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Combined use of *Trichoderma harzianum* and *Clonostachys rosea* to manage *Botrytis cinerea* infection in tomato plants

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Abstract *Botrytis cinerea* is a common pathogenic agent, causing a significant reduction in tomato quality and yield. In this study, the potential of two biocontrol fungal agents, *Trichoderma harzianum* and *Clonostachys rosea* to prevent and alleviate *B. cinerea* infection was assessed. To this end, the fungicidal effects of both biocontrol fungi were evaluated on ten common phytopathogens, including *B. cinerea*. Then, various ratios of *T. harzianum* and *C. rosea* mixtures were assessed to find the ratio that best promotes the growth of tomato seedlings and prevents the establishment of *B. cinerea*. The results showed that *T. harzianum* and *C. rosea* had significant fungicidal effects on all tested phytopathogens. Also, both biocontrol agents reduced the establishment of *B. cinerea*, *in vitro* assays, indicating they had significant fungicidal effects against this phytopathogen. However, when applied as a biofungicide to tomato plants, a combination of both biocontrol agents gave better control of *B. cinerea*. Investigating the molecular basis of resistance to *B. cinerea* induced by the mixture of *T. harzianum* and *C. rosea* revealed that the salicylic acid signaling pathway plays an important role in modulating the antioxidant enzyme activity. Taken together, the results of this research confirmed that both biocontrol fungal agents could induce the mechanism of *B. cinerea* resistance in tomato plants and shed light on the regulatory pathways associated with the resistance mechanism.

Keywords *Lycopersicon esculentum* · Gray mold · Salicylic acid signaling pathway · Disease management · Compound fungal agents

Fengshuo Li and Hossien Ghanizadeh contributed equally.

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Introduction

Tomato (*Lycopersicon esculentum* Mill.), is a plant member of the Solanaceae family and is native to South America (Guillemaud et al., 2015). Tomato is one of the most widely cultivated crops in the world due to its unique flavor and rich nutritional values

(Cheng et al., 2022). However, large areas of tomato production are prone to plant diseases, with *Botrytis cinerea* (gray mold) being a major cause of postharvest rot in tomatoes globally (Meng et al., 2022).

Botrytis cinerea releases a large number of spores on infected tissues, causing the pathogen to spread quickly to unaffected tissues (Wang et al., 2010). *Botrytis cinerea* can infect over 200 plant species (Williamson et al., 2007), and it has become a major limiting factor in tomato production (Cantu et al., 2009). The application of chemical fungicides is the most common method to prevent and control *B. cinerea* in agricultural production (Gullino et al., 1991). While chemical fungicides can provide good control of *B. cinerea*, long-term chemical fungicide applications can lead to fungicide resistance development and environmental pollution (Zubrod et al., 2019). Incorporating biological fungicides into disease management programs is one option to tackle the issues caused by the application of chemical fungicides.

Several studies have reported *Trichoderma* and *Clonostachys* as effective biological agents against *B. cinerea* (Cota et al., 2008; Meng et al., 2022; Saraiva et al., 2015; Silva et al., 2020). For instance, *T. harzianum* can reduce *B. cinerea* in tomato, lettuce and tobacco (De Meyer et al., 1998). Additionally, it was noted that *C. rosea* inhibited the establishment of *B. cinerea* and reduced the fruit decay rate (Peng & Sutton, 1990). *Trichoderma harzianum* belongs to the semi-fungal subphylum, *Pezizomycotina* and is a key soil antifungal strain (Otieno et al., 2003). *Trichoderma harzianum* has been used as a biocontrol agent against over 20 plant fungal diseases such as soybean stem rot, caused by *Sclerotinia sclerotiorum* and tomato fusarium wilt caused by *Fusarium oxysporum f. sp. lycopersici* (El Komy et al., 2015; Zhang et al., 2016). In addition to antifungal activity, *T. harzianum* was found to improve seed vigour, seedling emergence times, and the rate of photosynthesis (Fiorini et al., 2016; Vukelić et al., 2021). Currently, several commercially *T. harzianum*-based fungicides (e.g., Shibeijian, Trichodex and Topshield) are available that can control various plant diseases.

Clonostachys rosea is also a fungal biocontrol agent that is widely present in soils (Meng et al., 2022; Sun et al., 2020). *Clonostachys rosea* can effectively control various plant diseases, caused by *Fusarium* species, *S. sclerotiorum* and *B. cinerea* (Cota et al., 2009; Meng et al., 2022; Nobre et al., 2005), and prevents the establishment of *Aphanomyces Euteiches Dreehsler* in pea (Xue, 2003). Similar to *T. harzianum,* it has been shown that *C. rosea* can promote plant growth (Han et al., 2022).

It has been noted that a combination of biocontrol agents exerts more effective control against plant diseases than applying a single biocontrol agent (Alamri, 2014). For instance, a mixture of *Trichoderma viride, Pseudomonas fluorescens and Bacillus subtilis* gave better suppression of collar and root rot disease in physic nut (Latha et al., 2011), suggesting that using a mixture of strains can improve the efficacy of pathogen control (Burgess et al., 1999). Krauss et al. (2013) noted that a combination of *T. harzianum* and *C. rosea* could be used as a mixed biocontrol agent against *B. cinerea*; however, they noted that while there is competition between the two strains when used in mixtures, the antagonism between *C. rosea* and *Trichoderma* can be overcome by adjusting the mixing ratio of both biocontrol agents (Hoopen et al., 2010).

