Available online at www.sciencedirect.com
ScienceDirect
Biological Control 44 (2008) 24–31
Biological Control
www.elsevier.com/locate/ybcon

Antifungal activity of *Penicillium striatisporum* Pst10 and its biocontrol effect on Phytophthora root rot of chilli pepper

Yan Ma [a,b,1], Zhi-zhou Chang [b], Jiang-tao Zhao [c], Ming-guo Zhou [a,*]

[a] *College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China*
[b] *Institute of Agricultural Resources and Environments, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China*
[c] *Science and Technology Division, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China*

Received 21 March 2007; accepted 8 October 2007
Available online 13 October 2007

Abstract

Penicillium striatisporum Pst10 was isolated from the rhizosphere of chilli peppers. In dual culture agar plate assays, this isolate showed very high antagonistic effects on mycelium growth of *Phytophthora* spp., *Cladosporium cucumerium*, and *Sclerotinia sclerotiorum*. In *in vitro* assays, the toxicity of sterilized liquid culture filtrates (SLCF) of Pst10 grown in potato-dextrose broth (PDB) was tested against *Phytophthora capsici* mycelium growth and sporangia/spore formation or germination. The SLCF completely inhibited mycelium growth and even at a 100-fold dilution led to abnormal mycelium. A 20-fold dilution of SLCF inhibited formation and germination of sporangia and spores. Three antifungal substances were separated by thin-layer chromatography (TLC) from organic solvent extracts of liquid culture filtrate of Pst10. Composted pig manure slightly increased the colonization of the chilli rhizosphere by Pst10. In pot tests, the incidence of Phytophthora root rot of chilli was significantly reduced when artificially infested soil was treated with conidia and SLCF of Pst10.

Keywords: *Penicillium striatisporum*; *Phytophthora capsici*; Antagonistic mechanism; Biocontrol

1. Introduction

Phytophthora root rot (PRR), caused by *Phytophthora capsici* Leonian, is a common and destructive disease of greenhouse and field-grown chilli peppers (*Capsicum frutescens* L.) in China and world-wide. The disease causes serious economic losses of chilli peppers in more than 15 Provinces of China (Zuo, 2002). Shandong Province has suffered major PRR outbreaks in 1996, 1997, and 1998 (Lin et al., 2003). Chilli was grown on more than 1.3 million hectares in 2006 in China. Generally, the incidence of PRR in the pepper fields is 20–30% but in many continuous cropping fields it could be 40–60%, which leads to the estimated annual economic loss of ¥675 million or 96 million USD.

There are a few effective chemical control methods for PRR. There have been considerable efforts to find biological control agents for this disease and several potential candidates have been reported including: *Actinomyces* spp. (Zhu et al., 1995; Lee and Hwang, 2002; Hee et al, 2006; Ezziyyani et al., 2007), *Pseudomonas* spp. (Lee et al., 2003a,b,c; Jung and Kim, 2004; Paul and Sarma, 2006), *Bacillus* spp.(Dai and Guan, 1999; Lee and Hwang, 2002; Jung and Kim, 2003; Qiu et al., 2004; Jung and Kim, 2005), *Trichoderma* spp. (Liu and Lu, 2003; Ezziyyani et al., 2007) and even some fungal endophytes (Kim et al., 2007). The most promising have been the strains of *Penicillium* spp. (Anderson et al., 1988; De Cal et al., 1988, 1990; Fang and Tsao, 1989, 1992, 1995; Harrison and Stewart, 1988; Kharbanda and Dahiya, 1990; Melouk and Akem, 1987; Proksa et al., 1992; Nicoletti et al., 2004;

* Corresponding author. Fax: +86 25 84395641.
E-mail address: mgzhou@njau.edu.cn (M. Zhou).
[1] Present address: Crop Southern Protection and Food Research Centre, Agriculture and Agriculture-Food Canada (AAFC).

