining the efficient colonization of roots by *Trichoderma*. They achieve this by either increasing the plasticity of the host cell wall or causing irreversible deterioration of the cell wall structure.

As the fungal cell wall comprises mainly chitin, glucan, and proteins, mycoparasitism by *Trichoderma* involves the extensive use of CWDEs, viz., chitinases, glucanases, and proteases (Table 3). Use of a *Trichoderma* microarray to study the transcriptomic changes of genes in *T. atroviride* overgrown on a *Verticillium dahliae* colony revealed that there was total 143 differentially regulated genes (almost 98%) that belonged to the *T. atroviride* genome. The upregulated genes were all from classes of CAZymes and proteases, viz., serine, aspartic acid, and metallopeptidases genes that are crucial in weakening and disintegrating the fungal host cell wall (Morán-Diez et al., 2019). Therefore, it can be said that the differentially regulated genes of *T. atroviride* are unequivocally associated with the mycoparasitic and antagonistic activity against the targeted pathogen.

7 Enzymes for chitin degradation

It has been reported that the *Trichoderma* genome harbors a greater number of genes encoding chitinolytic enzymes, which is attributed to *Trichoderma*'s mycoparasitic nature. Fungal chitinases belong to the GH18 and GH20 families. GH18 chitinases can be further categorized into subfamilies A, B, and C. It has been observed that genes encoding chitinases from GH18 are significantly expanded in *T. atroviride*, *T. asperellum*, *T. atrobrunneum*, *T. gamsii*, *T. harzianum*, and *T. virens* (Kubicek et al., 2011). Chitin and chitosan (a partial or complete deacetylated derivative of chitin) comprise the chitinous layer of fungal cell wall. Chitosan in mycoparasitic fungi such as *Trichoderma* plays an important role in the scavenging of ROS produced by parasitized fungi. Kappel et al. (2020) observed that out of six genes encoding chitin deacetylase, the deletion of genes *cda1* or *cda5* in *T. atroviridae* led to severely impaired mycoparasitic ability. This result indicates that a decrease in or absence of chitin deacetylase enzymes results in a low level of chitosan in *Trichoderma*, and, therefore, the *Trichoderma* is not protected from ROS (Mukherjee et al., 2022). Further study led to the discovery of cell wall remodeling in *T. atroviridae* during mycoparasitic interaction *via*

TABLE 3 Enzyme profile of *Trichoderma* in mycoparasitic interactions.

Enzyme group	Enzyme name	Molecular weight (kDa)	Reference
Chitinases	Endoquitinase	33–37	Ulhoa and Peberdy (1991); De La Cruz et al. (1992); Harman (1993)
	Endoquitinase	52	Harman (1993)
	Endoquitinase	31–33	De La Cruz et al. (1992)
	Endoquitinase	46	Lima et al. (1997)
	Exoquitinase	40	Harman (1993)
	N-acetylglicosaminidase	102–118	Ulhoa and Peberdy (1991)
	N-acetylglicosaminidase	73	Harman (1993); Lorito et al. (1994)
	Exochitinase 1	–	Pellan et al. (2021)
	Exochitinase 2	–	
Glucanases	Endoglucanase (EG Th1)	23.5	Liu et al. (2013)
	Exoglucanase (ExG Th1)	61	
	Endo-1,3-β-glucanase	76	Lorito et al. (1994)
	Endo-1,3-β-glucanase	36	De La Cruz et al. (1995)
	Endo-1,3-β-glucanase	40	Noronha et al. (2000)
	Exo-1,3-β-glucanase	29	
	Endo-β-1,6-glucanase	46	Monteiro and Ulhoa (2006)
	Exo-1,3-β-glucanase	78	
	Exo-1,3-β-glucanase	110	Cohen-Kupiec et al. (1999)
	Exo-β-1,3-glucanase	83.1	Bara et al. (2003)
	α-1,3-Glucanase MUT1 (MutAp)	–	Grun et al. (2006)
	Endo-β-1,3-glucanase	–	Suriani Ribeiro et al. (2019)
	β-1,3-Glucanase	–	Senthilkumar et al. (2021)
Other enzymes from the *Trichoderma* secretome	α-Mannosidase	53.52	Monteiro et al. (2010)
	Acid phosphatase	41.71	
	α-1,3-Glucanase	71.79	
	Carboxypeptidase 2[a]	53.79	
	Glucosidase I	27.50	
	α-Mannosidase	53.52	
	Carboxypeptidase 2[b]	53.45	
	Endochitinase	41.71	
	Aspartate protease	–	Ramada et al. (2016)
	Serine protease	–	
	Trypsin-like protease	–	
	Endo-β-1,4-glucanase	–	
	β-Endo-1,3-glucanase	–	
	α-1,3-Glucanase		
	α-1,2-Mannosidase	–	

(Continued)

TABLE 3 Continued

Enzyme group	Enzyme name	Molecular weight (kDa)	Reference
	α-L-Arabinofuranosidase	–	
	α-Galactosidase	–	
	β-1,6-Glucanase	–	
	Endo-1,3(4)-β-glucanase	–	
	Endochitinase chit33	33	
	chit37 Endochitinase	37	
	chit42 Endochitinase	42	
	β-1,3-Exoglucanase	107.28	Kohler and Tisserant (2014)
	Neutral metalloprotease *NMP1*	–	Zheng et al. (2016)
	β-1,3-Exoglucanase	107.93	Blauth de Lima et al. (2017)
	Endochitinase	42	
	Endochitinase	34.026	
	Glucoamylase	66.25	
	Mutanase	67.63	
	Serine endopeptidase	42.47	
	β-Glucocerebrosidase	51.59	Nauom et al. (2019)
	β-1,3-Glucanase	40.1	
	1,4-α-Glucosidase	67.28	
	α-D-Galactosidase	48.25	
	1,2-α-Mannosidase	55.65	
	Peptidase M14	46.95	
	Endo-1,3-β-glucanase	92.19	
	Tyrosinase	46.95	
	Peptidase S8	92.55	

the upregulation of all six genes encoding chitosanase, especially toward the later stage of interaction. One interesting finding made during this study by Kappel et al. (2020) was CHS8, which is called as a hybrid synthase due to its similarity to both chitin synthases and hyaluronan synthases, and can utilize both UDP-*N*-acetylglucosamine and UDP-D-glucuronate as substrates. The authors, therefore, speculated that CHS8, along with CDA1, forms a chitin glycol-polymer layer that protects the *Trichoderma* cell wall during mycoparasitic interactions (Kappel et al., 2020).

8 Glucan degradation

The enzymes α- and β-glucanase are necessary for the deconstruction of the glucan layer. Fungal α-1,3-glucanases are members to the GH71 family. *T. harzianum* and *T. asperellum* explicitly secrete the exo-α-1,3-glucanases AGN13.1 and AGN13.2, respectively, in the presence of the *Botrytis cinerea* cell wall. Enzyme AGN13.1 is found to possess lytic properties against fungal cell walls and exhibit antifungal activity (Mukherjee et al., 2022).

β-1,3-Glucanases are classified into GH families, viz., 16, 17, 55, 64, and 81. The mycoparasitic *Trichoderma* genomes comprise a large number of genes encoding GH55 and GH64 family members (Kubicek et al., 2011). They play a significant role in the mycoparasitization of oomycete fungi (which have a cell wall composed of cellulose and β-1,3- and β-1,6-glucans). A study of *T. virens* mutants in which the *bgn3* gene, encoding β-1,6-glucanase, is overexpressed found that such mutants exhibited enhanced antagonism toward *Globisporangium ultimum*, whereas mutants overexpressing the genes for both β-1,3-glucanase and β-1,6-glucanase were found to exhibit enhanced inhibition of *G. ultimum* (Djonovic et al., 2007).

9 Protein degradation

Proteases are an important group of enzymes released by *Trichoderma* in the event of mycoparasitism. The differential regulation of several protease genes of *Trichoderma* is reported during mycoparasitism (Mukherjee et al., 2022). Overexpression of the *T. atroviride prb1* gene (encoding protease) reportedly provides increased protection against *R. solani* (Pozo et al., 2004). Cortes et al. (1998) observed that expression of the *prb* gene was induced before contact with the fungal host. Further study of gene behavior in nitrogen-limited conditions led to the finding that the promoter region of the *prb* gene contains a binding site for transcriptional activator of nitrogen catabolite-repressed genes, viz., *ARE1* (Olmedo-Monfil et al., 2002; Mukherjee et al., 2022). These findings caused Druzhinina et al. (2011) to hypothesize that, in the early stage of mycoparasitic interaction, the activity of proteolytic enzymes results in host-derived nitrogenous products, which are responsible for the activation of mycoparasitism-relevant genes *via* their binding to nitrogen sensors present on the *Trichoderma* cell surface.

10 Small cysteine-rich proteins as effectors of *Trichoderma*

Effectors released during *Trichoderma*'s interactions with plants or fungi participate in ROS scavenging, chitinase and glucanase production, fungal cell wall masking, protease inhibition, and the prevention of defense alarm activation in neighboring cells colonized by the invader (Rabe et al., 2013; Lanver et al., 2017). A study conducted by Mendoza-Mendoza et al. (2017) revealed the presence of 70–123 effector proteins in the soluble secretome of *Trichoderma*. However, not all these effectors possess a clear functional domain. Some of the effector proteins with known functional domains are discussed briefly herein.

11 Common in fungal extracellular membrane domain proteins

Common in fungal extracellular membrane is a protein domain containing eight cysteines, which distinguishes it characteristically from other known cysteine-rich proteins. CFEM domain proteins were first discovered in rice blast pathogen *Magnaporthe grisea* (Kulkarni et al., 2003) and are known to play important roles in fungal pathogenicity. The functions of CFEM domain proteins include plant-surface sensing, appressorium development, asexual development (Sabnam and Barman, 2017), iron assimilation (Nasser et al., 2016), and redox homoeostasis (Kou et al., 2017). Fifty soluble secreted proteins of *Trichoderma* have so far been found to contain CFEM domains.

In an experiment on a *Trichoderma*–maize co-culture conducted in a hydroponic system, a decreased abundance of several CFEM-containing secreted proteins was observed. On developing deletion mutants for two genes encoding CFEM domain proteins with IDs 92810 and 111486 (Joint Genome Institute (JGI) v2.0), Lamdan et al. (2015) observed an increased ISR response against necrotic phytopathogens. They suggested that an increased degradation or sequestering of CFEM domain proteins by host roots could be the reason for their loss of abundance in a *Trichoderma*–maize co-culture system. However, further study of CFEM domain proteins is needed to reveal their modeof action in *Trichoderma*–plant *or Trichoderma*–phytopathogen interactions.

12 LysM-like putative effectors

Chitin, a homopolymer of *N*-acetyl-D-glucosamine, represents the second most abundant organic matter after cellulose. Chitin is widely distributed in fungi as a major component of the cell wall, but is absent in plants. The presence of chitin in the plant system is recognized by specific lysin motif (LysM)-containing pattern recognition receptors (PRRs) in the plant cell surface, which trigger an innate immune response in plants (Marshall et al., 2011). The absence of these PRRs compromises the plant's defense against fungal pathogens. Therefore, the plant's ability to perceive chitin is very important in recognizing phytopathogenic fungi. However, successful plant colonizers have evolved strategies that overcome chitin-induced defense in plants. Alteration of cell wall composition and release of LysM-like effector proteins are some of the strategies adopted by micro-organisms. LysM-like effectors released by plant colonizers bind to the free chitin released in the plant apoplastic space during fungal growth and mask the colonizer's presence. In this way, they overcome chitin-induced plant defense. Genomic study of mycoparasitic and endophytic *Trichoderma* has revealed that they contain an increased number of genes encoding LysM-containing secreted and non-secreted proteins as well as chitinases. These proteins help in the penetration and establishment of *Trichoderma* within the plant system by binding themselves to the fungal chitin and thereby avoiding ligand–PRR binding (Hermosa et al., 2013). Moreover, it has also been suggested that proteins containing a LysM domain may provide a mechanism of self-protection against the *Trichoderma*'s own chitinases (Gruber and Seidl-Seiboth, 2012).

13 Hydrophobins

Hydrophobins are small, unique, surface-active fungal proteins with the ability to form an amphipathic membrane at the interface of hydrophilic and hydrophobic environments. Their β-structured core is composed of eight highly conserved cysteine residues linked by four disulfide bridges. Hydrophobin proteins have a large exposed hydrophobic area, which explains their high surface activity (Linder et al., 2005; Bayry et al., 2012). Class I hydrophobin molecules form rodlet layers on the fungal cell wall

by organizing themselves into a highly insoluble amphipathic membrane at the junction of the hydrophilic fungal cell wall and the hydrophobic environment. Class II hydrophobins form micro-aggregates to give rise to dimers and tetramers in a rodlet-like structure (Mendoza-Mendoza et al., 2017). Kubicek et al. (2008) carried out a comparative evolutionary study on class II hydrophobins produced by the ascomycetes group of fungi and noted that the genus *Trichoderma* ranked first in number and diversity of class II hydrophobins. Guzman-Guzman et al. (2017) reported that a class II hydrophobin, viz., TVHYDII1 of *T. virens*, contributes to the antagonistic activity of *T. virens* against *R. solani* and promotes *Arabidopsis* root colonization by *Trichoderma*. Huang et al. (2015) observed an up-regulation in hydrophobin synthesis and secretion in *T. asperellum* when placed in a 1% *Alternaria alternata* cell wall and 5% *A. alternata* fermentation broth, which is indicative of hydrophobins' role in mycoparasitism. Microarray analysis of *T. virens T87* genes revealed that genes encoding hydrophobins were largely downregulated during *Trichoderma*–tomato interaction (Rubio et al., 2012) and found to have a negative effect on the growth and development of tomato plants in *in vitro* conditions. This result may be indicative of limited root attachment of *T. virens T87* due to fewer hydrophobins, affecting its interaction with tomato plants. Interestingly, Przylucka et al. (2017) have observed upregulated *HFB7* genes of *T. virens* in interactions with tomato. *T. harzianum* secretes QID74, which is a hydrophobin-like cell wall protein with a high molecular mass. It is particularly involved in fungal cell wall protection, adherence to the host cell, and modification of the host root architecture by increasing lateral roots growth, which, in turn, ensures increased nutrient uptake. Effective utilization of these nutrients results in increased plant biomass, and promising results have been obtained in cucumber and tomato plants (Samolski et al., 2012).

14 Ceratoplatanin family proteins

Ceratoplatanins (CPs) are non-enzymatic unique fungal proteins similar to plant expansin proteins. *Trichoderma* CPs bind to chitin (Pazzagli et al., 2014), which may be helpful in opening the physical spaces of the parasitized fungal cell wall. Similarly, during colonization of the host plant, the behavior of CP proteins might be helpful in masking fungal cell wall chitin from detection by the host plant's receptors (Quarantin et al., 2016). CPs are also known as eliciting plant response-like proteins (EPLs) due to their role in the induction of SAR in plants (Gaderer et al., 2014). According to Gao et al. (2020), the number of EPL-encoding genes in *Trichoderma* species is either three or four. During *T. harzianum–S. sclerotiorum* interaction, the role of EPL1 was found to be significant for the expression of genes related to mycoparasitism and coiling around *S. sclerotiorum*. Moreover, EPL-encoding genes found to downregulate the expression of virulence genes present in *B. cinerea* necessary for botrydial biosynthesis. Therefore, EPLs have two major functions in *Trichoderma*–pathogen interaction, viz., the expression of mycoparasitism-related genes and protection of *Trichoderma* from secondary metabolites produced defensively by pathogens (Mukherjee et al., 2022).

In *Trichoderma*, the *Sm1* gene encodes a small, secreted protein belonging to the CP family. *Sm1* gene abundantly expressed throughout fungal development. Specific growth conditions modulate the expression of the *Sm1* gene in *Trichoderma*. Studies conducted by Vargas et al. (2011) concluded that the detection of sucrose-rich plant root exudates and their trafficking activates the expression of *Sm1* in *T. virens*. Djonovic et al. (2006) demonstrated the role of *Sm1* in *T. virens* as a non-enzymatic effector of plant defense. In addition, purified Sm1 protein was observed to trigger ROS production, thereby eliciting local and systemic resistance in the plant. Furthermore, these proteins are found to have no phytotoxic or antimicrobial function. *Trichoderma* has a wide host range, and therefore its interactions with its hosts have diverse consequences. Salas-Marina et al. (2015) observed that *Sm1*- and *Epl1*-deleted mutants of *T. virens* and *T. atroviride* led to decreased systemic resistance in treated tomato plants, and overexpression of these genes resulted in enhanced protection against phytopathogens. In a tripartite interaction involving *T. virens* Gv29-8, maize, and *Colletotrichum graminearum*, Crutcher et al. (2015) observed an enhanced expression of Sm2 protein by *T. virens*. The use of mutants developed by deletion of the Sm2-encoding gene revealed that they were able to induce same level of ISR in maize plants; however, the root colonization ability of *T. virens* was found to decrease significantly. Conversely, a study on the interaction of *Sm1*-deleted mutants of *T. virens*, I10 maize, and *Cochliobolus heterostrophus* revealed a decreased level of ISR, and an *Sm1*- and *Sm2*-deleted mutant caused a more severe reduction in the plant's defense response (Gaderer et al., 2015). The diverse responses obtained from these tripartite interactions could be due to the different lifestyles of the phytopathogens used for the study. The plant's immune response to biotrophs, hemibiotrophs, and necrotrophs upon infection could determine which *Trichoderma* elicitors/effectors are deployed. Indeed, the activity of the same *Trichoderma*-derived effector could be altered by the host plant's response, thereby explaining some of the distinct actions of *Sm1* and its paralogs depending on the three-way interaction.