Salicylic acid (SA) and jasmonic acid (JA) signaling pathways are primary resistance signaling pathways in plants (Zhao et al., 2018). The SA signaling pathway plays an important role in the plant's immune response to phytopathogens, and its mechanism of inducing resistance is of great significance for crop protection (Vlot et al., 2009). Exogenous SA treatment can control the occurrence of navel orange canker by inducing defense mechanisms, such as elevating H_2O_2 levels and promoting stomatal closure (Wang & Liu, 2012). In plants, the *nonexpressor of pathogenesis-related genes 1* (*NPR1*) gene as a major regulator of SA signaling, interacts with transcription factors such as TGA in the nucleus to promote the production of genes, inducing plant disease resistance (Kinkema et al., 2000). The JA signaling pathways also has a direct or indirect function in plant resistance signal transduction (Kunkel & Brooks, 2002). Both SA and JA signaling pathways can work synergistically/antagonistically to promote disease resistance mechanisms in plants. For example, it has been shown that *T. longibrachiatum* H9 induced cucumber resistance to *B. cinerea* by activating both JA and SA signaling pathways (Yuan et al., 2019).

In our preliminary investigation, we noted that mixtures of *T. harzianum* and *C. rosea* had a better growth promotive effect on tomato plants than single-bioagent treatment. These results implied that

some chemical substances might have been produced during the co-existence of *T. harzianum* and *C. rosea*. However, these results raised the question of whether combining both biocontrol agents can also improve disease control in tomato plants. In this research, we developed a combined biofungicide composed of *T. harzianum* and *C. rosea* and evaluated its impact on tomato growth and *B. cinerea* control.

Materials and methods

Microorganisms

Clonostachys rosea (CR) and *T. harzianum* (TH) strains were isolated from rhizospheric soil samples collected from a farm at Northeast Agricultural University, following the method outlined by Warcup (1950). The isolated *C. rosea* and *T. harzianum* strains were maintained on potato dextrose agar (PDA) cubes, sealed with paraffin wax, and stored at -80 °C under storage numbers CGMCC No.1977 and CGMCC No.12165, respectively. The strains were re-streaked onto fresh media every six months to retain active growth. Ten phytopathogenic fungi: 1) *B. cinerea* (OR137135; leaves; *Lycopersicon esculentum Mill.*); 2) *Fusarium verticillioides* (OR137142; corn ears; *Zea mays* L.); 3) *Rhizoctonia solani* (OR137143; leaves; *Oryza sativa* L.); 4) *Colletotrichum* sp. (OR137138; leaves; *Cucumis sativus* L.); 5) *Fusarium equiseti* (OR137139; stems; *Zea mays* L.); 6) *Fusarium solani* (OR137141; stems; *Solanum melongena* L.); 7) *Fusarium oxysporum* (OR137140; leaves; *Citrullus vulgaris Schrad.*); 8) *Cladosporium fulvum* (OR137137; leaves; *Lycopersicon esculentum Mill.*); 9) *Sphaerotheca fuliginea* (OR131269; leaves; *Cucumis sativus* L.); and 10) *Cercospora beticola* (OR137136; leaves; *Beta vulgaris* L.) were also isolated from infected plants, such as the leaves, roots, or fruits, using the method outlined by Ambikapathy et al. (2023). All isolated pathogenic agents were sent to Huada Gene Technology Co., Ltd. (Shenzhen, China) for identification. The sequences have been deposited with the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/).

Culture observation and molecular identification of *T. harzianum* and *C. rosea* strains

Trichoderma harzianum and *C. rosea* were inoculated from laboratory stocks onto sterilized PDA (Basu et al., 2015). To monitor colony morphology, both fungal strains were incubated under constant temperature (28℃) for 5 days. For this, small mycelium samples were intermittently removed with sterile needles, smeared onto slides and the morphology of the mycelium was examined using a microscope (YS100, Hitachi Co. Ltd., Japan). To validate the taxonomy of *T. harzianum* and *C. rosea,* mycelia were collected from PDA plates using a sterile hole-puncher (5 mm). Subsequently, the collected mycelia were ground into powder using liquid nitrogen. Genomic DNA was extracted using the method described by Zhang et al. (1996). Polymerase chain reaction (PCR) was performed to amplify the 18S rRNA regions. The gene regions studied are the binding internally transcribed spacer (ITS) and translation extension factor 1α (TEF1). The primers used for the ITS amplification were ITS1F (forward primer): 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4 (reverse primer): 5'-TCCTCCGCTTATTGATATGC-3' (Gardes & Bruns, 1993). The primers used for the TEF1 amplification were elongation-1F (forward primer): 5'-CATCGAGAAGTTCGAGAAGG; elongation-1R: (reverse primer) 5'-GCCATCCTTGGGAGATACCAGC (Hyun et al., 2009).

The PCR reaction (30 µl) contained 17.8 µl of molecular water, 3.0 µl of buffer (0.1 mol/L Tris–Cl), 2.0 µl of dNTPs (10 µmol/L), 3.0 µl of each forward (10 µmol/L) and reverse primers, 1.0 µl of DNA template and 0.2 µl of the enzyme. The PCR thermocycling program included initial denaturation at 95℃ for 5 min (1 cycle), 35 cycles of denaturation at 95℃ for 60 s, annealing at 55℃ for 30 s and extension at 72℃ for 60 s, followed by one final cycle of extension at 72℃ for 10 min. The PCR products were sent to Huada Gene Technology Services Co. Ltd., (Shenzhen, China) for sequencing. Sequencing was performed using the same forward and reverse primers outlined above. The sequence results were analyzed, aligned, and compared using the method described by Ghanizadeh et al. (2020), and phylogenetic analysis of DNA joint sequences associated with the ITS and TEF1 regions was performed to identify *T. harzianum* and *C. rosea* using MEGA 5.0.

The *in vitro* effects of *T. harzianum* and *C. rosea* on ten common phytopathogens

The isolated phytopathogenic fungi described above were cultured on PDA and kept in a constant temperature incubator (28℃) (LKYC-1102C, Lifu Technology Co. Ltd., Ningbo, China) for 8 days. Then, fresh mycelia from the phytopathogen cultures were obtained using a sterile hole-puncher (5 mm). The collected mycelia were placed on the centre of PDA media in a Petri dish containing the hyphae of *T. harzianum* or *C. rosea*. There were three replicates (i.e. Petri dishes) for each phytopathogen and biocontrol fungi combination (experimental unit). Petri dishes with phytopathogens only (no biocontrol fungi) were used as a control. At 10 and 20 days after incubation (DAI), the inhibition rates of *T. harzianum* and *C. rosea* were estimated using the following equation (Yu et al., 2011):

Phytopathogen inhibition rate (%) = (the diameter of the pathogen in control
−diameter of the pathogen in the experimental unit)
/(the diameter of the pathogen incontrol − the size of inoculation)
×100% (the size of inoculation [i.e., 5mm]).