1049-9644/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2007.10.005

Pascual et al., 2000; Sztejnberg et al., 1988), with *Penicillium oxalicum* Currie&Thom (Windels, 1981; Windels and Kommedahl, 1978, 1982; De Cal et al, 1997a,b, 1999; Larena et al., 2002; Sabuquillo et al., 2006) being the most intensely investigated.

Penicillium striatisporum Stolk was identified by Stolk (1969), but the only subsequent reports on this fungus were a Japanese patent in 1994 (Morino et al., 1994) and a report by Michael et al. (2005). Both studied medical aspects. There are no reports on the biological control potential of this fungus against plant pathogens. In this study, we report for the first time the efficacy of *P. striatisporum* as a biological control agent of *Phytophthora* spp. In order to determine the mode of action, we examined the culture filtrates of the fungus for inhibitory effects against *P. capsici* Leonian. As this fungus is a slow colonizer, we tested the effect of an organic fertilizer, composted pig manure, on the colonization ability of Pst10 in the chilli pepper rhizosphere. The strain designated as Pst10 appeared to have high potential as a biocontrol agent of Phytophthora root rot of chilli in preliminary potting tests.

Application of Pst10 for soil-borne disease control of plant had been authorized and approved by the Chinese Invention Patent Bureau. The objectives of this research was to investigate the potential of Pst10 as a fungal biocontrol agent against PRR in chilli pepper.

2. Materials and methods

2.1. Fungal cultures and growth media

The isolate Pst10 of *P. striatisporum* was originally isolated from the rhizosphere of chilli pepper in an experimental greenhouse in Nanjing, China. Pst10 has been deposited at China General Microbiological Culture Collection Center and assigned Accession No. CGMCC No.1533.

The fungal cultures of Pst10 and pathogenic fungi, *P. capsici* Leonian, *Phytophthora infestans* (Mont.) de Bary, *Phytophthora drechsleri* Tucker, *Phytophthora nicotianae* Breda de Haan, *Phytophthora megasperma* Drechs. f. sp. medicaginis, *Botrytis cinerea* (De Bary) Whetzel, *Sclerotinia sclerotiorum* (*Lib.*)de Bary, *Rhizoctonia solani* J.G. Kühn, *Fusarium graminearum* Schwabe, *Fusarium oxysporum* Schltdl. and *Pythium aphanidermatum* (Edson) Fitzp., *Magnaporthe oryzae* B. Couch, *Alternaria solani* Sorauer, *Cladosporium cucumerium* Ellis et Arthur, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen and *Candida albicans* (C.P. Robin) used in this study were maintained on potato-dextrose agar (PDA) slants at 4 °C and were grown on PDA plates at 25–26 °C in the dark.

2.2. Testing the antifungal spectrum of Pst10 using dual culture assays

The cultures of each of the test pathogens and Pst10 were grown on PDA plates at 25–26 °C for 5–7 days prior to the bioassay tests. The antifungal spectrum was determined by placing a 4-mm-diameter agar disk cut from the edge of an actively growing colony of Pst10 on one side of a PDA plate and an agar disk of the test pathogen of the same size on the opposite side of the same plate. For the yeasts, *S. cerevisiae* and *C. albicans*, cell suspensions obtained from 2-day-old cultures were spread around a Pst10 colony on PDA plate, respectively. Each combination was tested using five replicates. Zones of inhibition were determined after incubation in the dark at 25–26 °C for 8–10 days. The test was conducted twice.

2.3. Preparation of sterilized liquid culture filtrates (SLCF)

Erlenmeyer flasks (250 ml) containing 100 ml of potato-dextrose broth (PDB) were inoculated with four 8-mm-diameter mycelial disks obtained from the edge of vigorously growing colony of Pst10. Flasks were incubated in the dark at 25–26 °C on a rotary shaker at 160 rpm for 7 days. The liquid cultures were filtered through two-layers of cheesecloth in a funnel and the filtrates were centrifuged at 8000 rpm for 15 min. The supernatant was collected and sterilized by filtration through 0.22 μm Millipore membrane. The sterile filtrates were collected and stored at 4 °C before being used in the following experiment.