15 Swollenin

Swollenin is a soluble secreted protein first described in *T. reesei*. Swollenin and its orthologs are structurally characterized by the fungal carbohydrate-binding domain (CBD) in their N-terminal, which is followed by a region with domains 1 and 2, similar to plant expansin (Saloheimo et al., 2002). The presence of CBD in their N-terminal helps them to bind to the carbohydrate molecules present in the plant cell wall, and facilitates access to and colonization of the plant system. Brotman et al. (2008) observed that TasSwo—a swollenin protein secreted by *T. asperellum*—recognizes and binds to the cellulose present in the plant cell wall *via* the CBD and alters the architecture of the plant cell wall in favor of root colonization by *Trichoderma*. Furthermore, the authors demonstrated that the CBD present in these proteins acts as a

MAMP by inducing plant-innate immunity in cucumber to phytopathogens such as *Botrytis cinerea* and *Pseudomonas syringae*.

16 Wall stress responsive-component domain protein

Genomic study of *Trichoderma* has revealed the presence of several proteins with the cell wall stress-responsive component (WSC) domain. Although no direct information is yet available on how these proteins help *Trichoderma* in their interactions with plants or pathogens, similar proteins encoded in *Trichoderma* genome are also reported in other plant-beneficial endophytes. For instance, in *Piriformospora indica*, the WSC domain protein FGB1 performs the function of plant immunity suppressor by altering its cell wall composition and properties, and therefore aids its establishment within the host plant (Wawra et al., 2016). Moreover, as reported by Tong et al. (2016), these proteins may have a role in promoting cellular resistance, cell wall disruption, high osmolarity, the production of metal ions (Mg^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2}+, and K^{+}), and oxidation (Silva et al., 2019). In addition, under stressed conditions, WSC domain proteins, viz., FGB1 and WSC3, may be involved in β-glucan remodeling in the fungal cell wall (Wawra et al., 2019). A comparative secretome analysis of *Trichoderma* under salt stress conditions revealed that, in the presence of its plant host, the expression of WSC domain proteins in *Trichoderma* decreases, which may be indicative of the benefits derived by the fungus from its symbiont (i.e., the plant) through intensified root colonization (Rouina et al., 2022).

Other than the above-discussed secreted proteins, there are certain proteins identified in *Trichoderma* genomes, the function of which are not yet known. The presence of genes for different proteins, e.g., necrosis-inducing polypeptides (NPP1), killer-like toxins, GLEYA adherence proteins, and fungal ribonucleases (RNAses), are reported in different *Trichoderma* genomes. The expression of some killer-like toxin protein-encoding genes, such as *KP4*, hinders plant growth (Allen et al., 2008); however, as reported by Allen et al. (2011), the inclusion of this gene in transgenic plants is effective in protecting the plants from phytopathogens. Similarly, across *Trichoderma* species, the number of secreted GLEYA adhesin protein differs. According to previous studies, *T. guizhouense* harbors the maximum number of such proteins, i.e., three; *T. atroviride*, *T. gamsii*, and *T. parareesei* secrete two proteins; and *T. harzianum*, *T. virens*, and *T. reesei* contain only one such protein. The presence of necrosis-inducing proteins (NPP1) in both mycoparasitic and saprophytic *Trichoderma* genomes and their role in plant interaction is still not clear. However, it has been speculated that *NPP1* genes and their expression are not always related to necrosis in the host plant, but may also play a role in fungal growth and sporulation (Santhanam et al., 2013). Recently, the use of RNA-interacting proteins and RNA by fungi has been reported in the establishment of successful interactions with the host plant (Spanu, 2015). Among three different families of RNAses present in fungi, viz., non-specific RNases, RNase T1, and RNase T2, *Trichoderma* genomes are reported to express two RNAse families, i.e., RNase T2 and non-specific RNases. Although the T2 family RNases are known to perform functions such as nutrient acquisition, phosphate solubilization, defense against phytopathogens, self-incompatibility, and senescence (Deshpande and Shankar, 2002); however, their role in establishing interaction with plants is still not known.

17 Conclusion

Trichoderma is widely used across the globe due to its biocontrol and plant growth-promoting abilities. The interactions of *Trichoderma* spp. with host plants and pathogens at a molecular level will provide insights on the mechanisms that make *Trichoderma* a superior biocontrol agent. Mycoparasitism by *Trichoderma* is a complex process; therefore, a comprehensive study at the gene level is important to understand how the BCA safeguards itself from the defense strategies adopted by the parasitized fungi. Moreover, knowledge of secondary metabolites secreted by *Trichoderma* during their interaction with either the plant host or fungal host may be helpful in formulating effective bioactive molecule-based formulations that can provide enhanced protection to plants for a longer time. An understanding of the molecular dialogues between the host plant/fungus and *Trichoderma* is important to realizing the full potential of *Trichoderma* as a biocontrol agent.

Author contributions

MM prepared the original draft, and PD reviewed and edited the article. 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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Variation and stability of rhizosphere bacterial communities of *Cucumis* crops in association with root-knot nematodes infestation

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Introduction: Root-knot nematodes (RKN) disease is a devastating disease in *Cucumis* crops production. Existing studies have shown that resistant and susceptible crops are enriched with different rhizosphere microorganisms, and microorganisms enriched in resistant crops can antagonize pathogenic bacteria. However, the characteristics of rhizosphere microbial communities of *Cucumis* crops after RKN infestation remain largely unknown.

Methods: In this study, we compared the changes in rhizosphere bacterial communities between highly RKN-resistant *Cucumis metuliferus* (cm3) and highly RKN-susceptible *Cucumis sativus* (cuc) after RKN infection through a pot experiment.

Results: The results showed that the strongest response of rhizosphere bacterial communities of *Cucumis* crops to RKN infestation occurred during early growth, as evidenced by changes in species diversity and community composition. However, the more stable structure of the rhizosphere bacterial community in cm3 was reflected in less changes in species diversity and community composition after RKN infestation, forming a more complex and positively co-occurrence network than cuc. Moreover, we observed that both cm3 and cuc recruited bacteria after RKN infestation, but the bacteria enriched in cm3 were more abundant including beneficial bacteria Acidobacteria, Nocardioidaceae and Sphingomonadales. In addition, the cuc was enriched with beneficial bacteria Actinobacteria, Bacilli and Cyanobacteria. We also found that more antagonistic bacteria than cuc were screened in cm3 after RKN infestation and most of them were *Pseudomonas* (Proteobacteria, Pseudomonadaceae), and Proteobacteria were also enriched in cm3 after RKN infestation. We hypothesized that the cooperation between Pseudomonas and the beneficial bacteria in cm3 could inhibit the infestation of RKN.

Discussion: Thus, our results provide valuable insights into the role of rhizosphere bacterial communities on RKN diseases of *Cucumis* crops, and further studies are needed to clarify the bacterial communities that suppress RKN in *Cucumis* crops rhizosphere.

KEYWORDS

time dynamic, co-occurrence network, rhizosphere bacteria, Cucumis crops, root-knot nematodes

1 Introduction

Nematodes were reported to be the second most numerous in the animal kingdom residing in multiple ecosystems, with up to one million species (Bongers and Bongers, 1998; Mitreva et al., 2005). Root-knot nematodes (RKN) were among the top ten plant parasitic nematodes in the world found in almost all vascular plants, causing severe crop losses through direct injury and transmission of pathogenic microorganisms (Chalivendra, 2021). The most devastating RKN species were *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica*, causing hundreds of billions of dollars in crop losses each year (Nicol et al., 2011; Singh et al., 2015). Variety of the plant affected their response to RKN parasitism, which usually induced knotted gall tumors in the host plant (Jones et al., 2013). RKN penetrated root cells and induced the production of giant cells near the vascular bundle, from which nematodes to absorb nutrients by mouthparts (Moens et al., 2009).

Cucumber (*Cucumis sativus*), an economically important crop, in the Cucurbitaceae family is widely grown around the world (Kaur et al., 2023). The RKN was one of the most important diseases in cucumber (*Cucumis sativus*) growing regions around the world. It was estimated that RKN caused yield losses of 88% in cucumbers under greenhouse cultivation conditions (Expósito et al., 2019). The ubiquitous RKN developed different lifestyles and feeding strategies, which, made them difficult to manage (Topalović and Vestergård, 2021). Chemical nematicides have been widely used to control plant-parasitic nematodes, but the harmful effects of these chemicals on the environment have led to a significant reduction in their frequency of use. Currently, host plant resistance was an effective method for managing plant-parasitic nematodes, but in some cases, high genetic diversity within and between nematodes populations confounded the use of available resistant species and limited their effectiveness (Li et al., 2011). To date, there was no cultivated cucumber cultivars with RKN resistance (Weng, 2010). In contrast, a high resistance to RKN were found in African horn cucumber (*Cucumis metuliferus*), a relative of African-endemic *Cucumis* (Walters et al., 2006). The *C. metuliferus* was a member of the Cucumoraceae, which included several economically important crops such as cucumber, melon, squash and watermelon (Nakata et al., 2005). It was a potential genetic resource for Cucumoraceae crops improvement as it contains genes for resistance to a variety of Cucumoraceae crops pests and diseases (Yagi et al., 2014). And it has been reported to be resistant to RKN, gummy stem blight and *Fusarium* wilt (Chen et al., 2020). In this case, resistance to RKN in *C. metuliferus* was associated with reduced nematode penetration, developmental retardation and hypersensitive necrosis (Ye et al., 2017). However, the attempt of interspecific hybridization between *C. metuliferus* and *C. sativus* was not successful (Walters et al., 2006).

Biological control was a means of suppressing pests and pathogens by using other organisms that can be natural enemies, such as predators, parasitic organisms and competitors, with the advantages of environmental safety and precise targeting, of which microorganisms were receiving increasing attention (Vega, 2018; Chalivendra, 2021). Microorganisms that colonize plants in numbers that far exceed the number of plant cells were called the plant microbiome, which played an important role in plant growth and development (Mendes et al., 2013). There were several interaction ways between microorganisms and nematodes. Microbial competition for space, nutrients and water reduced nematode activity and reproductive capacity (Pal and McSpadden Gardener, 2006). And microorganisms produced and released certain antibiotics or toxins which may adversely affect the nematode during the infection stage (Lugtenberg and Kamilova, 2009). Many bacterial products can induce systemic resistance in plants to protect the whole plant from pathogenic bacteria and nematodes (Junaid et al., 2013). Some bacteria can also penetrate the cuticle and killed nematodes by the action of enzymes (Tian et al., 2007). In addition, microorganisms contributed to plant development by improving the solubility, uptake and absorption of nutrients, thus helping to increase the tolerance of plant roots to plant-parasitic nematodes (Schouteden et al., 2015). Many antagonistic microorganisms, including *Purpureocillium lilacinum*, *Streptomyces*, *Pseudomonas*, have been tested and widely used to suppress a number of plant pathogens and nematodes (Sarwar et al., 2018; Aiello et al., 2019; Jiao et al., 2019).

The assembly of the microbiome included dynamic changes in species composition and abundance, on the other hand, the steady-state composition of spatially distinct compartments. The rhizosphere, the narrow area around and affected by plant roots, was an important ecological niche in plant microbiome studies and one of the most complex ecosystems on Earth (Hinsinger et al., 2009; Raaijmakers et al., 2009). "Rhizosphere effect", a phenomenon in which the rhizosphere microbial community differed from the community in the bulk soil, implied that plant roots recruited specific microorganisms including nitrogen-fixing bacteria,

biocontrol microorganisms and protozoa, plant-promoting rhizobacteria (PGPR) from the bulk soil to the rhizosphere (Hein et al., 2008; Mendes et al., 2013). The interactions between these rhizosphere microorganisms that were beneficial to plant growth can impact their effects. In addition, the rhizosphere was the area where roots and their exudates affected various biological and ecological processes by interacting with microorganisms (Fang et al., 2013). Microbial interactions, complexity and diversity were of considerable importance to the formation and homeostasis of microbial communities (Cordovez et al., 2019). Also, microbial composition can be influenced by plant genotype, developmental stage (Bulgarelli et al., 2012; Zachow et al., 2014), fertilization management (Saha et al., 2008) and soil type (Schlaeppi et al., 2014).

Infestation by pathogens or RKN can disrupt the original rhizosphere microbial community structure of the crop and alter microbial diversity. A previous study of the *Arabidopsis* microbiome showed that plants can specifically recruit a set of beneficial microbes that induce resistance and promote growth in response to pathogen infection (Berendsen et al., 2018m). Besides, the Flavobacterium dominated the rhizosphere of resistant tomatoes after *Ralstonia solanacearum* infestation and tested that Flavobacterium could antagonise *Ralstonia solanacearum* (Kwak et al., 2018). The bacterial wilt outbreaks modified microbial composition and diversity as well as reduce the abundance of beneficial microorganisms in the soil (Wang et al., 2017). *Fusarium* wilt-diseased banana had a higher abundance and diversity of fungi or bacteria than disease-free soils, showing a change in the dominant phylum (Zhou et al., 2019). A study showed that tobacco composition of the root microbial community was significantly associated with RKN infection (Cao et al., 2022). In contrast, variations in the rhizosphere microbial community resulting from RKN infestation of *Cucumis* crops remain unclear.

Previous studies showed that *C. metuliferus* had the highest number of disease-resistance-associated NBS-LRR genes in Cucurbitaceae by assembling and analyzing the chromosomal level genome of *C. metuliferus* (Ling et al., 2021), and that root volatiles of *C. metuliferus* had potential applications against RKN (Xie et al., 2022), which implied the resistance to RKN in *Cucumis* crops was a complex and integrated mechanism. Therefore, this study compared and analyzed the response of rhizosphere bacterial community to RKN infestation in resistant *C. metuliferus* and susceptible *C. sativus*.

2 Materials and methods

2.1 Pot experiment and sample collection

C. sativus inbred line 9930 (cuc) and *C.metuliferus* inbred line CM3 (cm3) were provided by Institute of Vegetable and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS; Beijing, China). The *M. incognita* used in this study was obtained from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS; Beijing, China). The soil with a multi-year history of nematodes infestation was sampled from a greenhouse in Langfang city (Hebei, China). Then, the plants were maintained in a sterile mixture of soil: vermiculite: perlite (2:2:1, vol/vol/vol) in a glass room of a phytotron in April 2021 with day/night temperatures 28/18°C.

M. incognita was inoculated on the roots of the hollow cabbage. Approximately 45 days after inoculation, the *M. incognita* egg masses were picked with forceps and disinfected with 0.5% NaClO for 15 seconds. The egg masses were rinsed several times with sterile distilled water so that no NaClO remained on the surface, then placed in a sterile Petri dish with 20 mL of sterile water and incubated in a light-proof incubator at 28°C. The second-stage *M. incognita* juveniles (J2s) were counted using a stereomicroscope for bioassays.

The cuc and cm3 seeds were soaked in 0.5% NaClO for 2-3 minutes and then rinsed several times with sterile distilled water until no NaClO remained. The surface sterilised seeds were laid flat in a petri dish with sterile filter paper, soaked with a small amount of sterile distilled water (not over the seeds) and incubated in the dark at 28°C in an incubator. The seedling substrate and vermiculite were sterilised twice in an autoclave (121°C, 60 min) and the germinated seeds were sown into the sterilised substrate, which consisted of grass charcoal soil and vermiculite in a 2:1 volume ratio. Then the seedling cavity trays were grown in a greenhouse to obtain soil-grown seedlings for subsequent experiments.

The experiment consisted of five experimental groups, cm3, cuc, cm3 inoculated with nematodes (cm3J), cuc inoculated with nematodes (cucJ) and bulk soil. One sample (rhizosphere soil mix of five randomly selected plants) from each treatment at a time was taken and five replicates were set up. Each plant was inoculated with 500 *M. incognita* in cm3J and cucJ groups. Approximately 30 days (T1) and 60 days (T2) after inoculation, each plant was uprooted to prepare for rhizosphere samples. The roots without large pieces of soil were placed in a 250 ml sterilized conical flasks with phosphate buffer saline (pbs) buffer submerged. The conical flasks were placed on a shaker (20 min, 25°C, 160 r) to remove the soil still attached to the roots. Afterwards, the conical flasks were left to stand in a refrigerator at 4°C for 24 h. Finally, the lower soil solution was collected as the rhizosphere sample. In total, 40 rhizosphere and 10 bulk soil samples were collected.