The effects of *T. harzianum* and *C. rosea* inoculants on *B. cinerea*

The effect of *T. harzianum* and *C. rosea* inoculants on the establishment of *B. cinerea* was assessed. For each treatment group, disease-free tomato seeds (Dongnong 713, a tomato F_1 hybrid variety, developed by the Tomato Research Institute of Northeast Agricultural University in China) were immersed in 5 mL of sterile water and subsequently, placed on filter papers in sterile 90-mm Petri dishes (100 seeds per Petri dish) at room temperature. The filter papers were kept moist by adding 1 ml of sterile water daily until tomato seeds germinated. Germinated tomato seeds were planted in pots (5×8 cm size) filled with 200 g of media (peat moss: vermiculite = 3:1 sterilized for 30 min at 121℃ and 103 kPa) and kept in an incubator (HPG-280HX, Yongda Chemical Reagent Co. Ltd., Tianjin, China) at 28℃. Eventually, the tomato plants (four-leaf stage) were sub-irrigated with one of the treatment groups, namely, No.1 = *T. harzianum*, No.2 = *C. rosea*, No.3 = *T. harzianum*:*C. rosea* at a 2:3 ratio, No.4 = Shibeijian, a commercial biofungicide (active ingredient: *T. harzianum* chlamydospore) and No.5 = water (control treatment). The spores' suspension concentration of *T. harzianum* and *C. rosea* was 10^6 cfu/mL. Each treatment group was repeated three times, and each replicate consisted of 50 tomato seedlings. The tomato leaves were sprayed with a suspension containing *B. cinerea* spores (10^6 cfu/mL) 24 h after treatment (HAT). First, fourth and eighth days after the treatment (DAT), plant height (cm), incidence rate and disease index were measured to evaluate the effect of biocontrol fungi on tomato and *B. cinerea*. The activities of three key enzymes associated with plant disease defense system were also determined at 1, 4 and 8 DAT. The amount of proline (PRO) was measured using the method described by Bates et al. (1973). The activity of the antioxidant enzymes peroxidase (POD) and superoxide dismutase (SOD) was assessed following the method described by Radić et al. (2006) and Yu and Rengel (1999), respectively.

To assess the effect of biocontrol fungi mixtures on *B. cinerea* control in tomato plants, the incidence rate and disease index were calculated according to the following equations:

Incidence rate = Number of affected leaves/The total number of leaves × 100%
Disease index = sigma (disease series × disease leaf number)
/(total leaf number × highest disease series) × 100%

(disease series: Level 0, no necrotic spots; Level 1, 3 necrotic spots on a single leaf. Level 3, 4–6 necrotic spots on a single leaf; Level 5, 7–10 necrotic spots on a single leaf; Level 7, 11–20 necrotic patches on a single leaf, with some of them densely packed into patches. Level 9, single leaf with dense necrotic spots, covering more than 1/4 of the leaf area) (Chiang et al., 2017). All analyses were performed in triplicate.

To assess the chlorophyll fluorescence of tomato plants treated with either of the five treatment groups, plants were grown from seeds using the above-mentioned method. When tomato plants reached the 4–5 leaf stage, they were bagged for 30 min (dark adaptation) prior to measuring the chlorophyll fluorescence using an imaging fluorimeter (MAXI, Zealquest Scientific Technology Co., Ltd., Harbin, China). The initial fluorescence (F_o) was measured following irradiation with visible light (10 μmol/M^2/S) for 0.8 s. The maximum fluorescence (F_m) was obtained following irradiation with the saturated pulse light (8000 μmol/M^2/S) for 0.8 s. The maximum photochemical quantum yield of photosystem II (F_v / F_m = (F_m-F_o) / F_m) was automatically recorded by the fluorimeter, and the corresponding image was generated.

Effects of *T. harzianum* and *C. rosea* on salicylic acid and jasmonic acid signaling pathways

To understand the effects of *T. harzianum* and *C. rosea* on the signaling pathways of SA and JA in tomatoes, the content of both signaling molecules and the expression of the key genes involved in their signaling pathways were assessed. The content of SA and JA in tomato leaves was measured at 1, 4, 6, 8 and 10 DAT using SA ELISA Kit (Aidi Biotech Co. Ltd., Wuhan, China) and JA ELISA Kit (Aidi Biotech Co. Ltd., Wuhan, China), respectively, following the manufacturer's instructions.

The key genes associated with the SA and JA signaling pathways were identified using the KEGG pathway database (https://www.kegg.jp/pathway/ath04075). To evaluate the expression of the key genes, trizol reagent was used to extract total RNA from tomato leaves at 1, 4, 8 DAT. The extracted RNA was reverse-transcribed into cDNA using a kit (R101-01, Vazyme Co., Nanjing, China) following the manufacturer's instructions. The cDNA was used for a qRT PCR analysis to assess the expression of the key genes using a commercial kit (Q421-02, Vazyme Co., Nanjing, China). The qRT PCR reaction contained Mix ChamQ SYBR Color qPCR Master Mix (10.0 μl), forward and reverse primers (0.4 μl), 1.0μL of template cDNA and 8.2 μl of ddH_2O. The qRT PCR analysis was performed using the conditions outlined by Miao et al. (2022). The forward/reverse primers designed for the genes associated with the SA and JA signaling pathways are listed in Table S1. The expression level of the key genes was correlated to the physiological indices measured in tomato plants treated with the *T. harzianum* and *C. rosea* mix (No.3) and the control (No.5) treatment groups. The content of JA and SA, and the expression of the key genes associated with the JA and SA signaling pathways were assessed in triplicate samples.

