Combined Use of *Trichoderma atroviride* CCTCCSBW0199 and Brassinolide to Control *Botrytis cinerea* Infection in Tomato

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Abstract

Tomato gray mold caused by *Botrytis cinerea* is one of the main diseases of tomato and significantly impacts the yield and quality of tomato fruit. The overuse of chemical fungicides has resulted in the development of fungicide-resistant strains. Biological control is becoming an alternative method for the control of plant diseases to replace or decrease the application of traditional synthetic chemical fungicides and genus *Trichoderma* is widely used as a biological agent for controlling tomato gray mold. Brassinolide (BR) is a plant-growth-promoting steroid. To enhance the efficiency and stability of *Trichoderma* activity against *B. cinerea*, an optimal combination of *Trichoderma atroviride* CCTCCSBW0199 and BR that controls *B. cinerea* infection in tomato was identified. Strain CCTCCSBW0199 was found to have antagonistic activity against *B. cinerea* both in vitro and in vivo. In addition, a fermented culture of chlamydospores and metabolites, or metabolites only of strain CCTCCSBW0199 also reduced growth

of *B. cinerea*. BR reduced growth of *B. cinerea* and had no effect on the sporulation and growth of *Trichoderma* spp. An application of metabolites of a *Trichoderma* sp. + BR reduced gray mold on tomato leaves by approximately 70.0%. Furthermore, the activities of induced defense response-related enzyme, such as peroxidase, superoxide dismutase, catalase, and phenylalanine ammonia-lyase were increased in tomato plants treated with a *Trichoderma* sp. + BR. Our data suggested that applying a mix of metabolites of *T. atroviride* CCTCCSBW0199 + BR was effective at reducing gray mold of tomato and may lay a theoretical foundation for the development of novel biofungicides.

Keywords: *Botrytis cinerea*, brassinolide, cultural and biological practices, disease management, fungi, tomato gray mold, *Trichoderma atroviride*, vegetables

Tomato gray mold is a fungal disease caused by *Botrytis cinerea* that occurs worldwide, especially when the environmental temperature is at least 20°C and the humidity is high (Hannusch and Boland 1996; Ning 2013). Tomato gray mold results in defoliation and flower and fruit abortion, consequently affecting the yield and quality of tomato fruit (Wang et al. 2018). On average, tomato gray mold causes 20.0 to 30.0% yield loss, with up to 50.0% loss in the case of severe outbreaks (Zhao et al. 2014). Tomato gray mold is traditionally managed using chemical fungicides (Wang et al. 2018); however, long-term use of chemical fungicides leads to a high risk of the pathogen developing resistance, which seriously challenges sustainable control of the disease (Elad and Shtienberg 1995). Procymidone and carbendazim, for instance, are now rarely used to control the disease because *B. cinerea* has already developed strong resistance to these fungicides (Sun et al. 2010). Indeed, in some areas of China, the resistance rate of *B. cinerea* to these two fungicides is 100.0% (Liu et al. 2016). Therefore, the use of biological control to inhibit tomato gray mold has attracted much attention.

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Trichoderma spp. are widely used as biological control agents against plant diseases with minimal environmental risks. *Trichoderma atroviride* has been successfully used to control cucumber and tomato gray mold (Lei 1998). *Trichoderma* spp. protect host plants from infection by various pathogens via mechanisms that include nutritional competition, mycoparasitism, antibiotic activity, and induced resistance (Harman 2006). In addition, treatment of tomato with *Trichoderma* spp. results in altered expression of defense-related genes such as those involved in the jasmonic acid (Harel et al. 2014), salicylic acid, and abscisic acid pathways (Fernández et al. 2014). However, compared with soil applications, foliar applications of *Trichoderma* spp. are usually less effective at reducing disease; therefore, the combined use of *Trichoderma* spp. and other environmentally friendly biochemicals is proposed to be a good alternative for improving foliar applications of *Trichoderma* spp. for biocontrol (Harel et al. 2014).

Brassinolide (BR), a plant hormone that belongs to the brassinosteroid group, is involved in many cellular processes, including cell expansion and division, tissue differentiation, flowering, senescence, and abiotic stress responses (Xia et al. 2011). BR may also play an organ-specific role in tomato fruit development (Nomura et al. 2005). BR reportedly enhances plant resistance by improving plant osmotic regulation and increasing the activity of various protective enzymes such as peroxidase (POD) and superoxide dismutase (SOD) (Damghan 2009). Brassinosteroid hormones are important signaling molecules that stimulate the plant immune response (Yuan et al. 2018) and enhance plant growth and resistance to stress (Clouse and Sasse 1998; Mandava 1988; Vardhini et al. 2008). For example, upregulation of brassinosteroid cascade signaling is closely associated with disease resistance in barley (Ali et al. 2014) and plant growth and stress resistance in tomato (Vardhini et al. 2008).