2.2 DNA extraction and 16S ribosomal RNA gene amplification

Total bacterial genomic DNA from 0.5 g of soil for each sample was extracted using a Fast DNA SPIN extraction kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The DNA was stored at -20°C until further analysis. The quantity and quality of extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. The V3-V4 region of bacterial 16S rRNA genes was amplified by PCR using the forward primer 515F (5'-GTGCCAGCMGCCGCGGGTAA-3') and the reverse primer 907R (5'-CCGTCAATTCMTTTRAGT1T-3'). The primers were

incorporated into sample specific 7 bp barcodes for multiplex sequencing. After individual quantification, equal amounts of amplicons were pooled and pair-end 2x300 bp sequencing was performed on the Illumina MiSeq platform using the MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). The 16S sequence datasets for this study can be found in the NGDC with accession number: PRJCA014808 (https://ngdc.cncb.ac.cn).

2.3 Bioinformatic and statistical analysis

A total of 5,262,484 raw reads were obtained from 50 samples, with sequence lengths greater than 400 bp. After splicing the raw reads, double-ended primers were excised, and low-quality reads with a Q scores less than 30 were filtered. Operational taxonomic units (OTU) were clustered using usearch (v10.0) with a 97% similarity cutoff, and chimeric sequences were identified and removed using vsearch (v2.8) based on gold.fa (http://drive5.com/uchime/rdp_gold.fa). Each bacterial 16S rRNA gene was annotated in the greengenes database (gg_16s_13.5) using vsearch (v2.8). The OTU without annotation and annotations for Chloroplast, Mitochondria, Archaea were deleted. Bacterial reads were classified into 4531 OTU after quality filtering. Then, the OTU with relative abundance less than 0.001% were filtered and flattened according to sample_size 52353. We calculated bacterial alpha diversity using the Shannon indices and observed_otus by vsearch (v2.8), and bacterial beta diversity using principal co-ordinates analysis (PCoA) based on the Bray–Curtis dissimilarity matrix by edgeR and vegan packages. Data on the relative abundance differences between groups of bacteria at the phylum level were processed with the dplyr and reshape2 packages. The unique and shared OTU of different groups were plotted by the ggVennDiagram package (Gao et al., 2021). The volcano plot drawn with the edgeR and ImageGP packages showed OTU enrichment differences between groups. Bacterial co-occurrence networks of different groups at different stages visualised correlations with $|RHO| > 0.9$ and $P < 0.05$ using the ggCLusterNet package (Wen et al., 2022). Stamp, linear discriminant analysis effect size (LEfSe) analysis and circos plot were performed using the OmicStudio tools at https://www.omicstudio.cn/tool.

2.4 Isolation and screening of nematodes resistant bacteria

Samples from each group were mixed and enriched for bacteria by the following steps. 1) 100 ml of soil solution was added to 150 ml of pre-cooled distilled water, then slowly filtered through a double layer of gauze after mixing well. 2) The supernatant obtained by centrifugation of the filtered solution (700 r, 4°C, 5 min) was collected. 3) The precipitate was resuspended and centrifuged twice more, and the supernatants of the three times were combined. 4). After centrifugation (1000 r, 4°C, 10 min) of all the supernatants, the precipitate was resuspended with 15 ml of 0.8% NaCl. 5) The resuspension was centrifuged (11000 r, 4°C, 30 min) after the slow addition of 10 mL of Nycodenz (0.8 g/mL). 6) After centrifugation, it was divided into Nycodenz-soil mixed particles (lower layer), water (upper layer), and bacteria (middle layer). 7) The bacterial layer, resuspended by the addition of 10 mL of sterilised distilled water, was centrifuged (10,000 r, 4°C, 20 min) to obtain the Nycodenz-free bacteria.

The enriched bacteria were cultured on LB and R2A medium plates, and 20 single colonies of different sizes and morphologies were picked from each plate. Then 600 single colonies were obtained in 3 replicates. The nematicidal activity of the strains was tested in a 24-well plate. The 900 µL of bacterial broth was mixed with 100 µL of nematodes suspension (approximately 100 *M. incognita*) in individual wells. Additionally, 100 µL of nematodes suspension (approximately 100 *M. incognita*) mixed with 900 µL of sterilised distilled water and culture medium respectively were added to other wells to serve as untreated controls. The 24-well plates were incubated at 28°C for 24 h. Nematodes were considered dead if the bodies of nematodes were straight and did not move when stimulated with 0.5 M NaOH (Harada and Yoshiga, 2015). The corrected mortality rate was reference Yin (Yin et al., 2021). Bacteria with the corrected mortality rate greater than 85% compared to the corresponding medium were considered as antagonistic bacteria. Genomic DNA of strains were extracted using the TIANamp Bacteria DNA Kit (Tiangen, Beijing China) and the 16S ribosomal DNA was amplified by PCR using 2x Rapid Taq Master Mix (Vazyme, Nan- jing China) and primers 27F and 1492R (Alberoni et al., 2019). The following conditions were used: denaturation at 95°C for 3 min; followed by 34 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and extension at 72°C for 5 min. The product was stored at 4°C until sequencing. The sequence results were analyzed using the NCBI BLAST tool (https://www.ncbi.nlm.nih.gov/).

3 Results

3.1 Diversity and abundance of bacterial communities in different groups

At the operational taxonomic unit (OTU) level, alpha diversity of microorganisms in the rhizosphere of plants was measured using observed OTU and Shannon index. The Shannon index and the observed OTU index showed that the cucJ group was significantly higher than the cuc group and the bulk soil was significantly higher than the cuc and cm3 groups at T1 (Wilcoxon rank sum test $P < 0.05$; Figures 1A, B). However, the Shannon index was significantly higher in the bulk group than in the cm3 group (Wilcoxon rank sum test $P < 0.05$), but the observed OTU index was not significantly different at T2 (Wilcoxon rank sum test $P > 0.05$; Figures 1C, D).

To clarify the differences in the bacterial communities of the different groups, the beta diversity of bacteria was visualized by PCoA based on the Bray-Curtis distance metric. The extremely significant differences in bacterial community composition between bulk soil and plant rhizosphere (PERMANOVA test; $P = 0.000050$ for T1, $P = 0.00010$ for T2; Figures 1E, F) suggested that plants

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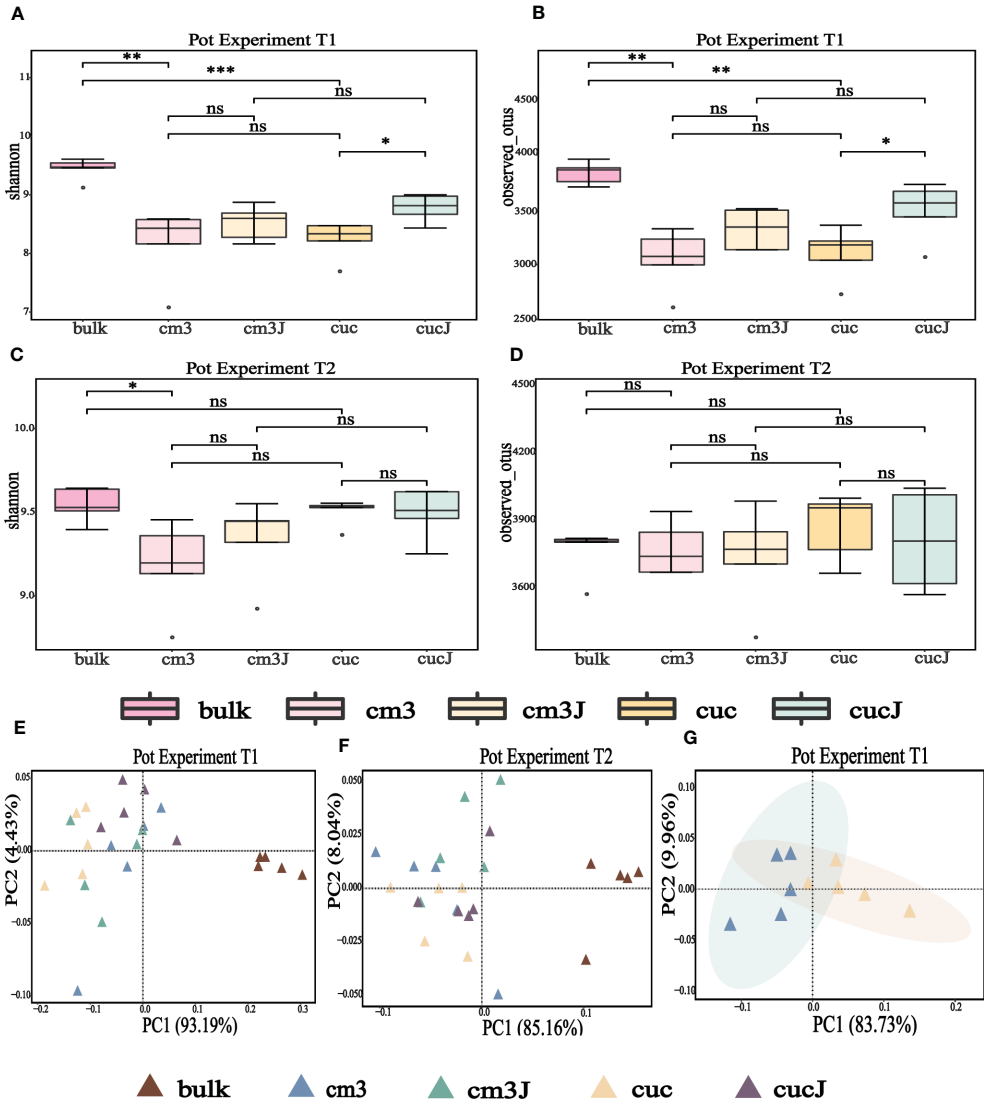

FIGURE 1
Alpha diversity (shannon indices and observed OTU) of bacteria in the rhizosphere at T1 **(A, B)** and T2 **(C, D)**. Differences between the cm3, cuc, cm3J, cucJ, and bulk soil were indicated in each figure panel (ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Principal co-ordinates analysis (PCoA) analysis of bacteria at T1 **(E, G)** and T2 **(F)** in the rhizosphere of different group on Bray–Curtis distance metrics.

influence bacterial communities. Also, PCoA analysis revealed a striking change in the composition of the bacterial communities of the cuc rhizosphere after infestation by RKN (PERMANOVA test; P = 0.0081; Figure 1G).

3.2 Changes in bacterial communities composition after inoculation with RKN

After RKN infestation, rhizosphere bacterial OTU species varied little, with 93% and 97% of the same OTU for cm3J and cm3 (Figures 2A, B), respectively, and 94% and 98% of the same OTU for cucJ and cuc (Figures 2C, D), respectively, in both periods. In order to gain insight into the taxonomic composition of rhizosphere bacterial community of different treatments, differences in the taxonomic composition of rhizosphere bacteria were compared at the phylum level (Figures 2E, F). The bacterial community at T1 was mainly composed of Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi, Gemmatimonadetes, Bacteroidetes and Acidobacteria. At T1, the abundance of Acidobacteria and Nitrospirae increased in cm3J compared with cm3. Compared with the cuc group, the relative abundance of Actinobacteria, Acidobacteria, TM7, Nitrospirae and Cyanobacteria increased in the cucJ group. The dominant bacterial community at T2 was the same as in T1 period, but no differences in composition ratios were observed. Only the relative abundance of Cyanobacteria increased in cucJ than in cuc. Obviously, the taxonomic ratios of the bacterial community between bulk soil and plant rhizosphere were quite different in each period.

Next, the differences in the content of the top 10 bacteria in relative abundance at the class level were compared across the groups

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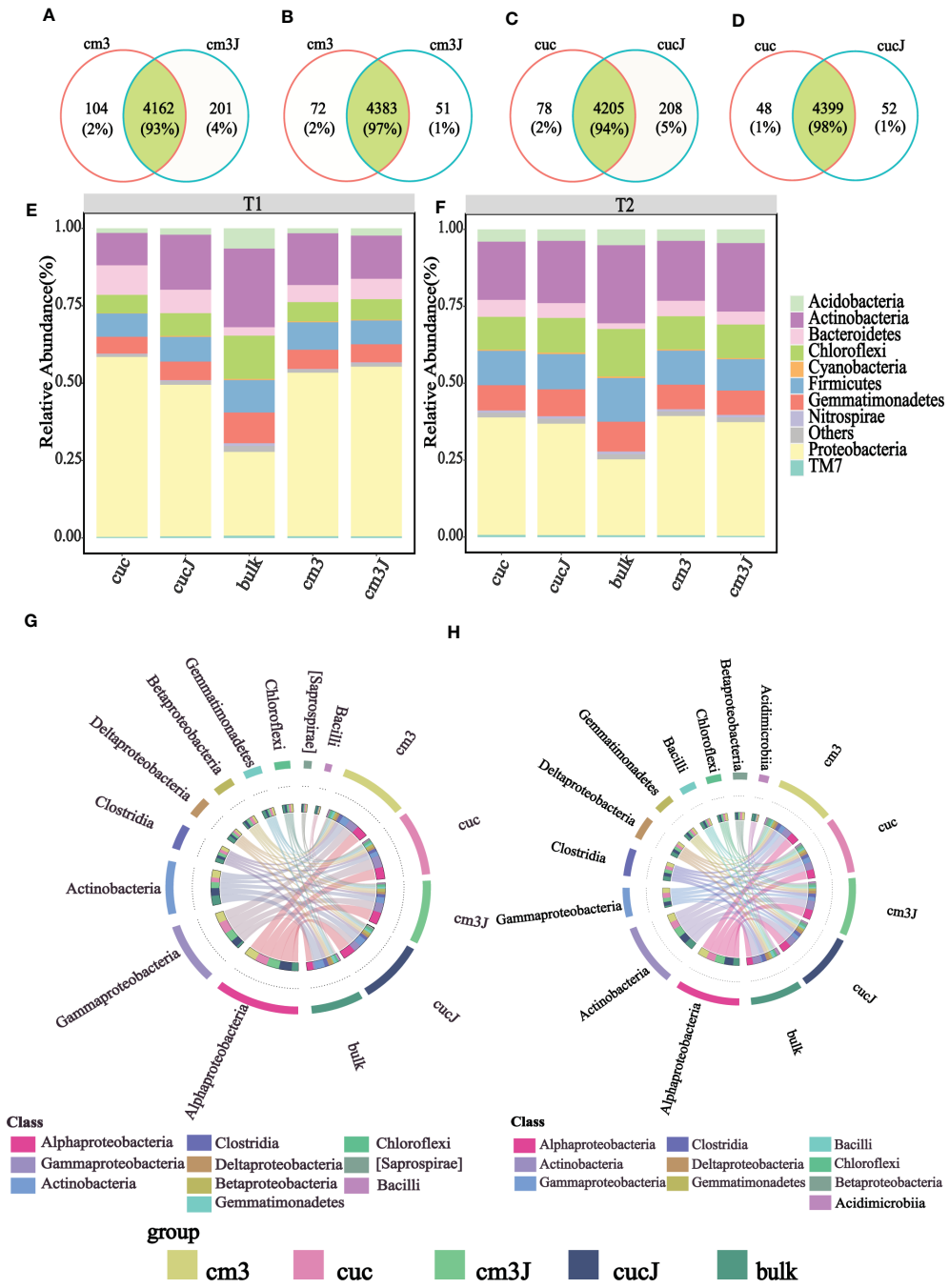

FIGURE 2
The cm3 and cm3J share the number of OTU species at T1 **(A)** and T2 **(B)** and cuc and cucJ share the number of OTU species at T1 **(C)** and T2 **(D)**. The relative abundance of major bacterial at T1 (phylum level; **(E)** and T2 (phylum level; **(F)** taxa present in the rhizosphere of different groups. Circos plot showing the taxonomical relative abundance of the top 10 bacterial microbiome at the class level at T1 **(G)** and T2 **(H)**. The thickness of each ribbon represents the relative abundance of bacterial assigned to different groups.

(Figures 2G, H). At T1, Gammaproteobacteria abundance appeared to decrease, but Actinobacteria and Bacilli increased to varying degrees in cucJ compared to cuc. Conversely, cm3J was more abundant in Betaproteobacteria but less abundant in Actinobacteria and Bacilli compared to cm3. At T2, Gammaproteobacteria and Betaproteobacteria decreased and Deltaproteobacteria increased in cucJ. Otherwise, Actinobacteria increased and Gammaproteobacteria, Chloroflexi, Betaproteobacteria and Bacilli decreased in cm3J.