Statistical analyses

All experiments were established using a completely randomized design with three replicates. Data were tested for normal distribution and variance equality before being subjected to variance analysis (ANOVA). Means were separated using Fisher's protected test at a 5% level of probability. All statistical analyses were conducted using SPSS 20.0 and Origin 2022 software.

Results

Trichoderma harzianum and *C. rosea* identification and sequence analysis (18S rRNA)

The colony morphology of *T. harzianum* and *C. rosea* after activation and rejuvenation is illustrated in Fig. 1a and c. Initially, the mycelia of *T. harzianum* were fine, with no changes in color up to 3 days after inoculation. As culturing progressed, the white flocculent mycelia gradually changed into green at 4 days after inoculation, and eventually at 8 days after inoculation, the mycelia turned dark green. *Clonostachys rosea* colonies were white, and the mycelia were initially longer and whiter. After a prolonged culture period, the mycelia gradually shortened and turned yellow at approximately 7 days after inoculation.

Amplification of ITS and TEF1 sequences from *T. harzianum* and *C. rosea* yielded products of about 500 bp and 300 bp, respectively, as estimated by agarose gel electrophoresis (Fig. S1). Phylogenetic trees of *T. harzianum* and *C. rosea* were constructed based on the ITS and TEF1 sequences (Fig. 1b and d). The strain of *T. harzianum* used in this research showed an overall 98% homology with the previously published *T. harzianum* sequences (MK322671.1; OM471990.1), indicating that our strain had ITS and TEF1 region sequences identical to those of the previously studied strain. The *C. rosea* strain from

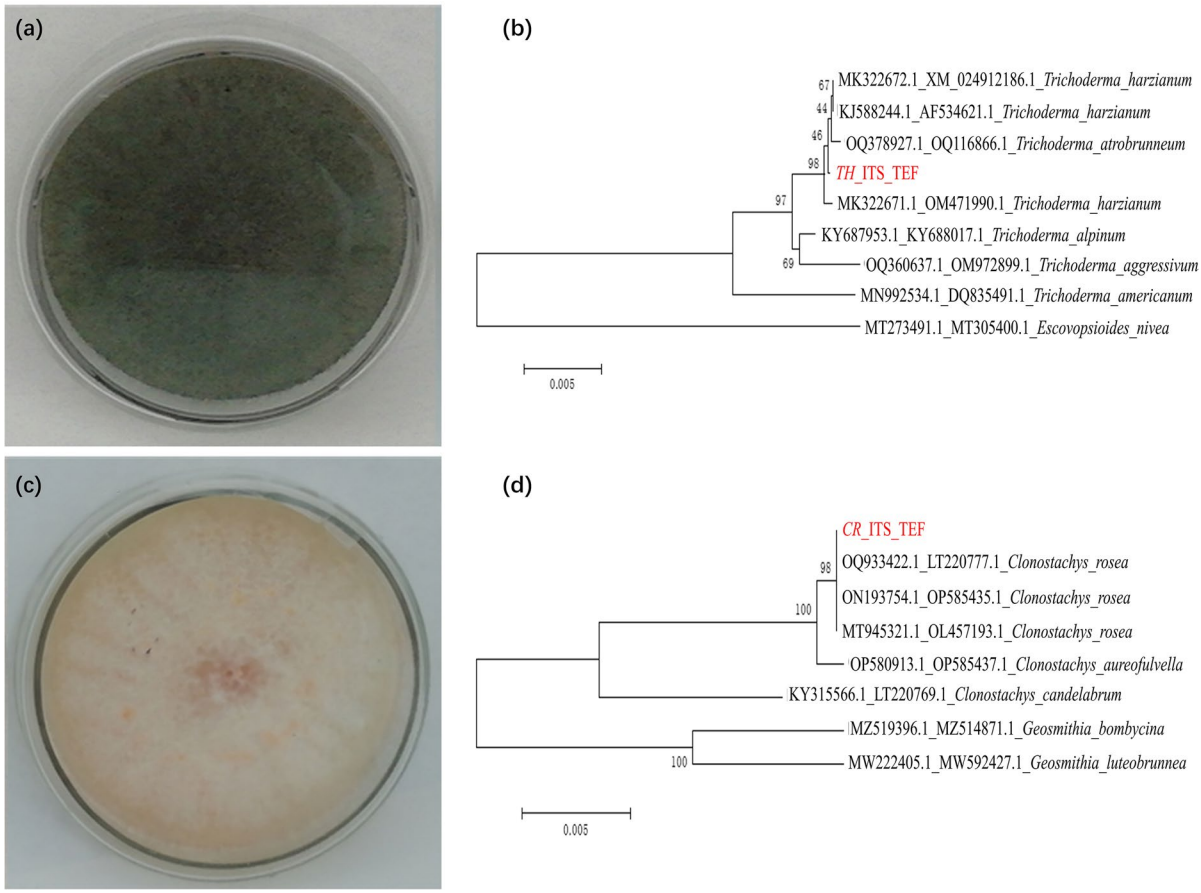

Fig. 1 Colony morphology for *T. harzianum* (**a**) and *C. rosea* (**c**); Phylogenetic trees of *T. harzianum* (**b**) and *C. rosea* (**d**) based on the ITS and TEF sequences. The strains used in this research are labeled as TH and CR, respectively

this research had 98% homology to the ITS and TEF1 sequences of the previously studied strains (OQ933422.1; LT220777.1), suggesting that our strain was conspecific with *C. rosea*.