There are few reports to date of combinations of BR and biological agents to manage disease. When BR was combined with a neem oil nuclear extract or Chinese herbal medicinal compounds, the treated plants were found to be more vigorous (i.e., taller) and their stem diameter, shoot fresh weight, and shoot dry weight were all greater. Such growth promotion in plants treated with combinations of biological compounds and BR has been suggested to be the result of increased levels of photosynthetic pigments (Wang et al. 2017).

Moreover, treatment of maize roots with *T. harzianum* significantly induced maize resistance to Curvularia leaf spot disease by upregulating BR signaling-related genes such as *BAK1*, *BRI1*, and *MYC* (Yu et al. 2016). However, to our knowledge, there has been no research on *Trichoderma* spp. + BR to control plant diseases. We postulated that a *Trichoderma* sp. + BR would synergistically control tomato gray mold. In this study, we evaluated the effect of a *Trichoderma* sp. + BR on the severity and incidence of gray mold caused by *B. cinerea* in tomato.

Materials and Methods

Materials. Twelve *Trichoderma* strains (JH3403, GDFS1009, ZJSX5003, H6, D9, CCTCCSBW0199, 1, HN524001, HN2J4010, HBJZ4007, HNCS3013, and CDCG3007) were provided by the Laboratory of Plant Pathology, Shanghai Jiao Tong University. *B. cinerea* was provided by the Chinese Academy of Agricultural Sciences (J. Zhang et al. 2017). BR powder was provided by Shanghai Lvze Bioengineering Co., Ltd. Seed of the tomato variety Zhongshu No. 4 were purchased from commercial seed shops.

***Trichoderma* cultivation, and preparation of conidial suspensions and fermented cultures.** *Trichoderma* strains were grown on potato dextrose agar (PDA) plates at 28°C for 7 days (Te'o et al. 2002) under a cycle of 14 h of light and 10 h of darkness (Li et al. 2015; Masih and Paul 2002). Conidial suspensions were obtained by harvesting spores from the PDA plates using 2 ml of sterile water, then adjusted to the required concentrations. Fermented cultures (FCs) were obtained by inoculating *Trichoderma* conidial suspensions into potato dextrose broth (PDB) (200 ml per 500-ml Erlenmeyer flask), followed by incubation at 28°C and 180 rpm for 5 days in a shaking incubator (Ho et al. 2003). FCs contained hyphae, chlamydospores, and metabolites. FCs containing chlamydospores and metabolites (FCCM) without hyphae were obtained by filtering through four layers of sterile gauze (Giese et al. 2006). FCs containing metabolites (FCM) without hyphae and chlamydospores were obtained by filtering through a 0.22- μ m Millex-GP Syringe Filter Unit (Millipore Sigma, Burlington, MA, U.S.A.) (Yau et al. 2011).

***B. cinerea* cultivation, and preparation of conidial suspensions.** *B. cinerea* was grown on PDA at 25°C for 7 days (Te'o et al. 2002) under a cycle of 14 h of light and 10 h of darkness (Li et al. 2015; Masih and Paul 2002). Conidial suspensions were obtained by washing the spores from PDA plates using 2 ml of sterile water.

Preparation of BR solution. The BR stock solution used was prepared from 99.9% BR powder, diluted with 95.0% alcohol to a concentration of 1 mg/ml (Kamuro and Inada 1991). From the stock solution (1 mg/ml), BR solutions of 1, 0.2, and 0.1 μ g/ml were prepared by diluting with sterile water.

Screening of antagonistic *Trichoderma* strains against *B. cinerea*. One mycelial disc (4 mm in diameter) each of a *Trichoderma* sp. and *B. cinerea* was excised from the growing edges of 7-day-old cultures and placed 2 cm apart on opposite sides of PDA plates (90 mm). The plates were incubated for 5 to 6 days at 20 and 28°C. A control of *B. cinerea* grown alone on PDA plates was used. The growth rate of *B. cinerea* was determined by measuring the colony diameter. The percent inhibition was calculated as follows: percent inhibition (%) = [(pathogen colony diameter in the control treatment – pathogen colony diameter in the challenge treatment)/pathogen colony diameter in the control treatment] $\times$ 100. (Cheng et al. 2012).

PDA plates amended with 10.0% *T. atroviride* CCTCCSBW0199 FCM were prepared and a disc of *B. cinerea* culture (4 mm in diameter) was placed in the center of the PDA medium and incubated at 25°C (optimum growth temperature for *B. cinerea*) for 5 to 7 days. Nonamended PDA served as the control. The colony diameter of *B. cinerea* was measured to identify the inhibitory effects of *Trichoderma* FCM on the pathogen.