2.4. Toxicity of SLCF to P. capsici

2.4.1. Effect on mycelium growth of P. capsici

Four-mm-diameter mycelium disks of *P. capsici* were placed in the center of a series of PDA plates containing 2, 0.67, 0.4, 0.2, 0.1, 0.067, 0.05 ml of SLCF in 20 ml of molten PDA medium (45–50 °C) resulting in 10, 30, 50, 100, 200, 300, 400 fold dilutions, respectively. A 0-fold dilution treatment consisting of 20 μl SLCF was directly dropped onto each disk in plate. The control treatment was not amended with SLCF. Each treatment had five replicates. The plates were incubated in dark at 25–26 °C for 20, 48 and 120 h before colony diameters were measured. Disks of *P. capsici* which showed no growth after 7 days of incubation were transferred to PDA plates and incubated at 25–26 °C for 72 h to determine if they were dead or just inhibited. This experiment was conducted three times. Percent inhibition (%) was calculated as equal to the (colony diameter of control − colony diameter of treatment)/colony diameter of control × 100.

2.4.2. Effect on sporangial/spore formation or germination of P. capsici

Fifteen mycelial disks (8-mm-diameter) of *P. capsici* were placed in glass dishes (9 cm in diameter) containing 20 ml sterile water and 1 ml of sterilized filtrate of Pst10. Five replicates were used for each dilution and were grown for 24, 48, and 72 h at 25 °C with continuous light. The control treatment consisted of 15 disks of *P. capsici* (8 mm in diameter) placed in glass dishes (9 cm in diameter) containing only 20 ml sterile water. Sporangium for-

mation and zoospores release were determined using a light microscope by checking five randomly selected fields in each disk.

To determine the effect of SLCF on sporangium and spore germination, 100 μl of sporangium or spore suspensions were spread on 20 ml PDA agar containing 1 ml SLCF. Five replicates were conducted for each treatment. PDA plates were incubated at 28 °C for 24, 72, and 120 h. After each incubation time, 100 sporangia or spores were counted randomly on each PDA plate under a light microscope and germination rate was calculated. Counting was conducted five times. The germination of the control sporangium or spore was determined in 100 μl suspensions spread on 20 ml PDA agar containing 1 ml of sterile water. This experiment was conducted twice. The germination rate (%) was calculated as the (number of germinated sporangia or spores/total number of sporangia or spores counted) × 100.

2.5. Preliminary separation of antibiotics produced by Pst10 grown in liquid culture

2.5.1. Extraction of liquid culture filtrates (LCF) with organic solvents

Several experiments were conducted to identify the active substances found in SLCF. Liquid cultures of Pst10 grown as described above were filtered through two-layers of cheesecloth in a funnel and centrifuged at 8000 rpm for 15 min and the supernatants were collected. Fifty milliliters of the supernatants were extracted with 150 ml of methanol or acetone, respectively, on a rotary shaker incubator, at 160 rpm for 1 h. After centrifugation at 6000 rpm for 15 min, the organic fractions and the residues were collected, respectively, and the supernatants were extracted once again with the same process. Finally, the supernatants of both extractions from the same solvent fractions were combined and concentrated at 45 °C on a rotary evaporator to a final volume of 5 ml. The residues from the same solvents were combined and vacuum-concentrated on a rotary evaporator to dryness and re-dissolved in 5 ml of sterile water.

Fifty milliliters of the supernatants were mixed with 150 ml of ethyl acetate, chloroform or *n*-hexane separately on a rotary shaker incubator at 160 rpm for 1 h and then allowed to remain stationary for 2 h. The organic fractions were collected and the aqueous fractions were re-extracted once more using the same process. The organic fractions or aqueous fractions of both extractions were combined and vacuum-concentrated to a final volume of 5 ml as described above.