The inhibitory effects of the two biocontrol fungi against ten common phytopathogenic fungi

Both biocontrol fungi exhibited significant inhibitory effects against all ten phytopathogens investigated in this research. As shown in Table 1, *T. harzianum* had the greatest (86.74%) and lowest (61.87%) inhibitory effects against *R. solani* and *F. oxysporum*, respectively. The inhibition rate of *T. harzianum* against *B. cinerea* was 85.47%, while an inhibition rate of 85.79% was recorded against *F. solani* for *T. harzianum.* The inhibition rate of *T. harzianum* against all phytopathogens, except *F. equiseti* and *C. fulvum* was over 80%. The inhibitory effects of *T. harzianum* against all ten phytopathogens were slightly lower at 10 DAI than at 20 DAI, but the difference was not significant.

The inhibition rate of *C. rosea* against all ten phytopathogens was greater at 20 DAI. Among all ten phytopathogens tested in this research, *C. rosea* exhibited the greatest inhibition rate against *Colletotrichum* sp. (77.12%). The lowest inhibition rate of *C. rosea* was recorded for *F. solani* at 10 (53.87%) and 20 (56.83%) DAI. The inhibition rates of *C. rosea* against *B. cinerea* were 74.93% and 78.27% at 10 and 20 DAI, respectively.

Table 1 Phytopathogen inhibition rates by *T. harzianum* and *C. rosea* (10th day and 20th day)

Names of pathogen	Phytopathogen inhibition rate by *T. harzianum* (%)		Phytopathogen inhibition rate by *C. rosea* (%)	
	10^{th} d	20^{th} d	10^{th} d	20^{th} d
Botrytis cinerea	85.47 ± 3.75 a	85.57 ± 6.35 a	74.93 ± 10.27 ab	78.27 ± 15.73 ab
Fusarium moniliforme	81.93 ± 2.95 ab	82.20 ± 5.53 ab	74.37 ± 9.64 abc	77.98 ± 7.29 ab
Rhizoctonia solani	86.74 ± 5.98 a	86.78 ± 10.31 a	76.11 ± 6.91 a	82.73 ± 5.00 a
Colletotrichum sp.	83.72 ± 3.72 ab	83.88 ± 6.65 ab	77.12 ± 7.41 a	79.09 ± 5.52 ab
Fusarium equiseti	72.39 ± 4.98 ab	72.49 ± 8.45 ab	54.57 ± 7.32 bc	59.47 ± 6.39 ab
Fusarium solani	85.79 ± 3.8 a	86.07 ± 6.52 a	53.87 ± 9.27 c	56.83 ± 14.08 b
Fusarium oxysporum	61.87 ± 4.57 b	62.04 ± 7.78 b	70.36 ± 4.97 abc	73.30 ± 8.74 ab
Cladosporium fulvum	76.61 ± 4.08 ab	76.71 ± 7.09 ab	58.75 ± 2.12 abc	60.38 ± 3.37 ab
Sphaerotheca fuliginea	82.12 ± 6.62 ab	82.14 ± 11.48 ab	69.53 ± 3.84 abc	71.90 ± 1.49 ab
Cercospora beticola	83.68 ± 5.16 ab	83.71 ± 8.9 ab	65.50 ± 6.55 abc	66.97 ± 8.83 ab

The data in the table represent the mean ± standard deviation. Mean values within each column followed by the same letters are not significantly different at a 5% probability according to Fisher's protected test

The effects of *T. harzianum* and *C. rosea* biological agents on the control of *B. cinerea*

The control efficacy of all treatment groups against *B. cinerea* infection in tomato plants and the related physiological indices were investigated (Fig. 2). The results showed that the mixture of *T. harzianum* and *C. rosea* (No.3 treatment group) greatly promoted plant height by 8 DAT, and the treated plants were 10.1 cm taller than those of the control group (Fig. 2a). Furthermore, the lowest incidence rate and disease index values were recorded for the tomato seedlings treated with the No. 3 treatment group (Fig. 2b, c). Compared to the control group, the incidence rate and disease index in tomato plants treated with the No.3 treatment group reduced by 80.14% and 73.84%, respectively, by 8 DAT. Furthermore, the results indicated that the mixture of *T. harzianum* and *C. rosea* gave the best control against *B. cinerea* and even a single bioagent (No.1 and No.2) performed better than the commercial biofungicide, Shibeijian (No. 4).

The activity of enzymes in tomato leaves treated with the biocontrol agent is illustrated in Fig. 2d-f. The results showed that the level of PRO had an overall increasing trend (Fig. 2d). In addition, at 8 DAT, the level of PRO in plants treated with the NO.1, NO.2 and NO.3 treatment groups was higher than those treated with the Shibeijian group (No. 4) or the control group (No. 5). The level of POD activity in all treatment groups had an increasing trend up to 4 DAT (Fig. 3e), with plants treated with the No.3 treatment group had 28.85% and 40.99% greater POD activity than those treated with the No.4 treatment group and control group, respectively. Overall, the level of SOD activity in tomato seedlings treated with the No. 3 treatment group was higher than those treated with other treatment groups (Fig. 2f).

The chlorophyll fluorescence images from tomato seedlings treated with different treatment groups are illustrated in Fig. 2g and h. The blue color indicates excited states of pigments in the photosystem II reaction centers in leaves, suggesting a higher rate of photosynthesis in plants. The leaves of tomato seedlings treated with the No.3 treatment group were bluer than the control and had an Fv/Fm value of 0.746, indicating that photosynthesis was enhanced in those plants. Among the treatments, the lowest Fv/Fm values were recorded for the control group (0.672).

Effects of *T. harzianum* and *C. rosea* on salicylic acid and jasmonic acid disease resistance signaling pathways

The effect of different treatment groups on SA and JA content, and signaling pathways are shown in Fig. 3. There was a generally increasing trend in the content of SA for all treatments, though the SA content was different among treatments throughout the study (Fig. 3a). The results showed that the content of SA in tomato plants treated with the No.3 treatment group was the greatest up to 8 DAT, when it reached a maximum value of 403 ng/g FW, which was 42.79% greater than that of the control group.