Effects of BR on *B. cinerea* growth in vitro. PDA plates amended with BR solution at 0.2 μ g/ml were prepared and inoculated with a disc of *B. cinerea* culture placed in the center of the plates. Nonamended PDA served as the control. All plates were incubated at 25°C for 5 days. The colony diameter of the pathogen was measured to identify the effects of BR on *B. cinerea* growth.

To observe the effect of BR on the hyphal morphology of *B. cinerea*, PDB was amended with BR at 0.2 μ g/ml (or not amended for the control). The broth was inoculated with a spore suspension of *B. cinerea* (1×10^6 spores/ml), then incubated at 25°C in an incubator with shaking at 180 rpm for 3 days. The hyphae of the pathogen were harvested from the broth using a pipette and rinsed with double-distilled water (ddH₂O). A small sample of hyphae was fixed in 2.5% glutaraldehyde (0.1 M) under a vacuum at 4°C for 24 h. The sample was dehydrated according to a described method (Wright 2000) and placed in acetone/epoxy resin (1:1) for 1 h followed by overnight incubation in acetone/epoxy resin (1:2). Subsequently, the sample was placed in pure epoxy resin for 8 h and then in a 60°C oven for 48 h of polymerization. Ultrathin slices (50 nm thick) of the sample were prepared using a Lycra ultrathin microtome. The sections were stained with uranium acetate for 5 min, washed with ddH₂O several times, then stained with lead citrate for 6 min. Finally the samples were washed in ddH₂O for 2 min, dried, and observed under a transmission electron microscope (120 kV Biology Transmission Electron Microscope; Tecnai G2 SpiritBiotwin) (Buckley et al. 1966).

Effects of BR on *T. atroviride* CCTCCSBW0199. To evaluate the effect of BR on mycelial growth of *T. atroviride* CCTCCSBW0199, PDA plates were amended with BR at 1, 0.2, and 0.1 μ g/ml. PDA alone served as the control. Discs (4 mm in diameter) of a *Trichoderma* sp. excised from the growing edge of a 7-day-old culture were placed in the center of the PDA plates. All plates were incubated at 28°C (optimum growth temperature for *T. atroviride*) for 5 days. The diameter of *Trichoderma* colonies was measured to identify the effects of BR on the growth of *T. atroviride* CCTCCSBW0199.

To evaluate the effect of BR on the sporulation of *T. atroviride* CCTCCSBW0199, PDB (100 ml per 250-ml Erlenmeyer flask) was amended with BR solution at 1, 0.2, or 0.1 μ g/ml; PDB alone served as the control. The broth was inoculated with a conidial suspension (1.0×10^6 spores/ml) and the flasks were incubated at 28°C and 180 rpm for 5 days. Hyphae were removed, and *Trichoderma* spores were counted using a hemocytometer.

Evaluations of BR contribution to the inhibitory effect of *T. atroviride* CCTCCSBW0199 on the growth of *B. cinerea*. Mycelial discs (4 mm in diameter) of both *Trichoderma* spp. and *B. cinerea* were placed 2 cm apart on PDA plates amended with different concentrations of BR (1, 0.2, and 0.1 μ g/ml). PDA without BR served as the control. *B. cinerea* grown alone on PDA plates was also prepared. All plates were incubated at 25°C for 5 days. The pathogen colony diameter was measured, and the percent inhibition was calculated as follows: percent inhibition (%) = [(single pathogen colony diameter – pathogen colony diameter in the treatment)/single pathogen colony diameter] $\times$ 100.

Greenhouse evaluations of the effect of *T. atroviride* CCTCCSBW0199 and BR on gray mold of tomato. Tomato seed were surface sterilized with 2.0% sodium hypochlorite for 3 min, rinsed with sterile water, and placed in sterile petri dishes containing moistened filter paper. The seed were allowed to germinate at room temperature for 2 days. The germinated seed were then transplanted into 15-cm-diameter pots. The growing media was composed of peat, vermiculite, and perlite at a ratio of 4:1:1. Each treatment included three pots and 5 plants/pot. All of the pots were maintained under a cycle of 14 h of light (25°C) and 10 h of darkness (15°C) in a greenhouse.

