Stem Blight Control of *Schizonepeta tenuifolia* Caused by *Phytophthora nicotianae* Using *Trichoderma* spp.

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Abstract: **Objective** To control stem blight disease of *Schizonepeta tenuifolia* caused by *Phytophthora nicotianae*. **Methods** The antagonist effect of 13 *Trichoderma* strains (including *T. viride* and *T. harzianum*) was evaluated upon mycelia growth of *P. nicotianae*. *Trichoderma* strains with high antagonistic activities against the pathogen were used to control stem blight of *S. tenuifolia* in the field. **Results** Of 13 *Trichoderma* strains tested, *T. viride* strain M3 showed maximum mycelia growth inhibition (83.2%) to the pathogen, followed by *T. viride* strain Tv04-2 (78.2%) and then *T. harzianum* strain ThB (65.0%), *in vitro*. Fungal cell wall degrading enzymes, protease, and β-1,3-glucanase were analyzed qualitatively and quantitatively in further study. *T. viride* strains M3, Tv04-2, and *T. harzianum* strain ThB efficiently against *P. nicotianae* were used to control stem blight of *S. tenuifolia* in the field, and *T. viride* strain M3 showed the best biocontrol potential. **Conclusion** *Trichoderma* spp. can be used as alternatives of pesticides to control stem blight, one of the serious soilborne diseases of *S. tenuifolia* caused by *P. nicotianae*. However, though *T. viride* strains Tv04-2 and *T. harzianum* strain ThB are also highly against *P. nicotianae in vitro*, the controlling efficacy of them on stem blight disease is not as excellent as *T. viride* strains M3 in the field.

Key words: β-1,3-glucanase; mycoparasitism; *Phytophthora nicotianae*; protease; *Schizonepeta tenuifolia*
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Introduction

Schizonepeta tenuifolia Briq. is a widely grown medicinal plant in China, and *P. nicotianae* causing stem blight is a most serious disease of *S. tenuifolia* (Zhang *et al*, 2009). Application of antagonists against the pathogen was the most economical, environment-friendly, and effective strategy.

Of the various biological control agents, *Trichoderma* spp. has been known to suppress many soil borne fungal diseases by various mechanisms, such as competition for nutrients and space (Wang *et al*, 1999; Sun, Yang, and Song, 2005), production of inhibitory volatiles and non-volatiles (Xian and Li, 2009), mycoparasitism involving the production of hydrolytic enzymes (Viterbo *et al*, 2002; Dutta and Chatterjee, 2004; Dhar, Mishra, and Chaudhary, 2006; Zhou *et al*, 2008), etc.

Also, many papers reported the study on antagonistic mechanisms and controlling of *P. nicotianae* in tobacco successfully (Wang *et al*, 2001; Li *et al*, 2002; Li *et al*, 2006; Chen *et al*, 2009). The present study has been designed to screen *Trichoderma* strains with high antagonistic activity against *P. nicotianae*, the pathogen of stem blight disease on *S. tenuifolia* under *in vitro* and field conditions.

Materials and methods

Materials

P. nicotianae was isolated from roots of diseased *S. tenuifolia* and maintained on V8 slants at 4 ℃ until use, which was identified by ZHANG Zheng. Thirteen *Trichoderma* strains (*T. viride* strains Tv04-1, Tv04-2, Tv04-3, Tv04-4, Tv04-6, M1, M2, M3, M4, M5, and *T. harzianum* strains ThB, Th3080, Th3093) were obtained from the Institute of Microbiology, Chinese Academy of Sciences and College of Plant Protection,

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Dual-culture method used for *Trichoderma* spp. and *P. nicotianae*

Dual-culture method was used to study the competition and mycoparasitism between the pathogen and the antagonists by inoculating them together on the Petri dish. The plates were then incubated at (25 ± 1) ℃ for 7 d, mycelia growth of both the pathogen and the antagonist was measured, and inhibitions of *Trichoderma* strains against the pathogen were calculated. Petri dish inoculated with the pathogen only was served as control.