In contrast to SA, a downward trend was recorded for the JA content in tomato plants for all treatment

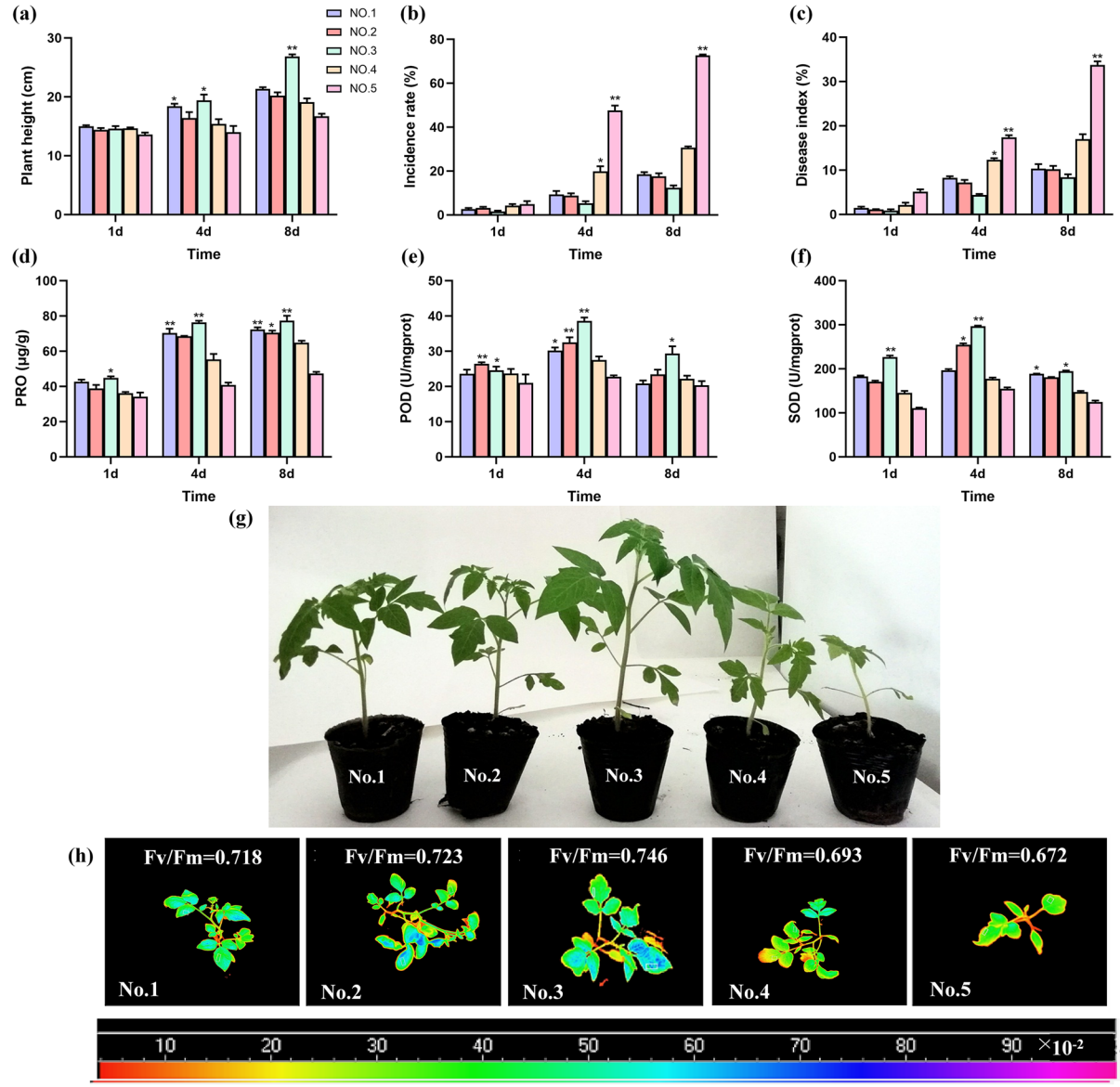

Fig. 2 Physiological indicators of tomato seedlings and enzyme activity in tomato leaves at 1, 4 and 8 days after treatment. (**a**) plant height, (**b**) incidence rate, (**c**) ddisease index, (**d**) proline (PRO), € peroxidase (POD), (**f**) superoxide dismutase (SOD), (**g**) plant morphology and (**h**) chlorophyll fluorescence images. Vertical bars represent the mean ± standard deviation. No.1–5 treatment groups, i.e., No. 1 = *T. harzianum (*TH), No. 2 = *C. rosea* (CR), No. 3 = TH + CR mixture, and No. 4 = Shibeijian (biofungicide) No. 5 = water (control)

groups with increasing time, and there was a small difference among all treatment groups at each time course (Fig. 3b). The signaling pathway diagrams of SA and JA, and the expression levels of the key genes associated with both compounds are shown in Fig. 3c and d, respectively. As shown in Fig. 3d, the expression levels of the *SlNPR1*, *SlPR1*, *SlJAZ1, SlMYC2* and *SlCOI1* genes increased significantly at 4 DAT, in plants treated with the No.1, No.2 and No.3, treatment groups. Particularly, at 4 DAT, a greater level of expression was recorded for the *SlNPR1*, *SlPR1* and *SlMYC2* genes in plants treated with the No.3 treatment group. At 8 DAT, all genes, but the *SlJAZ1* and *SlMYC2* genes were highly over-expressed in