To evaluate the effects of *T. atroviride* CCTCCSBW0199 + BR on tomato gray mold, *T. atroviride* FCCM of different concentrations were prepared, and BR stock solution at 1 mg/ml was amended with each *T. atroviride* FCCM to a final concentration of 0.2 μ g/ml. Sterile water served as a control. The following treatments were compared: (i) *T. atroviride* FCCM at 1×10^6 spores/ml, (ii) *T. atroviride* FCCM at 1×10^7 spores/ml, (iii) *T. atroviride* FCCM at 1×10^8 spores/ml, (iv) *T. atroviride* FCCM at 1×10^9 spores/ml, (v) *T. atroviride* FCCM at 1×10^6 spores/ml + BR at 0.2 μ g/ml, (vi) *T. atroviride* FCCM at 1×10^7 spores/ml + BR at 0.2 μ g/ml, (vii) *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 μ g/ml, (viii) *T. atroviride* FCC Maty 1×10^9 spores/ml + BR at 0.2 μ g/ml, and (ix) control = sterile water.

To evaluate the optimal *T. atroviride* CCTCCSBW0199 + BR application sequence, *T. atroviride* FCCM (1×10^8 spores/ml) and

BR at 0.2 µg/ml solution were prepared. The following treatments were compared: (i) *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 µg/ml (24 h later), (ii) BR at 0.2 µg/ml + *T. atroviride* FCCM at 1×10^8 spores/ml (24 h later), (iii) *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 µg/ml (at the same time), (iv) *T. atroviride* FCCM alone at 1×10^8 spores/ml, (v) BR alone at 0.2 µg/ml, and (vi) sterile water was sprayed as the control.

The treatment solutions were sprayed onto tomato seedlings at the three- to five-leaf stage using a spray bottle (10 ml/plant) (Chen et al. 1982; Sauer et al. 1986). After 24 h, a *B. cinerea* conidial suspension (1.0×10^6 spores/ml) was sprayed onto the leaves, and all plants were humidified to keep soil moisture at 20 to 30% for 5 days. Gray mold severity was assessed 5 days after inoculation (DAI) using a scale of 0 to 9, where 0 = no disease spots, 1 = the disease spot area accounted for 1.0 to 10.0% of the leaf area, 3 = the disease spot area accounted for 11.0 to 25.0% of the leaf area, 5 = the disease spot area accounted for 26.0 to 50.0% of the leaf area, 7 = the lesion area accounted for 51.0 to 75.0% of the leaf area, and 9 = the disease spot area accounted for more than 75.0% of the leaf area. The disease index and control efficiency of gray mold on the tomato leaves were calculated as follows: disease index = $100 \times [\sum (\text{number of leaves for each disease severity} \times \text{disease rank})] / (\text{total number of leaves} \times 9)$ and control efficiency (%) = $[(\text{disease index of the control} - \text{disease index of the treatment}) / \text{disease index of the control}] \times 100$.

Determination of defense response-related enzyme activity. Solutions of *T. atroviride* FCCM at 1×10^8 spores/ml and BR at 0.2 µg/ml were prepared and sprayed onto the leaves of three- to five-leaf tomato seedlings at the same time using a spray bottle (10 ml/plant). The following treatments were compared: (i) *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 µg/ml, (ii) *T. atroviride* FCCM alone at 1×10^8 spores/ml, (iii) BR alone at 0.2 µg/ml, and (iv) sterile water was sprayed as the control. After 24 h, a *B. cinerea* conidial suspension (1.0×10^6 spores/ml) was sprayed onto the leaves, and all plants were humidified to keep soil moisture at 20.0 to 30.0% for 5 days. Plant tissue homogenates were prepared as follows. Tomato leaves and phosphate buffer (0.1 mol/liter, pH of 7 to 7.4) in a 1:9 (wt/vol) ratio were ground in liquid nitrogen. An aliquot of the plant tissue suspension (0.1 g/ml) was centrifuged at 5,000 rpm for 10 min at 4°C, and the supernatant was collected. POD and SOD activity followed protocols recommended by Shanghai Cablebridge Biotechnology Co., Ltd. Catalase (CAT) and phenylalanine ammonia-lyase (PAL) activities were determined according to the protocols recommended by Shanghai Jianglai Biotechnology Co., Ltd. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of substrate (or the relevant group in a

substrate) in 1 min under specific conditions, and the activity is expressed as units per milligram of protein (Alici and Arabaci 2016; Jing et al. 2012).

Data analysis. The raw data such as pathogen colony diameters, spore numbers, and incidence numbers from the experiments were organized and saved in Microsoft Excel (version 2013). The pathogen percent inhibition, disease index of tomato leaves, control effects of different treatments, germination rates of *Trichoderma* spores, and disease incidence rates of tomato leaves were calculated with linear arithmetic functions in Microsoft Excel (version 2013). Analyses of variance (ANOVA) was performed to examine control effects of strain *T. atroviride* CCTCCSBW0199 at different concentrations, to compare percent inhibitions of BR + a *Trichoderma* sp. at different concentrations, and to test for control effects of BR + a *Trichoderma* sp. at various concentrations with the ANOVA least significant difference multiple comparisons command in SPSS (version 18.0). Each experiment was repeated three times, and all treatments were set up in biological triplicate.