Qualitative and quantitative tests of β-1,3-glucanase activity

The transparent circle method was used to detect the present of β-1,3-glucanase on the culture medium (6.80 g/L KH_2PO_4, 17.98 g/L K_2HPO_4, 5.00 g/L yeast juice, 4.00 g/L powdery of poria, 0.10 g/L aniline blue, 14.00 g/L agar) (Wang *et al*, 2007; Guo *et al*, 2008). Discs of *Tricroderma* spp. were inoculated on the centre of the medium. After incubated at (25 ± 1) ℃ for 4 d, transparent zone on plate was observed and measured.

Enzyme activity of β-1,3-glucanase was measured according to Yu, Xie, and Huang (2007) with minor modification. Czapek medium free of agar was used to prepare *Tricroderma* spp. shaking at 150 r/min and 25 ℃. After 4 d cultivation, culture of *Tricroderma* spp. was centrifuged at 10 000 r/min for 20 min, and the supernatant (the extract of crude enzymes) was stored at −20 ℃. 3,5-Dinitrosalicylic acid colorimeitry was used to measure enzyme activities of the crude extract. Take 100 μL laminarin (0.1%) that diluted with sodium acetate-acetic acid buffer (0.05 mol/L, pH 5.5), preheated at 37 ℃ water bath for 5 min, and added 100 μL crude enzyme extract. After well mixed, the mixture was put at 37 ℃ for 30 min. Reducing saccharides were evaluated by DNS method (Jijakli and Lepoivre, 1998), and 1 μg glucose generated every minute at 37 ℃ as one enzyme activity unit (1 U/mL).

Qualitative test of protease activity

Protease activity was evaluated by plate transparent zone method (Xu, Feng, and Xu, 1998). Disc of *Tricroderma* spp. was inoculated at the center of the medium plate containing 10 g/L gelatin and 15 g/L agar, and cultivated at 25 ℃ for 3 to 4 d. Then, 5 mL $HgCl_2$ (150 g/L) was used to treat the plate that growing with *Tricroderma* spp. for 15 min, and the diameter of transparent zone was measured.

Qualitative test of volatiles and non-volatiles

Production of volatile antibiotics was studied according to Cao *et al* (2007). Every 15 mL of V8 medium was poured in both the base and the lid of Petri dish, respectively. The lid was inoculated with the pathogen and the base inoculated with *Tricroderma* spp. The base and the lid were tightly enveloped by parafilm, and incubated at 25 ℃ for 4 d. Treatment without inoculated *Tricroderma* spp. was used as control, and three replicates were set for each treatment. The inhibitory percentage on mycelia growth of the pathogen was calculated based on colony diameter.

Production of non-volatile antibiotic was studied according to Liu and Wen (2005). Two layers of immobilon-Nc membrane were placed on V8 medium, *Tricroderma* spp. was inoculated on the center of membrane, and inoculated at 25 ℃ until the colony diameter reached about 5 cm. Then, membrane and *Tricroderma* spp. colony were removed, and the pathogen was inoculated at the center of medium plate, and incubated at 25 ℃ for 4 d. Pathogen inoculated on medium plate without metabolites of *Tricroderma* spp. was used as control, and three replicates were set for each treatment. Colony diameter was recorded every day, and mycelia growth inhibitions of *Trichoderma* strains against the pathogen were calculated.

Control of stem blight disease in the field

Potential antagonists *Tricroderma* spp. were finally subjected to the field trials in order to assess their efficacy in controlling stem blight disease caused by *P. nicotianae* on *S. tenuifolia*. Different biocontrol antagonists were prepared. Artificial medium (10 g cornmeal, 10 g wheat bran, and 7 mL distilled water) was taken into 250 mL flask, sterilized and inoculated with respective antagonists and incubated at (25 ± 1) ℃ for 10 d. Artificial medium (10 g oatmeal, 5 g cornmeal, 5 g wheat bran, and 8 mL V8 vegetable juice) was taken into 250 mL flask, sterilized and inoculated with pathogen and incubated at (25 ± 1) ℃ for 5 d. After incubation, both the pathogen and antagonists cultures were air-dried at room temperature naturally. When sowing of *S. tenuifolia* seeds, antagonists were applied to the 2−5 cm deep soil with 40 g/m^2. Fifteen days before high occurrence of stem blight disease on *S. tenuifolia*,

antagonists cultures were applied on the surface of the field soil with 40 g/m^2, and the pathogen culture was artificially inoculated on the surface of the field soil 15 d later. Ten days after inoculation, total number of plants and number of plants infected were recorded, and control of infection by *Trichoderma* spp. was calculated.