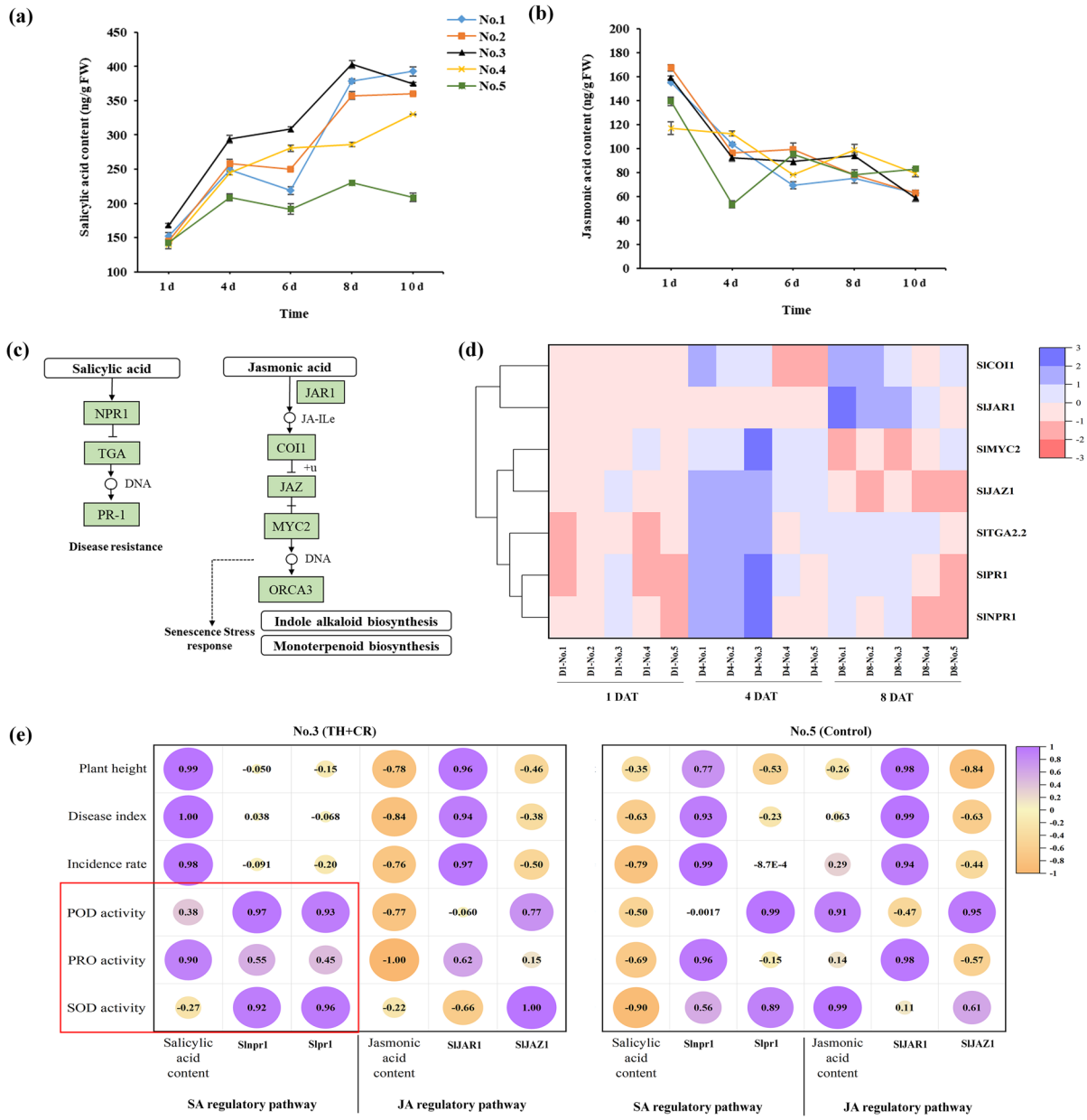

Fig. 3 The signaling pathways of salicylic acid (SA) and jasmonic acid (JA) in tomato plants after treatment. (**a**) SA content, (**b**) JA content, (**c**) the SA and JA signaling pathway diagram, (**d**) the heatmap of genes associated with the SA and JA signaling pathways, (**e**) correlation analysis of plant disease resistance indices and the SA and JA signaling pathways` genes. The treatment groups in the horizontal axes are: No. 1 = *T. harzianum* (TH), No. 2 = *C. rosea* (CR), No. 3 = TH + CR mixture, No. 4 = Shibeijian (biofungicide) and No. 5 = water (control). Vertical bars represent the mean ± standard deviation

plants treated with the No.1, No.2 and No.3, treatment groups. It was also noted that in plants treated with the No.3 treatment group, the SA content was strongly correlated with the PRO content and POD activity, while it was negatively correlated with SOD activity (Fig. 3e). Furthermore, the *SlNPR1* and *SlPR1* genes strongly correlated with plant defense-related physiological indices (red mark in Fig. 3e). In

particular, the *SlNPR1* and *SlPR1* genes were positively correlated with the activity of POD and SOD, and PRO content in the plants treated with the No.3 treatment group, and the correlation value was higher than those in the control group (No. 5). The content of JA was found to be negatively correlated with PRO content in the plants treated with the No.3 treatment group; however, a strong positive correlation was recorded between the *SlJAZ1* gene and the activity of POD and SOD.

Discussion

In this research, the antifungal activity of *T. harzianum* and *C. rosea* for *B. cinerea* management in tomatoes was investigated. Initially, both *T. harzianum* and *C. rosea* were isolated from soil samples, and their taxonomy was confirmed using morphological (microscopic) and genetic (18S rRNA) markers. Both techniques are commonly used in fungal identification (Anderson et al., 2003). Both *T. harzianum* and *C. rosea* showed broad-spectrum antifungal activity against ten common plant diseases, though the degree of inhibition was different among the tested phytopathogens. These results indicate that both biocontrol fungi can be used as a commercial biofungicide.

The inoculum ratio of fungal agents in combined biofungicides is critical in the efficacy of biocontrol, with suboptimal inoculation ratios resulting in antagonistic interactions between biocontrol agents (Hoopen et al., 2010). For instance, Krauss et al. (2013) noted that increasing the concentrations of *C. rosea* in a mixture of *C. rosea* and *T. harzianum* dramatically decreased the parasitic growth of *T. harzianum*. They suggested that the higher growth rate of *T. harzianum* resulted in more physical contact between hyphae and more exposure to mycoparasitism, which rendered *T. harzianum* more susceptible to hyphal damage. A screening approach using various ratios of biocontrol agents can aid the development of compatible combined biofungicides (Hoopen et al., 2010). In this regard, our study evaluated the effect of different biological agents on plant growth and *B. cinerea* control in tomatoes. Among the various microbial agents tested (data not shown), the *T. harzianum* and *C. rosea* mixture significantly promoted the growth of tomato plants and gave the best control against *B. cinerea* in tomato seedlings.