Results

Screening of antagonistic *Trichoderma* strains against *B. cinerea*. All *Trichoderma* strains tested inhibited the growth of *B. cinerea*, although inhibitions varied among the strains. Among the tested strains, *T. atroviride* CCTCCSBW0199 most significantly inhibited the growth of *B. cinerea*, by 69.8 and 73.6% at 20 and 28°C, respectively (Table 1). Similarly, FCM from strain CCTCCSBW0199 inhibited pathogen growth by 73.1% (Fig. 1). Consequently, all remaining experiments were performed with strain *T. atroviride* CCTCCSBW0199.

Effects of BR on *B. cinerea* growth in vitro. Vacuole size observed using transmission electron microscopy was larger in *B. cinerea* treated with BR solutions at 0.2 µg/ml compared with the control, indicating that BR interfered with the subcellular structure of the *B. cinerea* mycelium (Fig. 2).

Effects of BR on *T. atroviride* CCTCCSBW0199. The mycelial growth of a *Trichoderma* sp. was not affected by different BR concentrations (Fig. 3). In fact, BR at a concentration of 1 or 0.2 µg/ml stimulated the growth of *Trichoderma* mycelia compared with the control ($P < 0.05$). However, BR had no effect on sporulation of *Trichoderma* spp. (Table 2).

Determination of the effect of BR on *T. atroviride* CCTCCSBW0199-mediated inhibition of *B. cinerea* growth. The inhibitory rates of *T. atroviride* CCTCCSBW0199 + BR solution at 1, 0.2, and 0.1 µg/ml on the growth of *B. cinerea* were 70.3, 74.6, and 69.9%, respectively; higher than the *Trichoderma* sp. without BR control (67.0%). The results showed that BR at 0.2 and 1 µg/ml enhanced the inhibitory effect of *T. atroviride* CCTCCSBW0199 on *B. cinerea* growth, and that BR solution at 0.2 µg/ml + *Trichoderma* formulation was the most effective combination against the growth of *B. cinerea* (Table 3).

Determination of the effect of *T. atroviride* CCTCCSBW0199 FCCM and BR on tomato gray mold. An application of either *T.*

Table 1. Screening of *Trichoderma* strains with biocontrol activity against *Botrytis cinerea* growth

Strain number	20°C		28°C	
	Pathogen colony diameter (cm)	Percent inhibition (%) ^z	Pathogen colony diameter (cm)	Percent inhibition (%) ^z
JH3403	3.7 ± 0.1	59.2 C	3.4 ± 0	62.7 D
GDFS1009	3.6 ± 0.1	60.3 CD	3.5 ± 0.1	60.6 C
ZJSX5003	4.3 ± 0.1	52.2 A	3.9 ± 0	57.2 B
H6	4.0 ± 0	55.6 B	4.1 ± 0.1	54.4 A
D9	3.2 ± 0	64.1 FG	3.0 ± 0.1	66.3 G
CCTCCSBW0199	2.7 ± 0.1	69.8 H	2.4 ± 0.1	73.6 H
1	3.6 ± 0.1	60.3 CD	3.3 ± 0.1	63.5 DE
HN524001	3.2 ± 0	64.4 G	3.2 ± 0	69.6 EF
HNZJ4010	3.4 ± 0.1	62.5 E	3.0 ± 0.1	66.7 G
HBZJ4007	3.3 ± 0.1	62.9 EF	3.0 ± 0.1	66.7 G
HNCS3013	3.6 ± 0.1	60.3 CD	3.2 ± 0.1	64.0 E
CDCG3007	3.5 ± 0.1	61.1 D	3.1 ± 0.1	65.5 FG

^z Different uppercase letters in the same column indicate significant differences among treatments ($P < 0.05$).

Fig. 1. Inhibitory effects of *Trichoderma atroviride* CCTCCSBW0199 fermented cultures containing metabolites (FCM) on the growth of *Botrytis cinerea*. A, Potato dextrose agar (PDA) with FCM and B, control: PDA without FCM.

atroviride CCTCCSBW0199 FCCM or FCCM + BR significantly reduced the disease index of gray mold on tomato leaves ($P < 0.05$) (Table 4). Percent control of gray mold ranged from 61.1 to 71.3% for FCCM alone and 62.1 to 74.8% for FCCM + BR. The lowest concentration of FCCM reduced disease the most. Conversely, a higher concentration of FCCM (1×10^8 spores/ml) + BR reduced disease the most (Fig. 4; Table 4).