The concentrated aqueous and organic fractions were sterilized separately by filtration through 0.22 μm Millipore membrane and stored at 4 °C. Twenty microliters of concentrated organic or aqueous preparations were added to 20 ml molten PDA medium, mixed and poured into Petri dishes, five plates for each treatment. The controls contained an equal volume of each solvent or sterile water. One disk of *P. capsici* was placed in the center of each plate and the cultures were incubated as described above. Colony diameters were measured 72 h later. All bioassays of culture filtrates were repeated three times. The data were analyzed using the procedure of MINITAB.

2.5.2. Preliminary separation by TLC

Antifungal activity was detected in methanol, acetone, ethyl acetate and chloroform fractions of SLCF. The crude concentrated preparations were partially purified by TLC using GF_{254} silica gel plates (silica gel with green fluorescent indicator UV_{254}, manufactured by Qingdao Chemical plant). A 0.1 ml sample of each solvent fraction was spotted separately and the plates were developed in methanol–water (4:1, v/v) in closed tanks and examined under UV light at 254 nm. Four spots in vertical row of each fraction were observed with different R_f values and were scraped form the plate and extracted with methanol for 4 h. The silica gel in the extractions were removed by centrifugation at 6000 rpm for 15 min, and the supernatants for all four spots were collected separately and concentrated in vacuum to dryness and re-dissolved in 200 μl of methanol. A bioassay with *P. capsici* was conducted as described above.

2.6. Assessing the effect of composted pig manure (CPM) on colonization of Pst10

Fresh disks of Pst10 were transferred on PDA plates and grown at 25–26 °C for 10–15 days to produce conidia. The conidia were obtained by adding 15 ml sterile water to the cultures and agitated. The soil for potting experiment was obtained from a common vegetable farm and air-dried in laboratory.

Pots (12 cm × 12 cm × 10 cm) containing air-dried soil were placed in a glasshouse at 20–23 °C. Two seedlings of chilli cultivar Sujiao No. 2 with 2–4 leaves were transplanted into each pot with ten pots per treatment. The treatments consisted of:(1) air-dried soil with no additional treatment; (2) soil supplemented with 1% (g/g) CPM; (3) soil inoculated with a conidial suspension of Pst10 at final concentration of 4×10^6 conidia per gram air dry soil; and (4) soil inoculated with conidial suspensions of Pst10 at the same concentration as above and supplemented with 1% CPM (Pst10-CPM treatment).

The population density of Pst10 was measured 3, 10, 20 and 40 days after transplantation and inoculation of soil using the dilution plating method on selective medium. The medium was nutritional agar containing acetamide as a sole nitrogen source at 2000 ppm (Kelly and Hynes, 1985) because the wild strain of Pst10 could utilize acetamide very well. Populations of Pst10 were estimated as the number of colony forming units (CFU) per gram dry soil. The population density of the total number of fungi was determined by standard soil dilution plating assay on Martin agar medium (Martin, 1950). The entire experiment was conducted twice.

2.7. Effect of Pst10 on disease control

The ability of Pst10 to suppress PRR of chilli pepper in air-dried soil artificially infested with sporangia of *P. capsici* was examined on cultivar Sujiao No. 2. Conidial suspensions of Pst10 were prepared as described above. Five treatments consisting of infested and non-infested soil (500 g per pot) was transferred to ten pots per treatment and two seedlings with 2–4 leaves were transplanted to each pot. The treatments consisted of the following: (1) non-inoculated and inoculated soils; (2) infested soil mixed with 30 ml of SLCF of Pst10; (3) infested soil inoculated with conidial suspensions of Pst10; (4) infested soil mixed with 30 ml of SLCF and inoculated with conidial suspensions of Pst10; and (5) infested soil mixed with 30 ml of SLCF, inoculated with conidial suspensions of Pst10 and supplemented with 1% (m/m) CPM. A second application of SLCF was added 10 days after the first application. Pots were placed in a greenhouse with natural lighting for 14 days. The incidence of PRR was determined 7 and 14 days after transplanting and the percentage of plants with disease symptoms was calculated. This experiment was conducted twice.