To understand the disease resistance mechanisms induced by *T. harzianum* and *C. rosea*, we investigated the antioxidant enzyme activity in the treated plants and their correlations with the SA and JA disease resistance signaling pathways. Antioxidant enzymes are crucial in conferring tolerance against biotic and abiotic stresses in plants (Liu et al., 2013, 2020). The activity of antioxidant enzymes reflects the physiological state of plants (Li et al., 2023; Zhang & Feng, 2018). Hence, evaluating the activity of antioxidant enzymes can be used as an index to compare the effects of *T. harzianum* and *C. rosea* treatments on tomato seedlings, with high levels of enzyme activity implying that the biofungicide treatment provided effective control against phytopathogenic agents (Zhang & Feng, 2018). This research revealed that the protection conferred by the *T. harzianum* and *C. rosea* mixture against *B. cinerea* in tomato plants could be associated with activating antioxidant enzymes such as POD, PRO and SOD. In agreement with our results, Zehra et al. (2017) showed that the application of *T. harzianum* enhanced the tolerance of tomato plants against *Fusarium* wilt disease by inducing antioxidant defense systems. Zheng et al. (2018) also noted that *C. rosea* improved the tolerance to *B. cinerea* in tomato plants by activating of antioxidant enzymes, such as SOD. Antioxidant enzymes can enhance the efficiency of biofungicides by improving the response of the plant immune system rather than by killing the fungi (Meng et al., 2022).

Disease resistance mechanisms in plants can be broadly categorized into: 1- systemic acquired resistance (SAR) induced by pathogenic microorganisms and 2- the induced systemic resistance (ISR) promoted by microorganisms with biological control function (Cordier et al., 1998; Durrant & Dong, 2004; Nawrocka & Małolepsza, 2013). In both mechanisms, the induction of disease-resistance related genes in plants promotes the production of pathogenic-related proteins (PR proteins), increases the accumulation of phenolic substances and induces the activity of plant defense enzymes, enabling the plants to withstand the infection of pathogens (Pieterse et al., 2001; van Loon et al., 1998). However, ISR and SAR mechanisms do not use the same signaling pathways to promote disease resistance in plants. For instance, in *Arabidopsis thaliana*, it was found that the ISR mechanism triggered by non-pathogenic rhizobacterial strain *Pseudomonas fluorescens* was induced through the jasmonic acid /ethylene signaling pathway, while the SAR mechanism was induced through the SA

signaling pathway (Conrath et al., 2002). However, it has been shown that some biological control microorganisms can increase disease resistance in plants by increasing the expression of the SA signaling pathway (Jiang et al., 2018). Therefore, it is crucial to comprehend the pathways associated with plant disease resistance mechanism induced by microorganisms.

In previous research, Wang et al. (2019) confirmed that *C. rosea* can improve *B. cinerea* resistance in tomatoes through the JA and SA signaling pathways. Therefore, we explored if *T. harzianum* and *C. rosea* could also enhance the plant immune response through both signaling pathways. This research showed that the expression level of the *NPRl* gene was greater in plants treated with the *T. harzianum* and *C. rosea* mixture. The *NPRl* gene regulates the cross-talk between SA and JA signaling pathways (Pieterse & Van Loon, 2004). Hence, it plays a role in inducing disease resistance mechanisms in plants. Similarly, Zhang et al. (2010) concluded that the overexpression of the *NPR1* gene primarily induced citrus canker resistance. In this research, it was also noted that the expression levels of *SlNPR1* and *SlPR1*, two key genes modulating the SA signaling pathway, were significantly greater in tomato plants treated with the *T. harzianum* and *C. rosea* mixture. Enhancing the content of SA can induce disease resistance mechanism in plants through promoting the activity of antioxidant enzymes. For example, it has been shown that exogenous treatment of SA reduced improved resistance to *Penicillium expansum* by stimulating the activity of SOD and POD enzymes in sweet cherry fruit (Chan & Tian, 2006).

The JA signaling pathway can also play a role in inducing disease resistance and enhancing defense mechanisms in plants (Li et al., 2023; Zhao et al., 2003). However, this research showed that the SA signaling pathway played a greater role in regulating resistance to *B. cinerea* than the JA signaling pathway, in tomato plants treated with the *T. harzianum* and *C. rosea* mixture. Similarly, it has been shown that *Paenibacillus alvei* K165 promoted *Verticillium dahliae* resistance in *A. thaliana* by enhancing the defense mechanisms modulated by the SA signaling pathway (Tjamos et al., 2005).

Biofungicides can trigger pathogen-associated genes or plant-resistance proteins to activate plant defense against phytopathogens (Luo et al., 2018). Hence, applying biofungicide before plants are infected or at the early stage of infection can improve the control against phytopathogens by bolstering the immune system of plants (Meng et al., 2022; Zheng et al., 2018). Enhanced control against phytopathogens in plants can also be triggered by biocontrol fungal genes that promote disease resistance in the host plants (Islam & Sherif, 2020). Thus, future studies will investigate the genes associated with resistance to *B. cinerea* triggered by the *T. harzianum* and *C. rosea* treatment.

Conclusions

The results of this research revealed that applying the mixture of *T. harzianum* and *C. rosea* provided significantly enhanced resistance to *B. cinerea* in tomato plants under our experimental conditions. According to the correlation analysis of plant physiological parameters and resistance pathway indices, the fungal mixture induced the SA signaling pathway to promote resistance to *B. cinerea* in tomato plants.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval Ethics approval was not required for this research.

Conflict of interest All the authors declare that there is no conflict of interest.

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