3.3 Differences in rhizosphere bacteria of resistant and susceptible plants after inoculation with RKN

The difference in the rhizosphere enrichment of bacterial OTU in cm3J (Figure 3A; Supplementary Figure S1A) and cucJ (Figure 3B; Supplementary Figure S1B) compared with cuc, respectively. The cm3J enriched a quantity of OTU relative to cuc in both

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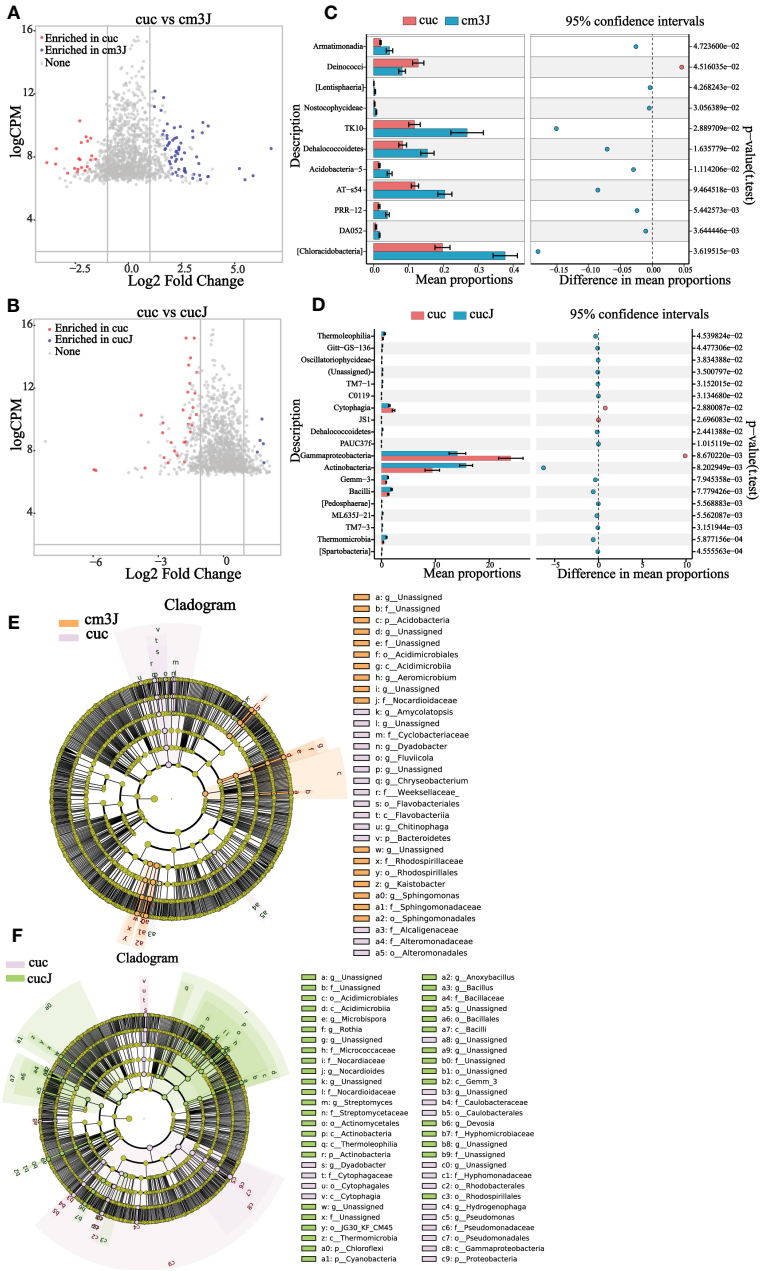

FIGURE 3
Cm3J **(A)** and cucJ **(B)** enriched OTU species compared to cuc at T1. STAMP analysis demonstrates differential enrichment of bacteria (class level) in the cm3J **(C)** and cucJ **(D)** at T1. Cladogram showing the bacteria phylogenetic structure of cm3J **(E)** and cucJ **(F)** with cuc respectively at T1.

periods, while cucJ only enriched a small amount of OTU in T1 period. Next, we compared the top 20 bacteria with differences in abundance in cm3J and cucJ with cuc, respectively. Specifically, the abundance of Armatimonadia, TK10, Dehalococcoidetes, Acidobacteria−5, AT−s54, PRR−12, DA052 and Chloracidobacteria was significantly higher in cm3J than in cuc (t.test, $P < 0.05$; Figure 3C) at T1. Only the abundance of Actinobacteria was significantly higher in cucJ than in cuc (t.test, $P < 0.05$; Figure 3D). Cm3J recruited more Chloroflexi and Alphaproteobacteria, while cucJ recruited more Thermomicrobia (Supplementary Figure S1C, D) at T2.

Compositional differences were assessed by calculating the linear discriminant analysis effect size (LEfSe) scores at different levels to find out the changes in the relative abundance of bacteria in resistant and susceptible plants after infection with RKN, respectively (Supplementary Figure S2). Under the condition of LDA threshold ≥ 3.0, cm3J was enriched Acidobacteria, Nocardioidaceae, Rhodospirillales, Sphingomonadales, Acidimicrobiia compared with cuc (Figure 3E) at T1. And cucJ was enriched with a considerable amount of bacteria compared to cuc including Bacilli, Cyanobacteria, Chloroflexi, Actinobacteria, Rhodospirillales, Hyphomicrobiaceae and Gemm_3

(Figure 3F). Thus, it can be found that cucJ enriched more bacterial after inoculation with RKN. Cm3J was mainly enriched with Chloroflexi, Actinobacteria, *Stenotrophomonas* and Alphaproteobacteria (Supplementary Figure S1E) at T2. Furthermore, there was only little difference between cucJ and cuc compared to rhizosphere bacteria. CucJ was mainly enriched with Thermomicrobia and Microbacteriaceae (Supplementary Figure S1F).

3.4 Changes in the co-occurrence network of bacterial communities

In exploring the interactions of rhizosphere bacterial communities of plants with different treatments at different growth stages, co-occurrence networks were constructed to demonstrate the differences in rhizosphere bacterial interactions. Firstly, it was straightforward to see that the bacterial network of the bulk soil (Figure 4E) was obviously different from the other groups. The taxonomic composition of the network showed no apparent differences among the cm3 (Figure 4A), cuc (Figure 4B), cm3J (Figure 4C) and cucJ (Figure 4D) groups, with most nodes belonging to Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Gemmatimonadetes, Chloroflexi, and Acidobacteria, respectively. The network differences among the groups at T1 were greater than those at T2, so specific analyses were performed for the bacterial networks of the groups at T1.

From cm3J to cucJ, the network complexity gradually decreased. There were 148 edges in the cm3J network, while 115 edges in the cucJ. The average degree and relative.modularity of cm3J were 3.6098 and 1.3185, edge density and diameter were 0.0446 and 1.9494 respectively, while the average.degree and relative.modularity of cucJ were 2.5556 and 0.6200, edge density and diameter were 0.0287 and 2.8704, respectively. More importantly, the positive correlation accounted for 74.32% and the negative correlation accounted for 25.68% in cm3J, while the positive and negative correlations in cucJ were 60.00% and 40.00%, respectively (Supplementary Table S1). Hub nodes were nodes that were more connected with other nodes in the network, and hub microorganisms were those that were closely connected with other microorganisms. The top five OTU with hub_score in the cm3J belonged to Firmicutes, Actinobacteria and Proteobacteria, respectively. Similarly, they belonged to Actinobacteria and Firmicutes respectively in the cucJ (Supplementary Figure S3).

3.5 Screening for RKN resistant bacteria

105 antagonistic bacteria were yielded screening of bacteria resistant to *M. incognita* using R2A and LB medium and classified in 10 genus (Figure 5A), of which 44.76% *Pseudomonas*, 15.24% *Priestia*, 16.19% *Stenotrophomonas* and 10.48% *Glutamicibacter* (Supplementary Table S2). More antagonistic bacteria were obtained in the cm3J than in the cucJ at T1, and both were less abundant than in the bulk soil. However, the cm3J obtained fewer species of antagonistic bacteria than the cucJ and the bulk soil (genus level). Similar screening results to T1 (Figure 5B) were showed at T2 (Figure 5C). Others, more antagonistic bacteria were obtained in cm3J and cucJ at T1 than T2. Notably, the antagonistic bacteria in the cm3J group were *Pseudomonas*, *Glutamicibacter*, and *Priestia*. The *Arthrobacter*, *Bacillus*, *Glutamicibacter*, *Priestia*, *Pseudomonas* and *Enterobacter* were identified in the cucJ group.

4 Discussion

4.1 Rhizosphere bacterial communities responded to RKN invasion in the early stages

Changes in soil microbial communities were the most important biological factors influencing the occurrence of soil diseases (Kloeppe et al., 1999; Larkin, 2008). In previous studies, the occurrence of RKN disease was found to be closely related to the interactions between soil microbial communities (Echeverrigaray et al., 2010). Plants infected with RKN responded with resistance by releasing many compounds into the rhizosphere (Čepulytė et al., 2018; Oota et al., 2020; Rutter et al., 2022). Nutrients and metabolites were released through root cells fed by nematodes through the symplast, thereby altering the composition of root exudates (Tian et al., 2015), which in turn may affect the microbial community. Similarly in this study, RKN invaded the plant roots and the rhizosphere bacterial species diversity and community composition changed. Moreover, changes in the rhizosphere bacterial community were more pronounced in the early stages of plant-root knot nematodes interaction, which was consistent with the findings of other studies (Poll et al., 2007). In the early stages of RKN infestation, the species diversity of rhizosphere bacteria increased in cucJ and cm3J, and the composition of the cucJ rhizosphere bacterial community was apparently altered. In addition, there were obvious differences in the rhizosphere bacterial co-occurrence network. These changes may be the result of a combination of attacks by RKN on rhizosphere microbial structures and the plant response in the early stages.

4.2 Stabilization of rhizosphere bacterial community structure to suppress RKN

In the study of rhizosphere bacterial community structure, it was found that the rhizosphere network was substantially more complex than the bulk soil, indicating that rhizosphere had greater potential for interaction and ecological niche sharing (Shi et al., 2016). Although the species diversity and community composition of both cm3J and cucJ rhizosphere bacterial communities changed in the face of RKN invasion, the cm3J community structure was more stable with only small changes and no obvious changes in species diversity and community composition, as well as fewer bacterial enrichment species. The homeostasis of soil microorganisms as a dynamic component of the plant-soil system was extremely important for the control of soil-borne diseases.

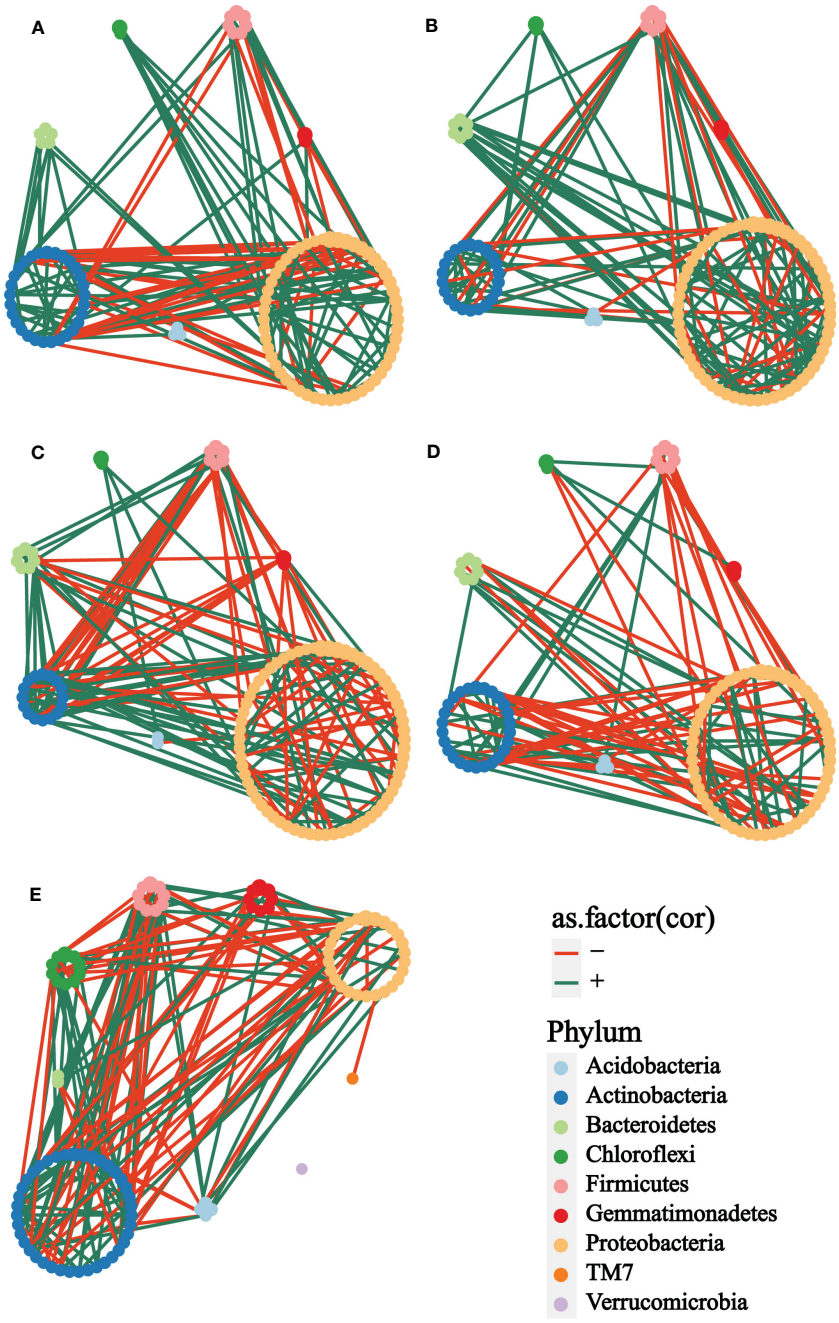

FIGURE 4
Co-occurrence network analysis of the rhizosphere microbial communities of different groups at T1. **(A)** cm3, **(B)** cuc, **(C)** cm3J, **(D)** cucJ, **(E)** bulk. The networks were colored based on the taxonomy taxa of bacteria at the phylum level. Edge s indicated correlations, which were divided into positive (green) or negative (red) correlations.

Cooperative and competitive interactions between microbial species and the modularity of the network influence the stability of the community (Faust and Raes, 2012; Coyte et al., 2015). Studies on the rhizosphere microbial community showed that the bacterial community structure of resistant genotypes was more stable than that of sensitive genotypes (Fu et al., 2019; Dong et al., 2021). Likewise, the bacterial community structure of cm3J was more stable than that of cucJ in this study.

The edges connecting two nodes representing different units in a microbial network indicate close associations between the abundance of these units in the whole sample, often interpreted as biological interactions. A great number of microbial interactions have also been confirmed experimentally in the *Arabidopsis* root community (Durán et al., 2018). In this study, the interactions and positive correlations between rhizosphere bacteria in cm3J were stronger, forming a more complex co-occurrence network. This indicated that the ecological interactions of cm3J rhizosphere dominant bacteria were more positive (Zhang et al., 2018; Lian et al., 2019). Elsewhere, the network complexity (Wagg et al., 2019) and hub taxa in supporting ecosystem function were important

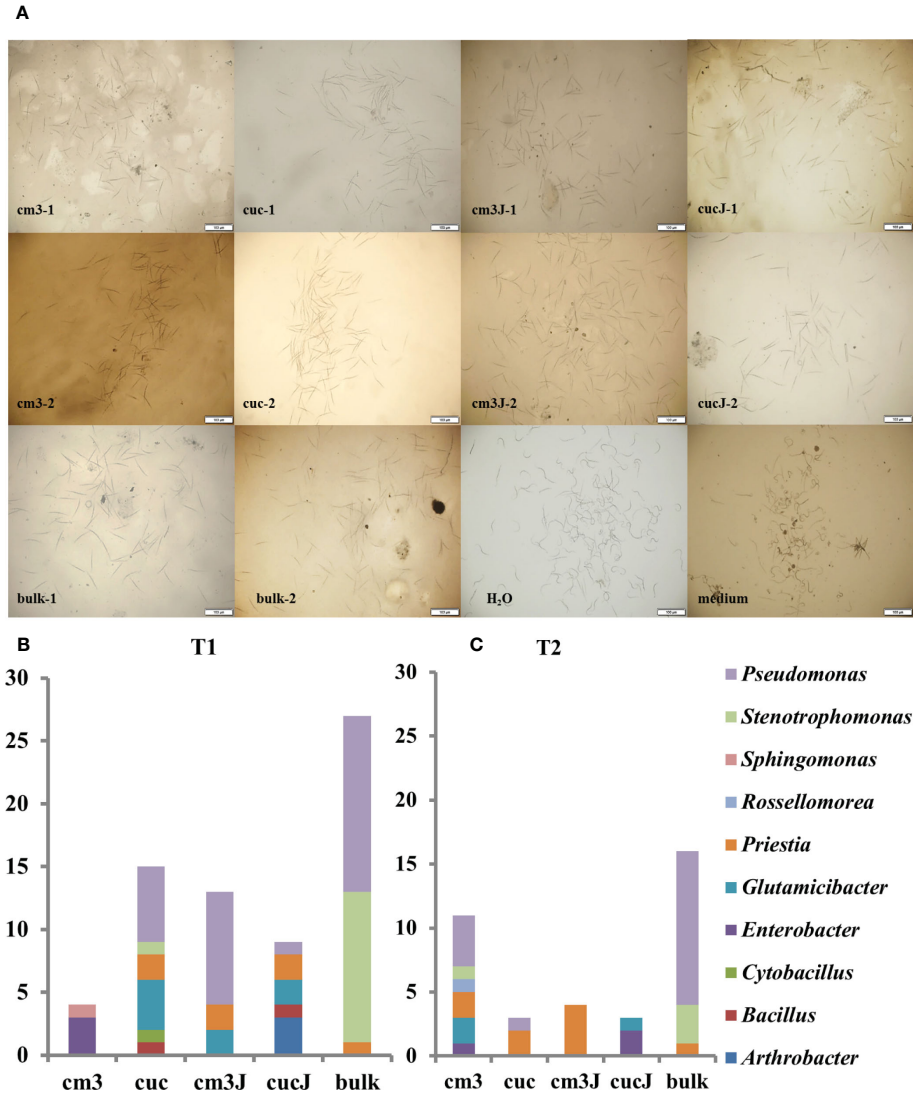

FIGURE 5
Effectiveness of antagonistic bacteria in killing nematodes **(A)**. Statistics of antagonistic bacteria genus in different groups at T1 **(B)** and T2 **(C)**. -1: T1; -2: T2.