An application of *T. atroviride* FCCM alone at 1×10^8 spores/ml followed by an application of BR at 0.2 $\mu\text{g/ml}$ (24 h later) and an application of a mix of *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 $\mu\text{g/ml}$ resulted in similarly high levels of control against the disease (Table 5). Therefore, in enzyme activity experiments, *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 $\mu\text{g/ml}$ were applied simultaneously for convenience.

Determination of defense-related enzyme activity. POD and CAT activities of the plants peaked 3 DAI. POD and CAT activities in extracts of tomato leaf treated with a *Trichoderma* sp. + BR reached 5.29 and 15.6 U/mg of protein, respectively, in contrast to 0.57 and 5.44 U/mg pf protein, respectively, detected for the control.

Table 2. Effects of brassinolide (BR) on *Trichoderma* sporulation

Treatment	Spore concentration (10^6 spores/ml) ^z
BR at 1 $\mu\text{g/ml}$	246.7 $\pm$ 12.3 A
BR at 0.2 $\mu\text{g/ml}$	226.7 $\pm$ 11.4 A
BR at 0.1 $\mu\text{g/ml}$	224.7 $\pm$ 11.2 A
Control	235.7 $\pm$ 11.8 A

^z Different uppercase letters in the same column indicate significant differences among treatments ($P < 0.05$).

Fig. 2. Effects of brassinolide (BR) on the mycelial ultrastructure of *Botrytis cinerea*. **A**, BR at 0.2 $\mu\text{g/ml}$, bar = 1 μm , and **B**, control (no BR), bar = 0.5 μm . Images show changes in size of the vacuole that is indicated by an arrow.

Fig. 3. Effects of applied concentrations of brassinolide (BR) on the growth of *Trichoderma atroviride* CCTCCSBW0199. CK (control) = no BR. Different uppercase letters indicate significant differences among treatments ($P < 0.05$) ($n = 3$).

Activities in the single treatments were lower than those with a *Trichoderma* sp. + BR for 3 DAI (Fig. 5A and B).

SOD activity peaked at 4 DAI and the greatest activity was detected in tomato leaves treated with a *Trichoderma* sp. + BR. SOD activity was 1.4-fold, 1.0-fold, and 2.0-fold higher than the SOD activities in the control for *Trichoderma* sp. alone, BR alone, and *Trichoderma* sp. + BR, respectively, at 4 DAI (Fig. 5C).

PAL activity detected in tomato leaves peaked at 2 DAI after treatment with *Trichoderma* sp. alone or *Trichoderma* sp. + BR. In these two treatments, PAL activity was 3.6-fold and 5.2-fold higher than the activity in the control at 2 DAI ($P < 0.05$). The PAL activity in extracts of tomato leaves treated with BR alone was lower than that in leaves treated with the *Trichoderma* sp. + BR at 2 DAI (Fig. 5D).

Discussion

In this study, FCM from *T. atroviride* CCTCCSBW0199 inhibited growth of *B. cinerea*, indicating that metabolites produced by this strain were antagonistic to *B. cinerea* growth. An aspinolide (AspC) from *T. arundinaceum* (Ta37) has been shown to downregulate the *B. cinerea* virulence genes *BcatrB*, *BMP1*, and *Bcpg1* in the presence of the sesquiterpenoid harzianum A or other unidentified secondary metabolites produced by *T. arundinaceum* (Malmierca et al. 2016).

Our study demonstrated that BR inhibits the growth of *B. cinerea*. BR promotes plant stem elongation and cell division and improves plant disease resistance, functioning as a defense signal for inducing expression of plant defense response-related genes (Li and Chory 1999; Zurek et al. 1994). This present study reports a direct inhibitory effect of BR on *B. cinerea* which may have caused the pathogen to be

Table 3. Inhibitory effects of applied concentrations of brassinolide (BR) + *Trichoderma atroviride* CCTCCSBW0199 against *Botrytis cinerea*

Treatment	Pathogen colony diameter (cm)	Percent inhibition (%) ^z
<i>T. atroviride</i> + BR at 1 $\mu\text{g/ml}$	2.4 $\pm$ 0	70.3 B
<i>T. atroviride</i> + BR at 0.2 $\mu\text{g/ml}$	2.0 $\pm$ 0	74.6 A
<i>T. atroviride</i> + BR at 0.1 $\mu\text{g/ml}$	2.4 $\pm$ 0.2	69.9 B
Control	2.6 $\pm$ 0	67.0 C

^z Different uppercase letters in the same column indicate significant differences among treatments ($P < 0.05$).