Statistical methods

The inhibition formula was as follows: Inhibition (*I*) = (diameter of control – diameter of treatment) / diameter of control × 100%. One-way analysis of variance and LSD method in SPSS 13.0 was used to analyze the significance of *Trichoderma* strains against the pathogen. Capital letters (such as A, B, etc.) were used to indicate the significance at 0.01 level, and lower letters (such as a, b, etc.) were used to indicate the significance at 0.05 level.

Results

Antagonist activities of *Trichoderma* spp. against the pathogen

Among all the antagonists used, *T. viride* strain M3 was the most potent one to control mycelia growth of the pathogen with 83.2% efficacy, followed by *T. viride* strain Tv04-2 and *T. harzianum* strain ThB, which showed 78.2% and 65.0% mycelia growth inhibition of the pathogen, respectively (Table 1).

Table 1 Effect of *Trichoderma* spp. on mycelia growth of *P. nicotianae*

Trichoderma spp. strains	Colony diameter of pathogen / cm	Mycelia growth inhibition / %
M3	0.90 ± 0.01	83.2 ± 0.2 Aa
Tv04-2	1.18 ± 0.06	78.2 ± 1.1 Aa
ThB	1.89 ± 0.05	65.0 ± 0.9 Bb
Tv04-1	1.95 ± 0.03	63.9 ± 0.6 BCb
Th3093	2.01 ± 0.06	62.8 ± 1.1 BCb
Tv04-4	2.25 ± 0.04	58.2 ± 0.7 BCDbc
Tv04-3	2.26 ± 0.08	58.0 ± 1.4 BCDbc
Th3080	2.29 ± 0.12	57.6 ± 2.2 BCDbc
M2	2.29 ± 0.18	57.5 ± 3.3 BCDbc
M1	2.38 ± 0.03	55.8 ± 0.5 BCDbc
Tv04-6	2.41 ± 0.25	55.3 ± 4.7 BCDbcd
M4	2.59 ± 0.05	51.9 ± 0.9 CDcd
M5	2.70 ± 0.10	49.9 ± 1.8 Dcd

Capital letters indicate the very significance difference, and lower letters indicate the significance difference, same as below

Hydrolases activity test

Mycoparasitism of *Trichoderma* spp. on plant pathogens was closely related to hydrolytic enzyme production. In this study, β-1,3-glucanase and proteases secreted by *Trichoderma* spp. showed significant inhibition on mycelia growth of the pathogen through degrading cell wall of the pathogen mycelia. For all *Trichoderma* spp. tested, *T. harzianum* strains M3 and Tv04-2 exhibited significantly higher β-1,3-glucanase activity, and *T. harzianum* strain ThB and *T. viride* strain Tv04-2 showed higher proteases activity, *in vitro* (Table 2).

Table 2 Assay of β-1,3-glucanases and proteases activity

Hydrolases	*Trichoderma* strains	Diameter of transparent cycle / cm
β-1,3-glucanases	M3	4.10±0.03 Aa
	Tv04-2	4.18±0.07 Bb
	ThB	2.43±0.11 Cc
proteases	M3	4.52±0.11 Aa
	Tv04-2	5.68±0.07 Bb
	ThB	5.28±0.06 Cc

β-1,3-Glucanase activities of *T. viride* strains M3, Tv04-2 and T. *harzianum* strain ThB were evaluated to be 19.26, 7.38, and 3.24 U/mL, respectively, which were positively correlated with diameter of transparent zone generated.

Volatiles and non-volatiles tests

The assay of volatile and non-volatile antibiotics suggested that three *Trichoderma* strains tested were capable of producing volatile and non-volatile antibiotics *in vitro* and that they showed adverse effects on mycelia growth of the pathogen. Results showed that both non-volatiles secreted by *T. viride* strain Tv04-2 and volatiles secreted by *T. harzianum* strain ThB had the most significant inhibition against mycelia growth of the pathogen; also we found that non-volatiles secreted by *Trichoderma* spp. were better than volatiles (Table 3).