(Toju et al., 2018). In contrast, the rhizosphere bacterial community of cucJ was more susceptible to disturbance. When infested by RKN, the cuc rhizosphere bacterial network transmitted the environmental disturbance to the whole network with a short time, in turn destabilizing the network structure. Network inference can provide insights into microbial community composition, but theoretical studies of the effects of some network properties on ecosystem stability still require experimental evidence (Faust, 2021).

After that, we found a close positive correlation between Bacteroidetes and Proteobacteria in the cm3J network. Among the soil microbiota, the Bacteroidetes tended to be a dominant phylum due to their ability to secrete a variety of carbohydrate-active enzymes (CAZymes) that targeted highly variable glycans in the soil (Larsbrink and McKee, 2020). Bacteroidetes were abundant pathogen suppressor members of the plant microbiome and contributed to rhizosphere phosphorus mobilization (Lidbury et al., 2021). Proteobacteria were also abundant, as typically observed in soil libraries (Janssen, 2006). And multiple positive interactions of Pseudomonadales and Rhizobiales with other bacteria, such as Sphingomonadales. The probiotic *Pseudomonas* (Gammaproteobacteria, Pseudomonadales) was versatile in terms of plant hosts, soil habitat and improving plant stress response (Kim and Anderson, 2018). The most known effects of *Pseudomonas* were the protection of plants from fungal diseases and the improvement of plant yield, as well as the recent discovery of interesting aspects regarding insecticidal activity (Ruffner et al., 2013). *Rhizobia* were a group of soil-borne bacteria that had the ability to fix atmospheric nitrogen for plant growth and promoted root growth (Masson-Boivin et al., 2009; Poupin et al., 2016; Garrido-Oter et al., 2018). *Sphingomonas* was the main group of rhizosphere and endophytic bacteria with multifaceted functions ranging from remediation of environmental pollution to production of highly beneficial phytohormones involved in rhizosphere remediation of organic matter (Zhang et al., 2013; Feng et al., 2019; Asaf et al., 2020). Additionally, Sphingomonadaceae, Brucellaceae, and

Bartonellaceae were also closely associated with other rhizosphere microorganisms. Brucellaceae and Bartonellaceae were associated with nematodes carriage (Bowman, 2011). In summary, the more positive correlation between probiotics and the close interaction with RKN-associated bacteria may be a reason for the resistance of cm3 to RKN infestation.

4.3 Recruiting beneficial bacteria

There was little change in bacterial species when RKN interacted with plant rhizosphere microorganisms; what changed was the abundance of some bacteria. In the early stage, the change in the abundance of bacteria in cucJ was an increase in Actinobacteria and Bacilli, a decrease in Bacteroidetes, Gammaproteobacteria and Betaproteobacteria. Actinobacteria played a role in soil nitrogen fixation, improving nutrient availability, and promoting the production of plant growth regulators (Bhatti et al., 2017). The Bacilli offered a number of advantages for their application in agricultural biotechnology, and some products based on Bacilli, especially *Bacillus*, have been marketed as microbial pesticides, fungicides or fertilizers (Perez-Garcia et al., 2011). Conversely, Actinobacteria and Bacilli decreased and Betaproteobacteria increased in cm3J. Cm3J was mainly enriched in Gammaproteobacteria and Betaproteobacteria. Differently, cucJ mainly recruited Actinobacteria, Saprospirae, Bacilli and Thermomicrobia.

In addition, Thiobacterales, iii1_15 and Kaistobacter may play a role in cm3J. In cucJ, Thermoleophilia, Streptomyces, Nocardioidaceae, Nocardioides, Micrococcaceae, Acidimicrobiales, Devosia, Gemm_3, Bacillaceae, Cyanobacteria and G30_KF_CM45 may play important roles. *Streptomyces* was the most abundant and important genus of actinomycetes. And *Streptomyces* had a beneficial symbiotic relationship with plants, promoting the nutrition and health of the latter (Pang et al., 2022). Nocardioidaceae can degrade a wide range of organic compounds, including aromatic and polyaromatic pollutants and toxic chemicals (Tóth and Borsodi, 2014). Plant-cyanobacterial interactions, as a beneficial symbiotic relationship, have long been demonstrated in rice growing areas. In addition, cyanobacteria may produce or secrete large amounts of biologically active compounds that have the ability to promote plant growth or may make plants more resistant to abiotic or biotic stresses (Bahareh et al., 2021). Most of the antagonistic bacteria screened were *Pseudomonas*, along with *Priestia*, *Stenotrophomonas*, and *Glutamicibacter*. A large number of *Pseudomonas* were screened in cm3J. *Stenotrophomonas* produced similar antibiotics and shared some enzymatic activities, which may make them attractive candidates for biological control of plant diseases and nematodes (Hayward et al., 2010).

We observed that RKN invasion caused recruitment and alteration of probiotic bacteria in both cm3J and cucJ. Cm3J was mainly enriched in the Proteobacteria and cuc was mainly enriched in the Actinobacteria and Bacilli. Moreover, the most abundant bacteria screened for antagonistic bacteria was *Pseudomonas* (Proteobacteria, Pseudomonadaceae). It was possible that *Pseudomonas* and other bacteria of the Proteobacteria played a part in RKN infestation of cm3J.

4.4 Hypothesizing the causes of differences in rhizosphere microbial communities

Root exudates of two crops may cause rhizosphere microbial differences. Root exudates include sugars, amino acids, organic acids, fatty acids, and secondary metabolites, which are important ways for plants to communicate with microorganisms and have a major influence on the composition of the rhizosphere microbiome (Bulgarelli et al., 2013; Sasse et al., 2018). Moreover, root exudates recruit microorganisms from the soil to the rhizosphere, where primary metabolites are mainly responsible for attraction and secondary metabolites are mainly responsible for screening the recruited microorganisms (Zhalnina et al., 2018). Furthermore, root exudates were strongly affected by plant species, developmental stage, root physiology, environment, soil type and stress type (Sasse et al., 2018). Beyond that, the assembly of microbial communities in plant roots also depends on microbial interactions (Bai et al., 2022). A study constructed a highly simplified maize SynCom, and single strain exclusion experiments on SynCom showed that no bacterial strains except Enterobacter cloacae caused SynCom collapse, indicating that Enterobacter cloacae was a key member in the community assembly process (Niu et al., 2017). In the case of cm3J and cucJ after infection with RKN in this study, it was possible that different root exudates and microbial interactions contributed to the differences in rhizosphere microbial communities.

5 Conclusion

In general, the interaction between RKN and rhizosphere bacteria was stronger in the early growth period, which provides a reference for the period of biocontrol. Secondly, a stable and well-connected rhizosphere bacterial community was positive for suppressing RKN infestation. Finally, *Pseudomonas* and other bacteria in the Proteobacteria in *Cucumis* crops showed clear changes in response to RKN invasion, which pointed the way for further research on biocontrol agents.

Data availability statement

The 16S sequence datasets for this study can be found in the NGDC with accession number: PRJCA014808 (https://ngdc.cncb.ac.cn).

Author contributions

BX and JL designed the experiment. ZM, YL, YY and JZ directed the experiment and the writing. XP and LS performed the experiment and processed the data. LS analysed the data and wrote the manuscript. JL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1163271/full#supplementary-material

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A cAMP phosphodiesterase is essential for sclerotia formation and virulence in *Sclerotinia sclerotiorum*

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Sclerotinia sclerotiorum is a plant pathogenic fungus that causes white mold or stem rot diseases. It affects mostly dicotyledonous crops, resulting in significant economic losses worldwide. Sclerotia formation is a special feature of *S. sclerotiorum*, allowing its survival in soil for extended periods and facilitates the spread of the pathogen. However, the detailed molecular mechanisms of how sclerotia are formed and how virulence is achieved in *S. sclerotiorum* are not fully understood. Here, we report the identification of a mutant that cannot form sclerotia using a forward genetics approach. Next-generation sequencing of the mutant's whole genome revealed candidate genes. Through knockout experiments, the causal gene was found to encode a cAMP phosphodiesterase (SsPDE2). From mutant phenotypic examinations, we found that SsPDE2 plays essential roles not only in sclerotia formation, but also in the regulation of oxalic acid accumulation, infection cushion functionality and virulence. Downregulation of *SsSMK1* transcripts in *Sspde2* mutants revealed that these morphological defects are likely caused by cAMP-dependent inhibition of MAPK signaling. Moreover, when we introduced HIGS construct targeting *SsPDE2* in *Nicotiana benthamiana*, largely compromised virulence was observed against *S. sclerotiorum*. Taken together, SsPDE2 is indispensable for key biological processes of *S. sclerotiorum* and can potentially serve as a HIGS target to control stem rot in the field.

KEYWORDS

***Sclerotinia sclerotiorum*, cAMP, cAMP phosphodiesterase, oxalic acid, host-induced gene silencing, pathogen control, next-generation sequencing, forward genetics**

Introduction

3',5'- cyclic adenosine monophosphate (cAMP) was first identified by Sutherland and colleagues in 1957 (Robison et al., 1971). It is a highly versatile secondary messenger playing essential roles in the regulation of many critical cellular processes across a wide range of organisms. For example, in bakers' yeast *Saccharomyces cerevisiae*, cAMP regulates

pseudohyphal morphogenesis (Pan and Heitman, 1999), mating (Arkinstall et al., 1991), glycogen utilization (Lemaire et al., 2004) and cell division cycle (Baroni et al., 1994). In mammalian cells, cAMP regulates processes including circadian clock (Fukuhara et al., 2004) and oxygen metabolism (Piccoli et al., 2006). In addition, cAMP-dependent signaling pathways are also essential for the survival and virulence of many pathogenic microbes. As an example, in *Candida albicans*, cAMP signaling cascade is involved in regulating key processes such as cell growth, filamentation, signal sensing, sexual mating and virulence (Huang et al., 2019).

Due to the important roles of cAMP, its biosynthesis and degradation must be strictly regulated to ensure a balanced signaling. cAMP is synthesized by plasma membrane-localized adenylate cyclase (AC) (Schwartz, 2001), which is activated by G protein-coupled receptors (GPCRs) in response to external signals, such as humidity, pH, etc. (Calebiro et al., 2009). Conversely, cAMP is degraded by phosphodiesterases (PDEs), cleaving the phosphodiester bond in cAMP to convert cAMP to AMP (Beavo et al., 2006). In mammals, there are over 10 different PDEs (Lugnier, 2006). Each PDE has a unique expression pattern and subcellular localization, which allows the regulation of specific cAMP and/or cGMP signaling pathways in different tissues and cell types (Degerman et al., 1997; Ekholm et al., 1997; Dousa, 1999).

In *S. cerevisiae*, PDE1 and PDE2 are the only two known PDEs with unrelated primary sequences, with low- and high-affinity respectively to their substrate. (Fujimoto et al., 1974; Sass et al., 1986; Nikawa et al., 1987). PDE1/CGS2 is the sole PDE in fission yeast *Schizosaccharomyces pombe*. Similar to *S. cerevisiae*, many phytopathogenic fungi also contain two PDEs, and PDE2 orthologs often play more crucial roles than PDE1 orthologs. For example, in grey mold pathogen *Botrytis cinerea*, deletion of *Bcpde2* results in severely compromised vegetative growth, conidiation, germination and virulence, while *Bcpde1* deletion mutant behaves like wildtype (Harren et al., 2013). Meanwhile, in rice blast fungus *Magnaporthe oryzae*, PDEH (high-affinity PDE2 ortholog) is the key regulator of cAMP and the loss of *PDEH* leads to dramatic defects in aerial hyphal growth and pathogenicity, while *pdel* (low-affinity PDE1 ortholog) mutant only shows mild defects (Ramanujam and Naqvi, 2010).

Sclerotinia sclerotiorum (Lib.) de Bary is a notorious soilborne plant fungal pathogen (Amselem et al., 2011; Xu et al., 2018). It has an extremely wide host range, capable of infecting over 600 plant species (Liang and Rollins, 2018), including many economically important crops such as canola, soybean, sunflower, and lettuce (Hegedus and Rimmer, 2005). The diseases caused by *S. sclerotiorum* are known as white mold or stem rot. It is a major threat to crop production worldwide, causing significant yield and quality losses (Bolton et al., 2006). *S. sclerotiorum* produces sclerotia, an overwintering structure that can survive in soil for many years (Adams and Ayers, 1979). Sclerotia can germinate carpogenically to release airborne ascospores as primary inoculum for new infections through reproductive apparatus apothecia (Erental et al., 2008). Sclerotia can also germinate as mycelia, infecting adjacent plants (Erental et al., 2008). Successful infection relies on the formation of infection cushions (also called compound appressoria), which enables penetration of plant tissues (Huang et al., 2008).

Various environmental factors, such as temperature, light and pH, affect sclerotia formation (Erental et al., 2008), along with genetic factors. The MAP kinase signaling cascades (MAPK cascades) are crucial in many cellular processes (Cargnello and Roux, 2011). They are conserved and involved in the development of *S. sclerotiorum*. *S. sclerotiorum* possesses three MAPKs (Amselem et al., 2011), with SsSMK1 being the most extensively studied. Our recent study has shown that the SsSMK1 cascade (SsSTE50-SsSTE11-SsSTE7-SsSMK1) is necessary for the development and virulence of *S. sclerotiorum* (Tian et al., 2023). However, the expression of *SsSMK1* is inversely associated with cAMP levels in *S. sclerotiorum*. Increased cAMP levels inhibit sclerotia development by interfering with *SsSMK1* transcription (Chen et al., 2004), demonstrating a negative impact of cAMP on Sclerotinia biology and MAPK signaling.

Interestingly, in *S. sclerotiorum*, cAMP seems to have diverse roles in its biology. Studies have shown that an adenylate cyclase loss-of-function mutant with cAMP synthesis deficiency, *sac1*, is impaired in growth, pathogenicity and sclerotia development (Jurick and Rollins, 2007). However, sclerotia development is inhibited when the endogenous and exogenous cAMP levels are elevated (Rollins and Dickman, 1998). The exact mechanism by which cAMP regulates key biological processes in *S. sclerotiorum* are still not fully understood. How cAMP homeostasis is controlled is also unclear.

In this study, we describe the identification of *Sspde2* from our forward genetics screen aiming to find mutants with sclerotia development defects (Xu et al., 2022). *Sspde2* mutants showed prolonged oxalic acid production, dysfunctional infection cushions, and compromised pathogenicity through cAMP-dependent MAPK inhibition. By performing host-induced gene silencing (HIGS) targeting *SsPDE2*, we observed largely compromised virulence of *S. sclerotiorum* on tobacco leaves. Thus, *SsPDE2* has the potential to be used as a HIGS target for the control of stem rot in plant hosts.