Table 4. Disease index and percent control of applied concentrations of *Trichoderma atroviride* CCTCCSBW0199 fermented cultures containing chlamydospores and metabolites (FCCM) + brassinolide (BR) solution at 0.2 $\mu\text{g/ml}$ against gray mold on tomato leaves^z

Treatment	Disease index	Percent control (%)
<i>T. atroviride</i> FCCM at 10^6 spores/ml	8.8 A	71.3 A
<i>T. atroviride</i> FCCM at 10^7 spores/ml	11.3 C	65.4 B
<i>T. atroviride</i> FCCM at 10^8 spores/ml	12.2 B	61.1 D
<i>T. atroviride</i> FCCM at 10^9 spores/ml	12.0 C	63.3 C
<i>T. atroviride</i> FCCM at 10^6 spores/ml + BR at 0.2 $\mu\text{g/ml}$	12.9 B	62.1 C
<i>T. atroviride</i> FCCM at 10^7 spores/ml + BR at 0.2 $\mu\text{g/ml}$	9.6 A	69.2 B
<i>T. atroviride</i> FCCM at 10^8 spores/ml + BR at 0.2 $\mu\text{g/ml}$	8.2 A	74.8 A
<i>T. atroviride</i> FCCM at 10^9 spores/ml + BR at 0.2 $\mu\text{g/ml}$	10.4 B	69.1 B
Control	32.7 D	...

^z Different uppercase letters in the same column indicate significant differences among treatments at the 0.05 level.

more vulnerable to *Trichoderma* parasitism. This finding was supported by the induction of enlarged empty vacuoles in *B. cinerea* mycelia by BR (Fig. 2). Deformed vacuoles might lead to the loss of a variety of functions such as those related to the accumulation of amino acids and inorganic polyphosphates, pH balance, an optimal cellular ion concentration, osmotic pressure regulation, and removal of cellular toxic substances (Cramer et al. 1980; Shubin et al. 2016).

The growth and sporulation of *Trichoderma* spp. was not affected by different concentrations of BR compared with the control. In contrast, *Trichoderma* mycelia and spores grew better under some concentrations of BR solution than in the control. The influence of BR on the growth and sporulation of *Trichoderma* spp. was dependent on the BR concentration applied. For example, the greatest degree of *Trichoderma* growth and sporulation was promoted at BR concentrations of 0.2 and 1 µg/ml, respectively. Under other conditions, the effects of BR on the growth and sporulation of *Trichoderma* spp. were not significant. The reason for this difference is unclear but it may be related to the fact that *Trichoderma* fungi have different sensitivities to BR stress (Osiewacz 2002).

BR had different effects on the growth of *B. cinerea* and *Trichoderma* spp. Study revealed that a BR solution resulted in 20.0% inhibition of *Bipolaris maydis* (Ni et al. 2017). Research indicated that fungicide oxime ethers acyl hydrazone B1 inhibited the growth of *Rhizoctonia solani* by 80% but did not inhibit growth of *Botrytis cinerea* (Jiao et al. 2011). Thus, it is possible that BR could be likened to a fungicide; its inhibition effect is genus specific and different fungi have different sensitivities or specific responses to BR stress.

The higher inhibitory rates of the *Trichoderma* sp. + BR indicated that they had a synergistic inhibitory effect on the pathogen, although the synergistic action depended on the concentration of BR. Greenhouse evaluations supported the synergistic effect of a *Trichoderma* sp. + BR except at the lowest concentration of FCCM. When FCCM was applied alone, the lowest concentration resulted in the highest percent control of gray mold. A possible reason for this observation is that control of tomato gray mold was unstable with FCCM alone and the synergistic effect depended on the concentrations of

Trichoderma spp. and BR used. Specifically, *Trichoderma* FCCM at 1×10^8 spores/ml + BR solution at 0.2 µg/ml inhibited 74.8% of pathogen growth, suggesting that this was the best combined formulation under our conditions. However, the mechanism of synergistic effect remains unclear.

A sequential application of *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 µg/ml (24 h later) and *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 µg/ml showed no significant differences on the percent control. Hence, for convenience, simultaneous application of *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 µg/ml was used in subsequent experiments.

Table 5. Disease index and percent control of application sequences of *Trichoderma atroviride* CCTCCSBW0199 fermented cultures containing chlamydospores and metabolites (FCCM) + brassinolide (BR) at 0.2 µg/ml solution against gray mold on tomato leaves

Treatment ^y	Disease index	Percent control (%) ^z
<i>T. atroviride</i> FCCM at 10^8 spores/ml + BR at 0.2 µg/ml (24 h later)	11.1	74.6 A
BR at 0.2 µg/ml + <i>T. atroviride</i> FCCM at 10^8 spores/ml (24 h later)	13.3	69.3 B
<i>T. atroviride</i> FCCM at 10^8 spores/ml + BR at 0.2 µg/ml (at the same time)	11.1	74.4 A
Control	43.3	...
<i>T. atroviride</i> FCCM alone at 10^8 spores/ml	23.3	46.2 C
BR alone at 0.2 µg/ml	26.7	38.3 C