Control of stem blight disease in the field

Controlling stem blight disease caused by *P. nicotianae* on *S. tenuifolia* was conducted in the field for two successive years with the application of biocontrol agents (Table 4, Fig. 1). Of three antagonists tested, *T. viride* strain M3 showed maximum control rate of the pathogen infection, 80.56% in 2008 and 76.83% in 2009.

Though *T. viride* strain Tv04-2 and *T. harzianum* strain ThB showed significant inhibition on mycelia growth of the pathogen *in vitro*, control rates of the pathogen

Table 3 Effect of volatile and non-volatile substances secreted by *Trichoderma* spp. on mycelia growth of *P. nicotianae*

Treatments	Volatile substances		Non-volatile substances	
	Colony diameter of the pathogen / cm	Growth inhibition / %	Colony diameter of the pathogen / cm	Growth inhibition / %
CK	5.13±0.24 Aa	—	5.13±0.24 Aa	—
M3	4.82±0.05 Aa	6.0	3.66±0.05 Bb	28.7
Tv04-2	4.22±0.08 Bb	17.7	3.48±0.15 Bb	32.2
ThB	3.80±0.28 Bc	25.9	3.81±0.08 Bb	25.7

Table 4 Controlling efficacy of antagonistic *Trichoderma* spp. on stem blight of *S. tenuifolia* in the field

Year	Treatments	Total number of plants	Number of infected plants	Infection / %	Control rate of infection / %
2008	Control	126	97	76.98 ± 3.39 Aa	—
	M3	144	28	19.44 ± 1.71 Bb	80.56
	Tv04-2	87	56	64.37 ± 0.68 Cc	35.63
	ThB	103	43	41.75 ± 3.61 Dd	58.25
2009	Control	372	323	86.83 ± 2.41 Aa	—
	M3	315	73	23.17 ± 3.25 Bb	76.83
	Tv04-2	417	263	63.07 ± 3.07 Cc	36.93
	ThB	276	151	54.71 ± 2.01 Dd	45.29

Fig. 1 Bio-control efficacy of *T. viride* strain M3 against stem blight caused by *P. nicotianae* on *S. tenuifolia* (A) and control (B) only inoculated with *P. nicotianae*

A: most of *S. tenuifolia* plants growing well in the field, though part of them also infected by *P. nicotianae*, the degree of damage was not as heavy as control that inoculated with the pathogen only. B: many *P. nicotianae* infected *S. tenuifolia* plants died without the protection of *Trichoderma* spp.

infection in the field by *T. harzianum* strain ThB were 58.25% in 2008 and 45.29% in 2009, respectively, followed by *T. viride* strain Tv04-2 35.63% in 2008 and 36.93% in 2009.

Discussion

Potential antagonism of *Trichoderma* spp. includes competition, antibiosis, mycoparasitism, etc, and mycoparasitism of plant pathogens by *Trichoderma* spp. has been well documented. Mechanism of hyper-parasitism includes various kinds of interactions like coiling of hyphae around the pathogen, penetration, production of haustoria, and lysis of hyphae, etc. Mycoparasitic *Trichoderma* spp. may recognize cell wall components, hydrolyse, and utilize them as substrate (Harman *et al*, 2004). Some *Trichoderma* spp. can colonize and penetrate plant root tissues and initiate a series of morphological and biochemical changes and induced the plant defense responses (Ozbay and Newman, 2004).

Antibiotics, such as volatiles or non-volatiles, can be secreted by antagonistic agents, such as *Trichoderma* spp., which were also demonstrated by the results in this study. They exerted strong inhibition on the pathogen by producing compounds like harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, and heptelidic acid *etc*. (Evident *et al*, 2003; Benítez *et al*, 2004).

It is evident from the results that antagonistic

agents showing excellent antagonist activity *in vitro* condition may not have effective control of infection in the field. So, it was necessary to evaluate the biocontrol capability of antagonistic agents against the pathogen in the field. It may be advantageous to identify an isolate with high enzyme activities on cell wall of plant pathogen. Clone and transfer of the hydrolase gene from *Trichoderma* spp. to target plants, and constitutive expression of high amounts of lytic enzymes will enhance the resistance of host plants to pathogen steadily. Furthermore, if expressed products of the transferred gene have biocontrol capability against a wide range of pathogen, transfer of the target hydrolase gene to the plants would have a very important significance.

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