Materials and methods

Fungal strains and culture conditions

Fungal cultures were grown on potato dextrose agar (PDA, Shanghai Bio-way technology) at room temperature and stored on PDA slants at 4 °C or as sclerotia. For transformant screening, hygromycin B (Sigma) was added at a final concentration of 50 μg/ml. Bacteria used in this study were grown in Luria-Bertani (LB, Bio Basic) medium.

Colony morphology and growth rate determination

All strains with different genotypes were grown on PDA plates for 2-3 days. Then, mycelial agar disks were taken from the colony margin with a sterilized pipet tip end (5 mm diameter), transferred to the center of fresh PDA plates (90 mm diameter) and incubated

at room temperature. The colony diameter was measured every 12 hours until mycelia reached the edge of the petri plate. The images of colony morphology were taken 7- and 14-days post inoculation for sclerotia observation.

Acidification assay

Fungal strains were inoculated on PDA medium supplemented with 50 mg/L bromophenol blue (BPB). OA production (media acidification) is indicated by a change in PDA-BPB medium from blue to yellow.

Target gene knockout

The homologous recombination based method was used to generate *Sspde2* gene replacement cassette as previously described (Xu et al., 2022). All primers used for PCR are listed in Supplementary Table S1.

Plant infection assays

Mycelial plugs (2 mm or 5 mm in diameter) of 2-day-old cultures were inoculated on unwounded or wounded Arabidopsis (*Arabidopsis thaliana*, ecotype Col-0) or tobacco (*Nicotiana benthamiana*) leaves placed on moistened paper towels in petri dishes. Inoculated leaves were incubated in a growth chamber (23° C; 16 h day/8 h night regime). The lesion sizes were quantified by ImageJ software. The virulence test was repeated at least three times with similar results.

Infection cushions observation

Fresh mycelial plugs (5 mm in diameter) with growing hyphal tips were placed on the glass slides on moistened paper towels in petri dishes and incubated at 23°C for 36 h. The formation of infection cushions was monitored by a ZEISS light microscope.

RNA extraction and RT-PCR analysis

To examine the transcripts of *SsSMK1* in WT and *Sspde2* mutants, actively growing mycelia of different genotypes were inoculated on individual PDA plates overlaid with the cellophane for 2-3 days before reaching the edge of the plates. About 100 mg of fungal hyphae from each genotype were collected, RNA was extracted using EZ-10 Spin Column RNA Mini-preps kit (Bio Basic). cDNA was generated by Easy Script™ reverse transcriptase (ABM). Real-time PCR (RT-PCR) was performed using SYBR premix kit (TaKaRa) to quantify the expression of *SsSMK1* in WT and *Sspde2* mutants. The *S. sclerotiorum* gene *ACTIN* (*Sscle14g099090*) was used as internal control to normalize the expression. RT-PCR assay was repeated twice, each with three biological replicates. Primers used for RT-PCR are listed in Supplementary Table S1.

Transient expression of HIGS construct in *N. benthamiana* and *SsPDE2* expression examination in infected leaves

For HIGS vector construction, 958 bp sense fragment of *SsPDE2* was fused with intron 3 fragment of the malate synthase gene of *A. thaliana* (ms-i3; GenBank accession number AB005235), as described previously (Tinoco et al., 2010) through double-joint PCR. The fused fragments were cloned into plant expression vector pCambia1300 to generate the intermediate constructs, pCa-S-i. Then antisense fragment of *SsPDE2* were ligated into pCa-S-i vectors to create the final pCa-*PDE2*-RNAi construct. The primers used for making HIGS constructs were listed in Supplementary Table S1.

The binary pCa-*PDE2*-RNAi construct was introduced into *Agrobacterium tumefaciens* GV3101 through electroporation. The resulting agrobacteria with the optical density (OD_{600}) at 0.8 were infiltrated into the right halves of four-week-old *N. benthamiana* leaves through the blunt tip of plastic syringes as described before (Wu et al., 2019). The empty vector (EV) pCambia1300 was infiltrated into the left halves as control. After infiltration, the plants were kept in dark for 3 days to induce the expression of the RNAi constructs. Then both left and right sides of infiltrated *N. benthamiana* leaves were inoculated with *S. sclerotiorum* WT strain 1980 to examine disease progression.

To assess trans-species RNAi, about 100 mg of necrotic tissues from infected tobacco leaves expressing EV or pCa-*PDE2*-RNAi construct were collected for total RNA extraction, cDNA generation and RT-PCR analysis as described above. *ACTIN* was used as internal control. RT-PCR assay was repeated twice, each with three biological replicates.

Results

A UV mutant of *S. sclerotiorum* exhibits no sclerotia, aberrant oxalic acid production and largely compromised virulence

We previously performed a forward genetic screen with ascospores of *S. sclerotiorum* (Xu et al., 2022). MT2 was identified as a mutant that failed to form sclerotia (Figure 1A). When vegetative growth was examined on petri plates, MT2 showed 13% reduction in mycelial growth as compared to wildtype (WT) strain *S. sclerotiorum* 1980 (Figure 1B).

To test whether MT2 is defective in pathogenicity, we first examined its oxalic acids (OA) levels, as OA secretion by Sclerotinia is a key virulence determinant of the pathogen (Cessna et al., 2000). When MT2 was grown on potato dextrose agar (PDA) supplemented with bromophenol blue (violet when pH > 4.6; yellow when pH < 3.0), both WT and MT2 plates changed color rapidly from violet to yellow within 3 dpi (days post inoculation). WT plate returned to light violet within 10 dpi, likely due to OA degradation. However, MT2 plates

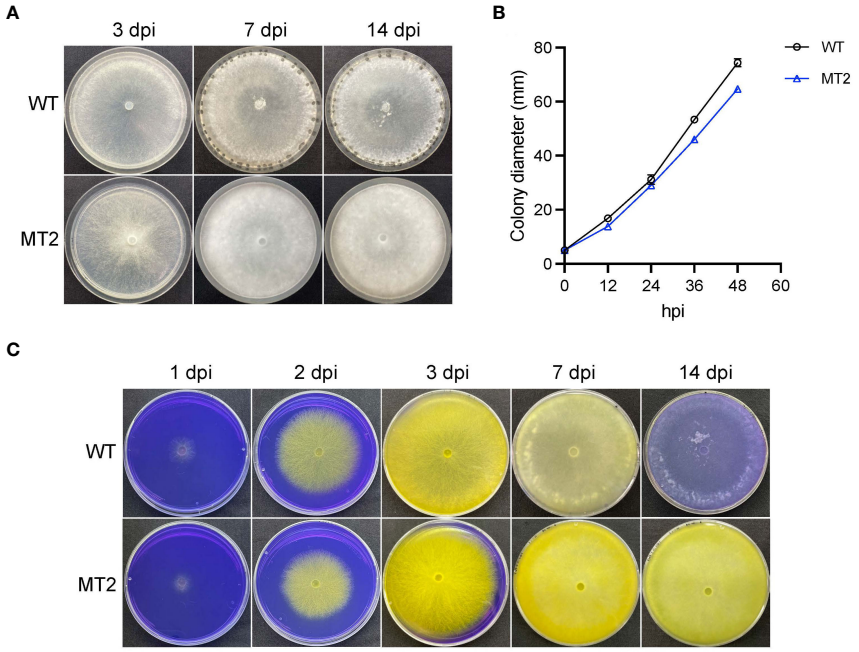

FIGURE 1
MT2 exhibits no sclerotia formation and sustained oxalic acid levels. **(A)** Colony morphology of WT and MT2. Both strains were cultured on PDA plates for 3, 7 and 14 days, respectively, before the pictures were taken. **(B)** Colony diameters of WT and MT2 on PDA plates over a period of 48 h The diameters were measured every 12 hours. **(C)** Acidification assay. Yellow color indicates acidification. Photos were taken at 1, 2, 3, 7 and 14 dpi, respectively.

remained yellow even two weeks after inoculation, suggesting a sustained OA levels in MT2 (Figure 1C).

To test the virulence of MT2, we used detached WT leaves of both *Nicotiana benthamiana* and Arabidopsis Col ecotype. MT2 caused no lesions when its mycelia were inoculated on Arabidopsis leaves (Figure 2A). It formed much smaller lesions on *N. benthimiana* (Figure 2C). However, with pre-wounding, the pathogenicity of MT2 on Arabidopsis and tobacco leaves was somewhat restored (Figures 2B, D), suggesting that the impaired pathogenicity of MT2 is due to both penetration and post-penetration defects during infection.

Infection cushion formation of *S. sclerotiorum* plays an indispensable role in its pathogenicity. WT formed mature infection cushions on glass slides within 24 hpi, shown as pigmented hyphal aggregates (Figure 2E). However, MT2 overproduced infection cushions. These infection structures could be observed at almost every hyphal branch tip (Figure 2E). Due to the penetration defects of MT2, these over-accumulated infection cushions are likely malfunctioned.

MT2 contains a mutation in a cAMP phosphodiesterase encoding gene

To identify the causal mutation responsible for the MT2 defects, the full genome of MT2 was sequenced by next -generation sequencing (NGS). Using the pipeline we established for mutation analysis (Xu et al., 2022), six significant SNPs (single nucleotide polymorphisms) leading to nonsynonymous changes were captured (Figure 3A). We first knocked out two candidate genes, *Sscle04g033830* and *Sscle15g106030*, and observed no phenotypic defects compared with WT (data not shown). After analyzing the NGS data for INDELs (insertion and deletion mutations), we found two of them with frameshift consequences (Figure 3A). One of them, *Sscle06g053640*, encodes a cAMP phosphodiesterase, SsPDE2. Previous studies in *Botrytis cinerea* showed that deletion of *Bcpde2*, the ortholog of SsPDE2, resulted in no sclerotia formation and significantly reduced virulence (Harren et al., 2013). Further analysis revealed that the frameshift mutation in *Sscle06g053640* resulted in a premature stop codon (Figure 3B). Considering the similar defects between MT2 and *Bcpde2*, *Sscle06g053640* became the prime candidate for MT2, and MT2 was renamed as *Sspde2-1*.

Knocking out *SsPDE2* yielded mutants with phenotypes like MT2

A targeted gene knockout method based on homologous recombination (Figure 3C) and protoplast purification was used to obtain pure *Sscle06g053640* deletion mutants in the WT background. Two independent pure deletion alleles, *Sspde2-2* and *Sspde2-3*, were obtained and verified by PCR. A 905-bp fragment within the *Sscle06g053640* gene was present in WT but absent in both transformants (Figure 3D). The presence of selection marker gene hygromycin phosphotransferase (*HPH*) in the transformants was also confirmed by the amplification of a 799-bp product (Figure 3D).

When these two knockout mutants, *Sspde2-2* and *Sspde2-3*, were examined together with MT2, all displayed similar phenotypes including no sclerotia formation and slightly reduced vegetative growth

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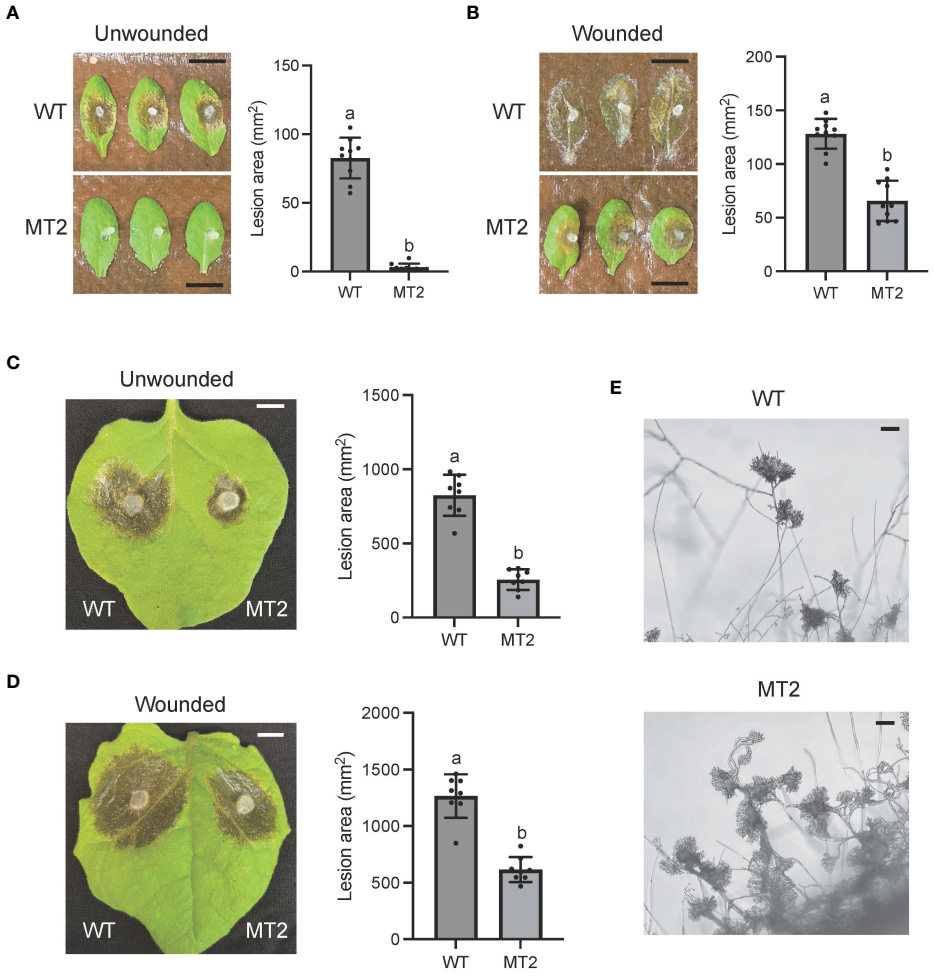

FIGURE 2
MT2 exhibits largely compromised virulence in both Arabidopsis and *N. benthamiana*. **(A, B)** Left: Pathogenicity assay for WT and MT2 on unwounded **(A)** and wounded **(B)** leaves of Arabidopsis respectively. Representative photos were taken at 36 hpi. Right: quantification of lesion areas with WT and MT2 on unwounded **(A)** and wounded **(B)** leaves of Arabidopsis respectively. The dots represent the values of lesion areas measured by ImageJ. The statistics analysis was carried out by One-way ANOVA. Letters represent statistical significance ($p < 0.01$). Error bars represent means ± Standard Deviation (SD, n= 8). The scale bar is 1 cm. (**C and D**) Left: pathogenicity assay for WT and MT2 on unwounded **(C)** and wounded **(D)** leaves of *N. benthamiana* respectively. Representative photos were taken at 48 hpi. Right: quantification of lesion areas with WT and MT2 on unwounded **(C)** and wounded **(D)** leaves of *N. benthamiana*, respectively. The dots represent the values of lesion areas measured by ImageJ. The statistics analysis was carried out by One-way ANOVA. Letters represent statistical significance ($p < 0.01$). Error bars represent means ± SD (n= 8). The scale bar is 1 cm. **(E)** Infection cushions formation on glass slides. The images were taken at 36 hpi. The scale bar is 100 µm.

(Figures 4A, B). Meanwhile, *Sspde2-2* and *Sspde2-3* behaved similarly as MT2 in OA accumulation (Figure 4C), colonization on detached leaves of Arabidopsis (Figures 5A, B) and *N. benthamiana* (Figures 5C, D) and infection cushion formation (Figure 5E). Therefore, we conclude that *SsPDE2* is the causal gene for the MT2 phenotypes.

The defects of *Sspde2* are associated with cAMP-dependent MAPK inhibition

Previous study has shown that the addition of compounds which increase either endogenous or exogenous cAMP levels inhibits sclerotial development, and this cAMP-dependent inhibition is through interfering with mitogen-activated protein kinase (MAPK) signaling which regulates numerous cellular growth and developmental processes (Lewis et al., 1998). As a predicted cAMP degrading enzyme, the disruption of *SsPDE2* may lead to elevated cAMP levels, thereby blocking MAPK activation. To test this, the transcripts of MAPK signaling marker gene *SsSMK1*, an ERK-type MAPK required for sclerotial formation in *S. sclerotiorum* (Chen et al., 2004), was examined by Real time-PCR (RT-PCR). As shown in Figure 5F, *SsSMK1* expression was reduced by 50% in *Sspde2* mutants compared with that in WT, demonstrating that loss of *SsPDE2* is indeed associated with cAMP-mediated downregulation of MAPK activity.