^y *T. atroviride* FCCM at 10^8 spores/ml + BR at 0.2 µg/ml (24 h later) means using BR at 0.2 µg/ml 24 h later than *T. atroviride* FCCM at 10^8 spores/ml; BR at 0.2 µg/ml + *T. atroviride* FCCM at 10^8 spores/ml (24 h later) means using *T. atroviride* FCCM at 10^8 spores/ml 24 h later than BR at 0.2 µg/ml; and *T. atroviride* FCCM at 10^8 spores/ml + BR at 0.2 µg/ml (at the same time) means using them at the same time.

^z Different uppercase letters in the same column indicate significant differences among treatments at the 0.05 level.

Fig. 4. Control efficiency of applied concentrations of *Trichoderma atroviride* CCTCCSBW0199 fermented cultures containing chlamydospores and metabolites (FCCM) + brassinolide (BR) solution at 0.2 µg/ml against gray mold on tomato leaves 5 days after inoculation with *Botrytis cinerea*. A, *T. atroviride* FCCM at 10^6 spores/ml; B, *T. atroviride* FCCM at 10^7 spores/ml; C, *T. atroviride* FCCM at 10^8 spores/ml; D, *T. atroviride* FCCM at 10^9 spores/ml; E, *T. atroviride* FCCM at 10^6 spores/ml + BR at 0.2 µg/ml; F, *T. atroviride* FCCM at 10^7 spores/ml + BR at 0.2 µg/ml; G, *T. atroviride* FCCM at 10^8 spores/ml + BR at 0.2 µg/ml; H, *T. atroviride* FCCM at 10^9 spores/ml + BR at 0.2 µg/ml; and I, control (sterile water).

Fig. 5. Effects of *Trichoderma atroviride* CCTCCSBW0199 fermented cultures containing chlamydospores and metabolites (FCCM) + brassinolide (BR) at 0.2 µg/ml on defense enzymes associated with resistance against *Botrytis cinerea* on tomato leaves. **A**, Peroxidase (POD) enzyme activity; **B**, catalase (CAT) enzyme activity; **C**, superoxide dismutase (SOD) enzyme activity; and **D**, phenylalanine ammonia-lyase (PAL) enzyme activity. Time points are days after treatment with *T. atroviride*, BR, *T. atroviride* + BR, or control (CK; sterile water).

The *Trichoderma* sp. + BR stimulated higher activities of the anti-oxidant enzymes SOD, POD, CAT, and PAL in tomato leaves than treatment with either *Trichoderma* spp. or BR alone, suggesting that the combination of a *Trichoderma* sp. + BR stimulates the expression of antioxidant-enzyme-related genes. Z.-r. Zhang et al. (2017) showed that the activity of PAL and POD enzymes was consistent with the change of gene expression. Meanwhile, this phenomenon might explain the mechanism of the synergistic effect. SOD is known to scavenge free radicals accumulated in infected plants, thereby preventing damage to plant cell membranes and delaying senescence (Giannopolitis and Ries 1977). POD is a key enzyme in lignin synthesis that can strengthen the plant cell wall structure and provide protection against pathogenic infection (Hegedüs et al. 2001). CAT plays a key role in the clearance of hydrogen peroxide in plants (Asghari and Aghdam 2010) and as a receptor for salicylic acid to enhance plant resistance to diseases (Li and Li 2007). PAL activity is closely associated with levels of plant phytoalexins, lignins, and phenolic compounds (Aghdam et al. 2012). We suggest that the increased activity we detected in these antioxidant enzymes with the use of a *Trichoderma* sp. + BR led to better host plant resistance to gray mold infection in tomato.

To the best of our knowledge, this study is the first to report the combined application of *T. atroviride* CCTCCSBW0199 and the plant growth regulator BR to synergistically prevent tomato gray mold. The synergistic action of *T. atroviride* FCCM at 1×10^8 spores/ml + BR at 0.2 µg/ml in the control of tomato gray mold caused by *B. cinerea* was confirmed. The genus *Trichoderma* was previously demonstrated to have a strong capacity to induce resistance in tomato (Martínez-Medina et al. 2013), and BR significantly promotes tomato growth and resistance to stress (Vardhini et al. 2008, 2010). The application of a *Trichoderma* sp. + BR enhanced the growth and sporulation of *Trichoderma* spp. at specific concentrations, inhibited growth of *B. cinerea*, and increased resistance to gray mold in tomato. Therefore, this combination may lead to a more stable control efficiency against *B. cinerea* infection in tomato.

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