The deletion alleles of *Sspde2* failed to complement the sclerotia formation defect of MT2 by hyphal fusion

To further confirm that *SsPDE2* is responsible for the MT2 phenotypes, we performed a hyphal fusion assay. In most

A

Reference gene	Nucleotide change	AA change	Predicted function
Sscle04g033830	C31T	L11F	short chain dehydrogenase /reductase
Sscle06g050900	G4922A	G1641E	Uncharacterized protein
Sscle06g053640	T189 deletion	F63 frameshift	3'5'-cyclic nucleotide phosphodiesterase
Sscle09g069060	G1196A	G399E	Sugar/inositol transporter
Sscle10g075940	C838T	H280Y	Uncharacterized protein
Sscle10g078880	T1020 deletion	F340 frameshift	Uncharacterized protein
Sscle15g106030	A1983C	L661F	Oligopeptide transporter
Sscle16g110530	C412T	R138C	Uncharacterized protein

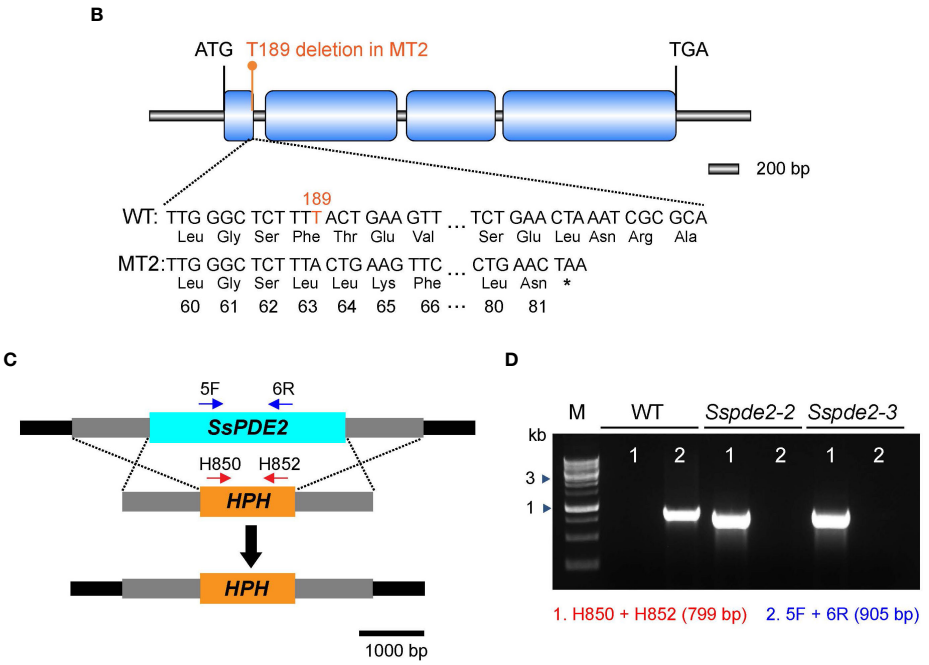

FIGURE 3
MT2 carries a mutation in *SsPDE2*. **(A)** List of candidate mutations of MT2 from NGS data analysis as compared with the reference WT *S. sclerotiorum* Strain 1980. *SsPDE2* (*Sscle06g053640*) is highlighted in red. **(B)** Diagram of the frameshift mutation in *Sscle06g053640*, including exons, introns, start codon and stop codon. The deleted nucleotide is highlighted in orange. The premature stop codon caused by the frameshift is shown as *. The diagram was drawn using Illustrator for Biological Sequencing (IBS) (Liu et al., 2015). **(C)** The *SsPDE2* locus and gene replacement design. The *SsPDE2* and *HPH* genes are presented as light blue and orange rectangles, respectively. The primers indicated by red and blue arrows in the diagram were used for knockout mutant screening. The scale is shown at the bottom. **(D)** PCR verification of *Sscle06g053640* (*SsPDE2*) gene deletion. Genomic DNA isolated from WT and mutant alleles *Sspde2-2* and *Sspde2-3* were used as PCR templates. Positions of the two pairs of primers for checking the insertion of *HPH* and *SsPDE2* deletion are indicated in **(C)** and the sizes of amplified bands are shown in brackets. M lane is DNA marker.

multinucleate fungal species, such as *Neurospora crassa* (Beadle and Coonradt, 1944) and *Aspergillus nidulans* (Timberlake and Marshall, 1988), hyphal fusion can occur between two or more genetically distinct individuals. This results in the formation of heterokaryons, which allow for genetic exchange and complementation among different nuclei within the heterokaryons. As *S. sclerotiorum* is able to form stable heterokaryons (Ford et al., 1995), mycelial fusion experiment can be performed to examine the genetic relationship between mutants. When MT2, *Sspde2-2* and *Sspde2-3* were cultured with different combinations on PDA plates for 7 days (Figure 6), none of these combinations gave rise to sclerotia. However, for the control plate when MT2 and R240, a non-sclerotial forming mutant found from the same screen and carries mutation in different genes (unpublished data from our lab), were fused, sclerotia formation was rescued. This suggests that *Sspde2* deletion alleles failed to complement MT2, with providing further evidence that *SsPDE2* is responsible for the MT2 phenotypes.

SsPDE2 is predicted to be a high-affinity cAMP phosphodiesterase

SsPDE2 is 944-aa in length and has a central cAMP/GMP phosphodiesterase catalytic domain (Figure 7A). This PDE domain

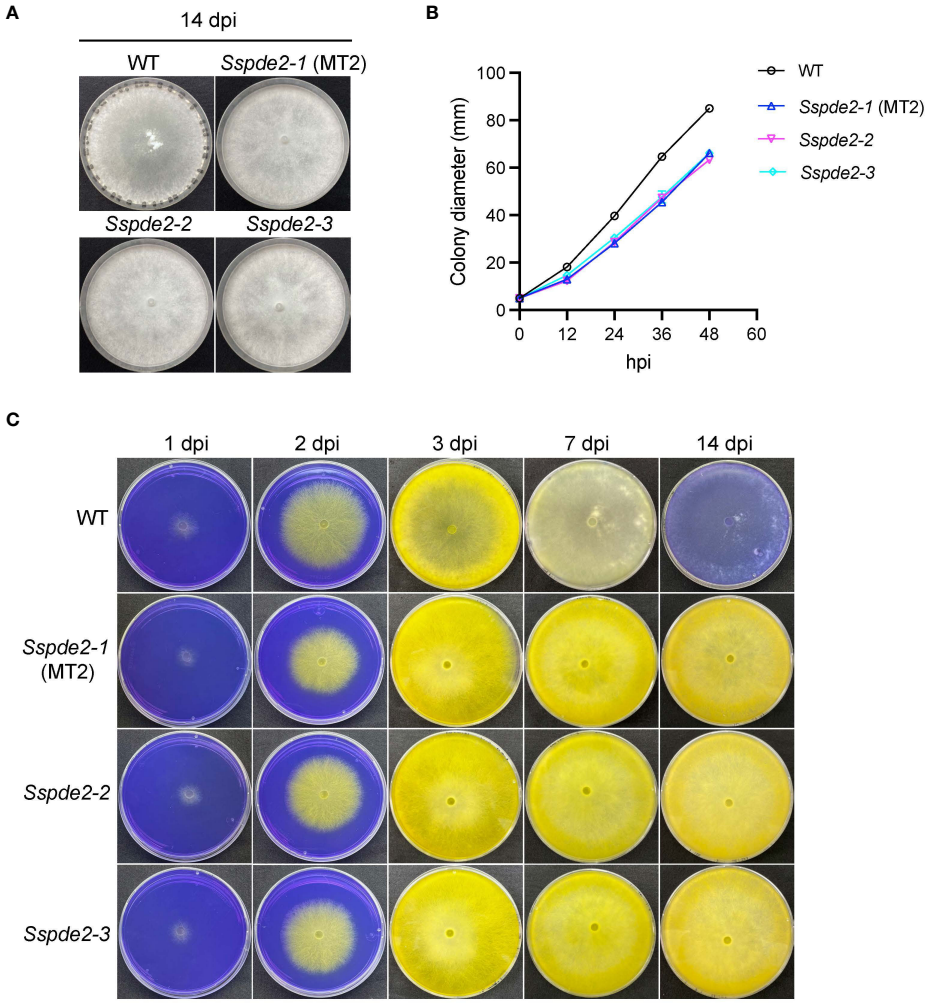

FIGURE 4
Sspde2 deletion alleles behave similarly as MT2 in vegetative growth, sclerotia formation and OA production. **(A)** Colony morphology of WT, *Sspde2-1* (MT2), *Sspde2-2*, and *Sspde2-3*. All genotypes were cultured on PDA plates. Photos were taken at 14 dpi. **(B)** Colony diameters of the indicated genotypes on PDA plates over a period of 48 h The diameters were measured every 12 hours. **(C)** Acidification assay. Photos were taken at 1, 2, 3, 7 and 14 dpi, respectively.

shares high sequence similarity with its orthologous proteins in other filamentous fungi and all PDE2 proteins carry a conserved PDE class I motif (Ramanujam and Naqvi, 2010) at the predicted PDE active sites (Figure 7A). As many PDE proteins possessing class I motif have been proven to be a high-affinity cAMP PDE and control the basal levels of intracellular cAMP, such as ScPDE2 in bakers' yeast (Sass et al., 1986) and MoPDEH in *M. oryzae* (Ramanujam and Naqvi, 2010), SsPDE2 is likely also a high-affinity PDE that can degrade cAMP and regulate cAMP levels in *S. sclerotiorum*.

Like bakers' yeast *S. cerevisiae*, *S. sclerotiorum* also has two phosphodiesterases, PDE1 and PDE2. SsPDE1 is 482 aa in length and has a cAMP PDE catalytic domain (Figure 7B). Different from SsPDE2, SsPDE1 possesses a conserved class II PDE motif at the predicted PDE active sites (Figure 7B). Since class II PDEs have low cAMP affinity, SsPDE1 may be a low-affinity PDE in *S. sclerotiorum*.

Although both SsPDE1 and SsPDE2 are designated to be PDEs, they are dissimilar in sequences. Phylogenetic analysis of PDE1/2 orthologs in plant pathogenic fungi and yeast showed that PDE1s and PDE2s diverge early in evolution and fall into distinctive clades (Figure 7C). Within each clade, PDEs in pathogenic fungi are more closely related than those in yeast (Figure 7C), suggestive of possible conserved roles of PDEs in phytopathogenic fungi.

HIGS of *SsPDE2* attenuates *S. sclerotiorum* virulence in tobacco

Host-induced gene silencing (HIGS) is a strategy that has been developed for plant disease control. In HIGS, plants can be engineered to express double-stranded RNAs (dsRNAs) that can target specific pathogen genes. When the pathogen is in contact with the host that can generate the dsRNAs, the dsRNAs can be up-taken by the pathogen through trans-kingdom RNAi (Wang and Dean, 2020), triggering the degradation of the corresponding target genes.

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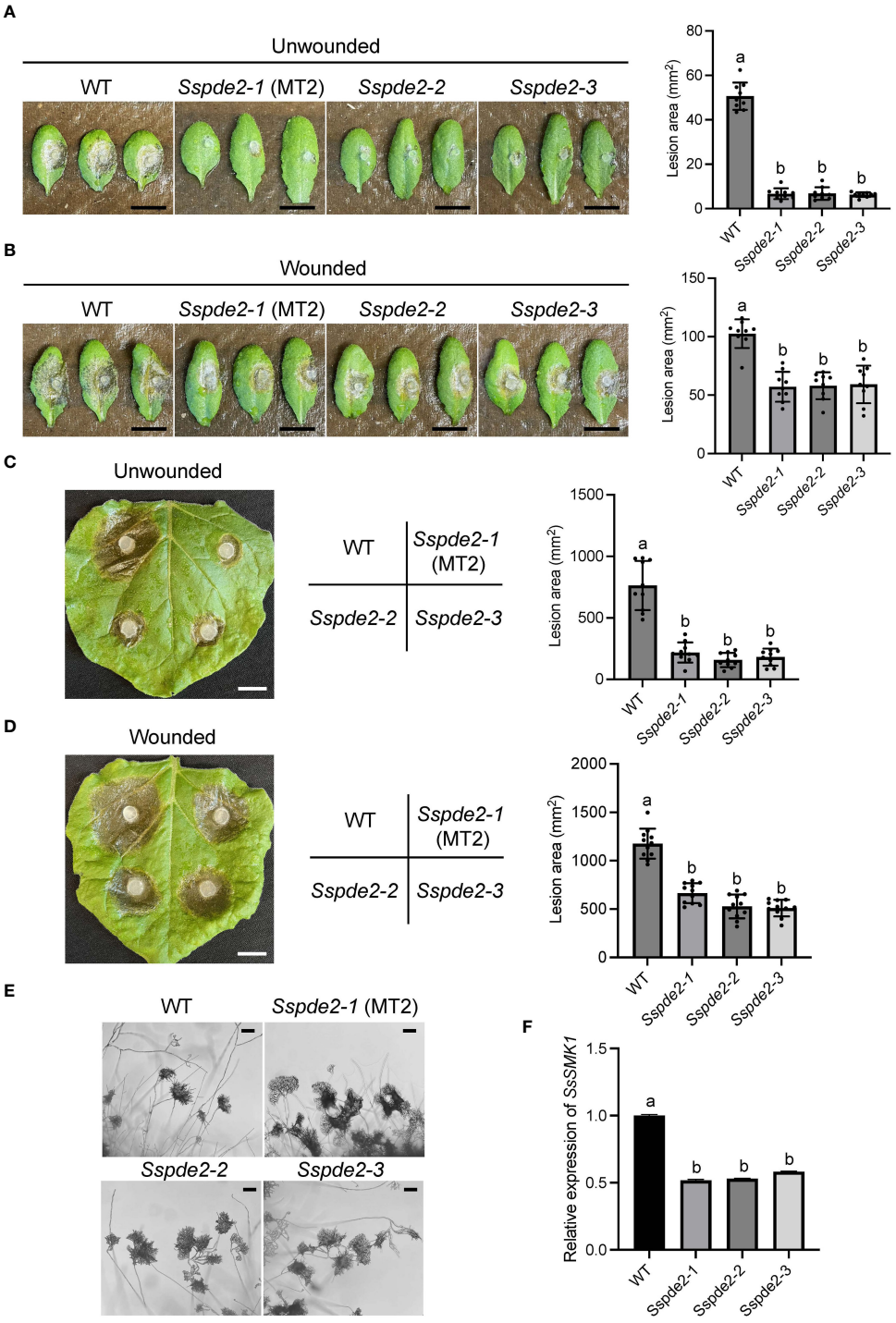

FIGURE 5
Sspde2 deletion alleles show similar defects as MT2 in virulence, infection cushion formation and MAPK transcriptional regulation. **(A, B)** Left: Pathogenicity assay for WT, *Sspde2-1* (MT2), *Sspde2-2*, and *Sspde2-3* on unwounded **(A)** and wounded **(B)** leaves of Arabidopsis respectively. Representative photos were taken at 36 hpi. Right: quantification of lesion areas with the same sets of genotypes on unwounded **(A)** and wounded **(B)** leaves of Arabidopsis respectively. The dots represent the values of lesion areas measured by ImageJ. The statistics analysis was carried out by One-way ANOVA. Letters represent statistical significance ($p < 0.01$). Error bars represent means ± SD (n= 9). The scale bar is 1 cm. **(C, D)** Left: pathogenicity assay for WT, *Sspde2-1* (MT2), *Sspde2-2*, and *Sspde2-3* on unwounded **(C)** and wounded **(D)** leaves respectively of *N. benthamiana*. Representative photos were taken at 48 hpi. Middle: Indication of inoculated genotypes on tobacco leaf shown in the left. Right: quantification of lesion areas with the same sets of genotypes on unwounded **(C)** and wounded **(D)** leaves of *N. benthamiana*, respectively. The dots represent the values of lesion areas measured by ImageJ. The statistics analysis was carried out by One-way ANOVA. Letters represent statistical significance ($p < 0.01$). Error bars represent means ± SD (n= 9). The scale bar is 1 cm. **(E)** Infection cushions formation on glass slides. The images were taken at 36 hpi. The scale bar is 100 μm. **(F)** Relative gene expression of *SsSMK1* in WT and *Sspde2* mutants. The statistics analysis was carried out by One-way ANOVA. Letters represent statistical significance ($p < 0.01$). Error bars represent means ± SD.

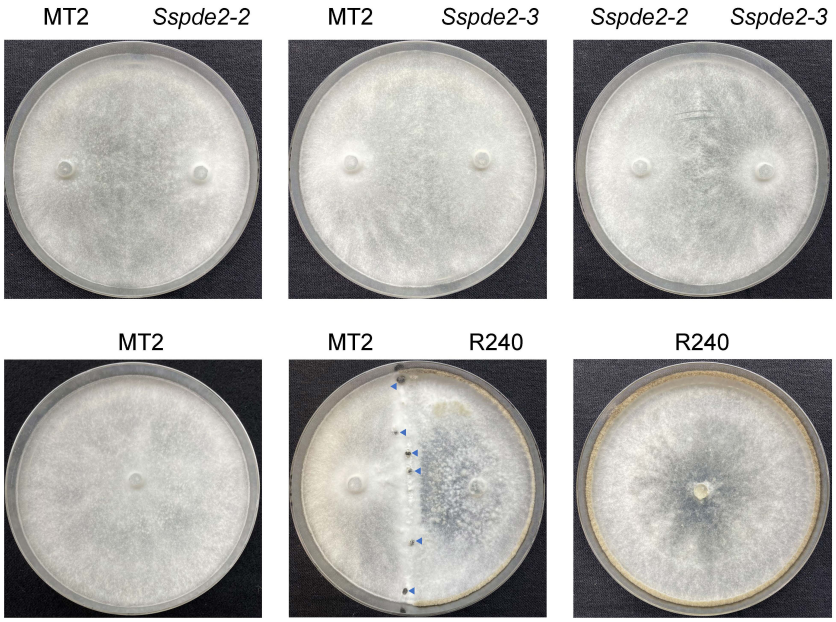

FIGURE 6
Sspde2 deletion alleles failed to complement the sclerotia formation defect of MT2 by hyphal fusion. All genotypes with different combinations were inoculated on two sides of the plates for genetic complementation test. R240 was used as a positive control. Representative photos were taken at 7 dpi. Blue arrows indicate the sclerotia formed in the middle.

HIGS has been shown to be effective against a wide range of plant pathogens, including fungi (Nowara et al., 2010; Song and Thomma, 2018; Spada et al., 2021) and nematodes (Iqbal et al., 2020). HIGS has been attempted in *S. sclerotiorum* previously to target well-studied pathogenicity genes, such as OA biosynthesis gene *SsOAH1* (McCaghey et al., 2021). Here, we tested whether *SsPDE2* can be used as a HIGS target. When empty vector (EV) and HIGS construct pCa-*PDE2*-RNAi were infiltrated into opposite sides of the same *N. benthamiana* leaf followed by *S. sclerotiorum* inoculation, the lesion area was largely reduced on the side expressing *SsPDE2* HIGS construct compared to the EV control (Figure 8A). Moreover, the gene expression level of *SsPDE2* in *S. sclerotiorum* WT strains inoculated onto *N. benthamiana* leaves expressing pCa-*PDE2*-RNAi was reduced by 70% compared with those leaves expressing EV (Figure 8B), demonstrating the successful trans-species RNAi of *SsPDE2*. Thus, *SsPDE2* can serve as a HIGS target for disease control against *S. sclerotiorum*.

Discussion

In this study, we identified a putative high-affinity phosphodiesterase, SsPDE2, by a forward genetics approach. SsPDE2 is required for multiple developmental pathways and pathogenicity as mutations in *SsPDE2* result in no sclerotia formation, aberrant oxalic acid production and largely attenuated virulence in *S. sclerotiorum*.

As a predicted hydrolytic enzyme, the major biological role of PDE2 is to break down cAMP by hydrolyzing the phosphodiester bond to yield AMP (Beavo et al., 2006). Therefore, disruption of *PDE2* causes hyperaccumulation of cAMP (Park et al., 2005). Earlier studies have shown that addition of compounds which can increase cAMP levels, such as Caffeine (inhibiting phosphodiesterase activity) and NaF (activating AC), blocks scleroial initiation (Rollins and Dickman, 1998). This is consistent with our observation where *Sspde2* mutants fail to form sclerotia. Meanwhile, *Sspde2* mutants show the same colony morphology as WT *S. sclerotiorum* grown on PDA plates supplemented with 10 mM cAMP (Chen et al., 2004; Chen and Dickman, 2005). Thus, the inability in sclerotia formation in *Sspde2* is likely caused by highly elevated cAMP levels, and SsPDE2 therefore serves as a key cAMP homeostasis regulator in *S. sclerotiorum*.

In mammalian cells, cAMP binds to Rap1, a small monomeric GTPase, and inhibits the activity of MAPK cascades (Hu et al., 1997). This also occurs in *S. sclerotiorum*. It has been shown that addition of cAMP not only inhibits sclerotia formation, but also Ss*SMK1* transcription, supporting a negative role of cAMP on MAPK signaling (Chen et al., 2004; Chen and Dickman, 2005). SsSMK1 is an ERK-type MAPK that plays important role in many cellular processes in *S. sclerotiorum*. The disruption of *SsSMK1* results in reduced hyphal growth, impaired sclerotia development and attenuated pathogenicity (Chen et al., 2004; Tian et al., 2023). As *SsSMK1* downregulation was observed in *Sspde2* mutants, and considering the inhibitory effect of cAMP, it is likely that *SsPDE2* promotes *SsSMK1* transcription by reducing cellular cAMP levels.

Moreover, in this study, we found that *Sspde2* mutants exhibit prolonged acidification compared with WT. This could be ascribed to mis-regulated OA production and catabolism as it is known that increasing cellular cAMP levels enhances OA accumulation (Rollins and Dickman, 1998; Chen and Dickman, 2005). OA has been extensively studied for its role in the virulence of *S. sclerotiorum*. During the initial stages of infection, OA can act as a necrotizing

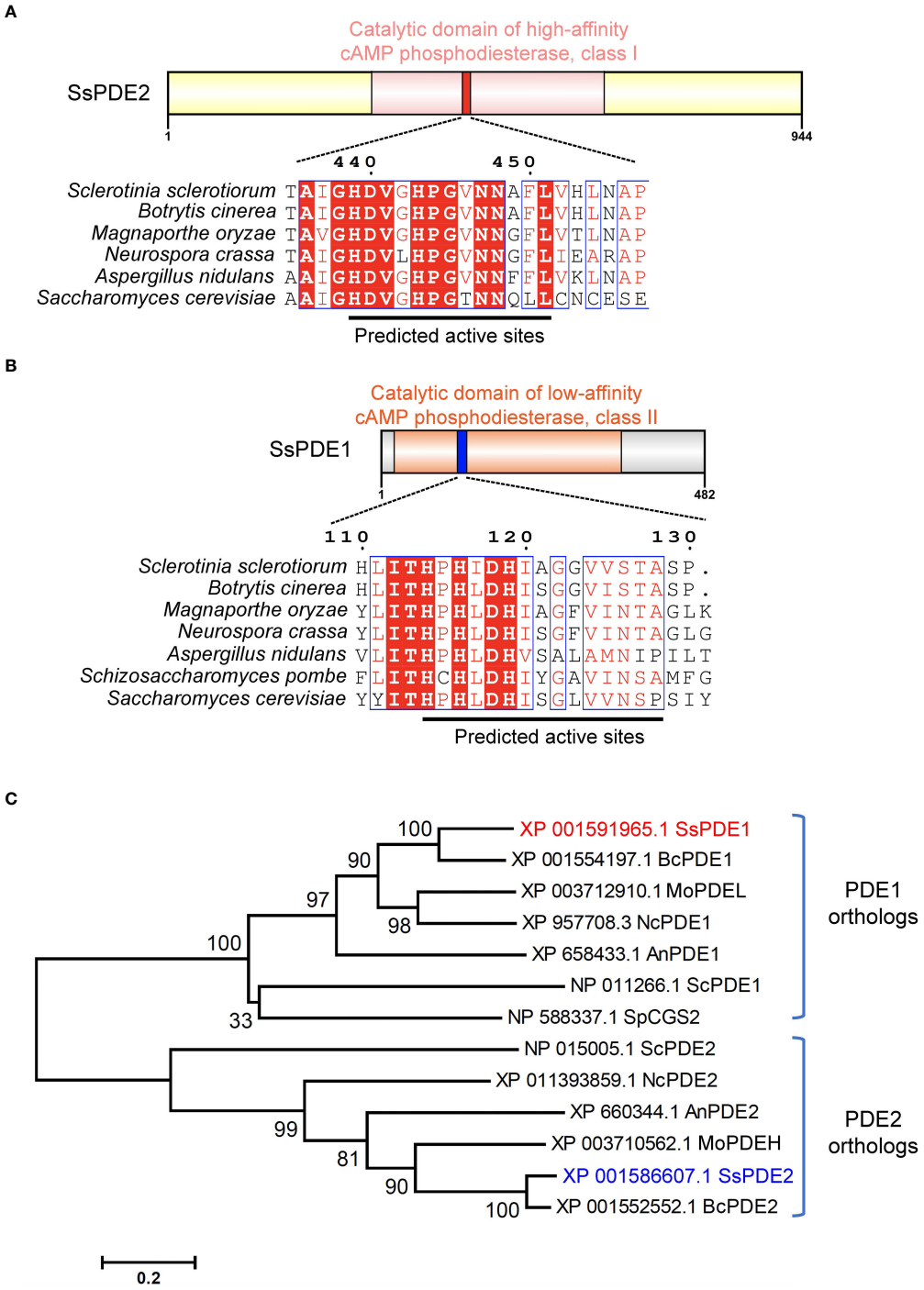

FIGURE 7
SsPDE2 is predicted to be a high-affinity cAMP phosphodiesterase. **(A, B)** Sequence analysis of SsPDE1 and SsPDE2. The catalytic domains of PDEs were analyzed by InterPro (https://www.ebi.ac.uk/interpro/). The diagrams were drawn using IBS (Liu et al., 2015). Sequence alignments of PDE1/2 orthologs in *S. sclerotiorum* and other filamentous fungi were conducted by CLUSTALW and alignment images were made using ESPript 3.0 (Robert and Gouet, 2014). Identical or conserved amino acids are either highlighted by red shadows or boxed. And the predicted PDE active sites were indicated by black lines. **(C)** Phylogenetic analysis of SsPDE1/2 proteins from *S. sclerotiorum* and other fungi. The tree was built using MEGA6, Neighbor-joining method and evaluated by bootstrap. The bootstrap values from 1,000 replicates are labelled above the branches. SsPDE1/2 are highlighted in red and blue, respectively. The accession numbers of PDEs used to build the tree are labeled on the right. The scale bar is shown at the bottom.

virulence factor by lowering the pH of the plant tissues, assisting fungal penetration and colonization (Kim et al., 2008; Liang et al., 2015). Therefore, *S. sclerotiorum* pathogenesis requires the accumulation of high OA levels. However, in this study, sustained OA levels in *Sspde2* does not seem to aid fungal colonization. On the contrary, *Sspde2* has significantly compromised virulence. This negative effect of OA towards *S. sclerotiorum* pathogenesis can be explained by the study of an oxalate decarboxylase SsODC2, which

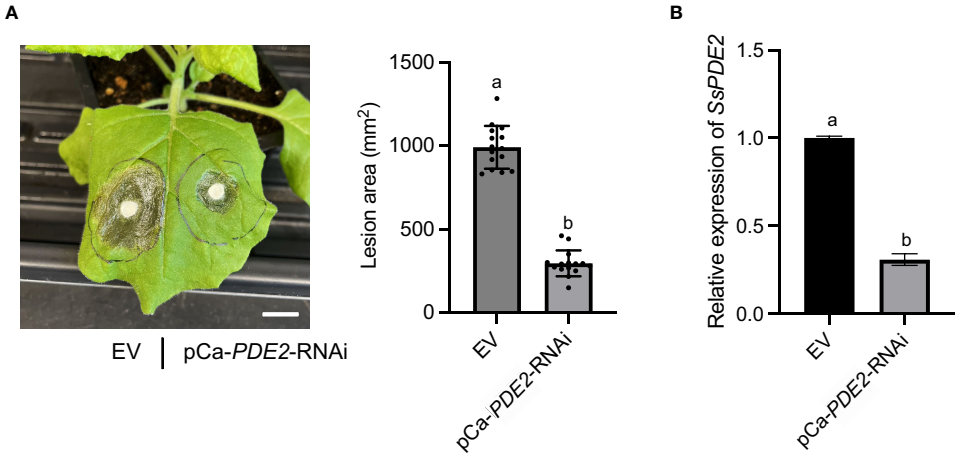

FIGURE 8
HIGS of *SsPDE2* confers resistance against *S. sclerotiorum* infections. **(A)** Left: Pathogenicity assay of *S. sclerotiorum* WT strain on leaves expressing EV (left) and pCa-*PDE2*-RNAi (right) constructs. The representative photo was taken at 48 hpi. Right: Quantification of lesion areas. The dots represent the values of lesion areas measured by ImageJ. The statistics analysis was carried out by One-way ANOVA. Letters represent statistical significance ($p < 0.01$). Error bars represent means ± SD (n= 15). The scale bar is 1 cm. **(B)** Relative gene expression of *SsPDE2* in EV and pCa-*PDE2*-RNAi expressing *N. benthamiana* leaves inoculated with *S. sclerotiorum* WT strains. The statistics analysis was carried out by One-way ANOVA. Letters represent statistical significance ($p < 0.01$). Error bars represent means ± SD.

catabolizes OA into carbon dioxide and formate. *Ssodc2* loss-of-function mutant was less efficient at compound appressorium differentiation and exhibited reduced virulence despite OA hyperaccumulation *in vitro* (Liang et al., 2015). Therefore, the role of OA in pathogenesis is complex. Proper amount of OA is required for epidermal cell disruption and penetration. When OA levels exceed certain threshold, especially with extended length of time, it may expose the fungus to toxicity, compromising the pathogen.

Like other filamentous fungi, *S. sclerotiorum* has two PDEs, SsPDE1 and SsPDE2. Their respective orthologs, BcPDE1 and BcPDE2, have been studied in another closely related sclerotia-forming pathogen *B. cinerea*. Deletion of *BcPDE2* resulted in significantly impaired hyphal growth, conidiation, spore germination, sclerotia formation and virulence. However, *bcpde1* deletion mutant is WT-like (Harren et al., 2013). Here, we found that *Sspde2* displayed similar defects as *Bcpde2* in sclerotia development and virulence, but not in vegetative growth. Only 13% growth retardation was observed in *Sspde2* compared with WT. It is possible that different from the negligible role of BcPDE1 in *B. cinerea*, SsPDE1 may contribute to the hyphal growth in *S. sclerotiorum*. This can be tested in the future by obtaining *Sspde1* mutant.

HIGS is a technique in plant disease control that involves the use of host-delivered dsRNA to silence specific genes in plant pathogens (Kong et al., 2022). HIGS can be achieved against many eukaryotic pest which has the RNAi pathway, (Shabalina and Koonin, 2008) as small interfering RNAs (siRNAs) can move from host plants to invading pathogens through cross-kingdom RNAi (Ming Wang et al., 2016). HIGS has been attempted in various plant-pathogen interactions and has been proven to be efficient in many cases (Nowara et al., 2010; Zhang et al., 2016; Spada et al., 2021). Although HIGS has the potential to be broadly applied in plant protection, one limitation is the identification of optimal targets to silence. Since our study has shown that SsPDE2 is essential for many processes in *S. sclerotiorum*, especially in virulence, silencing *SsPDE2* could potentially impair the ability of *S. sclerotiorum* to cause diseases in plants. Indeed, when we expressed cross-kingdom RNAi construct targeting *SsPDE2* in tobacco leaves, largely compromised virulence of *S. sclerotiorum* was observed. Therefore, *SsPDE2* has the potential to be used as HIGS targets for controlling stem rot in plants. Moreover, the conservation of *SsPDE2* in many necrotrophic fungal pathogens raises the possibility that *SsPDE2* may also be used to manage other diseases caused by similar fungi.

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

YX performed most of the experiments and wrote the manuscript draft. XL and YZ supervised the work and revised the manuscript. YQ identified the MT2 mutant in the screen. 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/fpls.2023.1175552/full#supplementary-material

SUPPLEMENTARY TABLE 1
List of primers used in this study.

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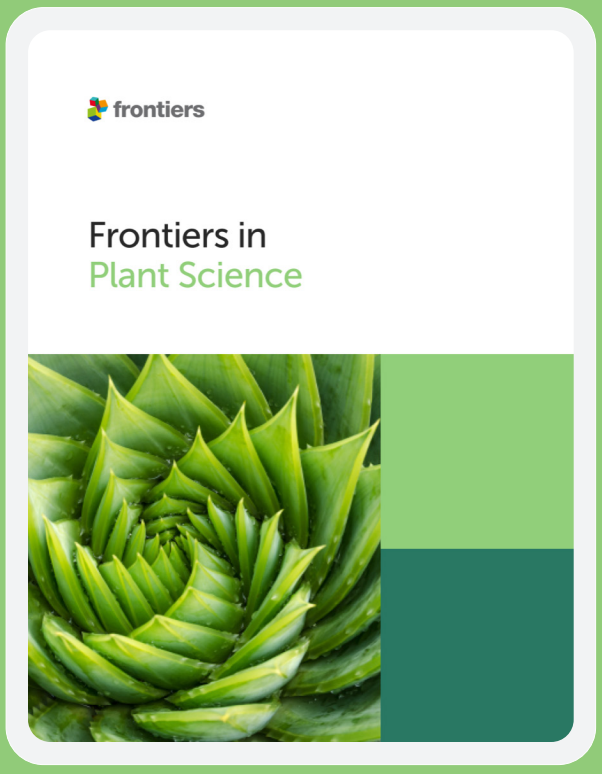

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