3. Results

3.1. Antifungal properties of Pst10

In dual culture agar plate assays, Pst10 inhibited the growth of several species of Phytophthora, including *P. capsici*, *P. infestans*, *P. drechsleri*, *P. megasperma*, *P. nicotianae* and it also inhibited *C. cucumerium* and *S. sclerotiorum*. The average zone of inhibition was 30 mm. However, Pst10 had no inhibitory effect on *B. cinerea*, *R. solani*, *P. aphanidermatum*, *M. oryzae*, *F. graminearum*, *F. oxysporum* or *A. solani*, suggesting that the inhibitory compounds secreted by Pst10 have selective activity against some fungi since Pst10 did not inhibit the growth of *S. cerevisiae* but strongly constrained the growth of *C. albicans*.

This appears to be the first report that *P. striatisporum* can antagonize *Phytophthora* spp., *S. sclerotiorum* and *C. cucumerium*. Antibiotic activity was detected in liquid culture after 3–4 days of incubation using mycelial growth inhibition of *P. capsici* as a bioassay. Maximum concentration of antifungal substances was observed in liquid culture after 8–10 days of incubation. The inhibitory activity against *P. capsici* did not decrease on the PDA plates after 30 days.

3.2. Antagonism of Pst10 SLCF against P. capsici

3.2.1. Effect on mycelium

Table 1 shows that mycelium of *P. capsici* grew slowly on PDA plates containing 30–100 fold dilutions of SLCF. Furthermore, the mycelium appeared to be abnormal in comparison to controls in that it was unusually thicker in diameter, the hyphal tips were twisted and was more heavily branched (Fig. 1). The extent of deformity decreased as the filtrates were diluted. For example, the number of branches of hyphae grown on PDA plates containing 100-fold dilutions of SLCF was notably less than that of hyphae grown on PDA plates containing 50-fold dilutions of SLCF. In addition, the diameter of the deformed hyphae grown on PDA plates containing 50-fold dilutions of SLCF was thinner than that of the deformed hyphae grown on PDA plates containing 30-fold dilutions of SLCF. Mycelial disks of *P. capsici* treated with 20 μl of SLCF did not grow. Similar results were observed with mycelial disks placed on PDA plates containing 10-fold dilutions of SLCF. Removing disks of no-growth from plates containing SLCF and soaking in sterile water amended with 0.7% NaCl for 12 h and then transferring them to fresh PDA plates did not restore growth after 3 days of incubation suggesting that the mycelium was killed by the compounds produced by Pst10. Examination of the plugs with a light microscope revealed that the mycelium was intact but that the cytoplasm was highly vacuolated as shown in Fig. 1.

3.2.2. Effects of Pst10 on sporangium and spores of P. capsici

Sporangia were not produced or formed within 24 h when fresh mycelium disks of *P. capsici* were treated with 20 ml sterile water containing 1 ml of SLCF of Pst10. Over 120 sporangia were found in the control treatment. A 20-

Table 1
Toxicity of sterilized liquid culture filtrate (SLCF[a]) of Pst10 added to potato-dextrose agar (PDA)[b] medium to mycelium growth of *Phytophthora capsici* in *in vitro* assays[c]

Incubation time (h)	% Inhibition[d] with various dilution-folds of SLCF							
	0	10	30	50	100	200	300	400
24	100a	100a	100a	100a	100a	43b	29c	21c
48	100a	100a	100a	70b	57bc	30c	20c	17c
120	100a	100a	91a	69b	53bc	12c	4d	1e

[a] SLCF, sterilized liquid culture filtrate.
[b] The inoculated PDA plates containing different amounts of SLCF were incubated in the dark at 25–26 °C for 24, 48, and 120 h before measuring the colony diameter.
[c] Values were the means of five replicates and if followed by the same letter within each row did not differ significantly at $p = 0.05$. MINITAB procedure was used for statistical treatment.
[d] % Inhibition = (colony diameter of control − colony diameter of treatment)/(colony diameter of control) × 100%.

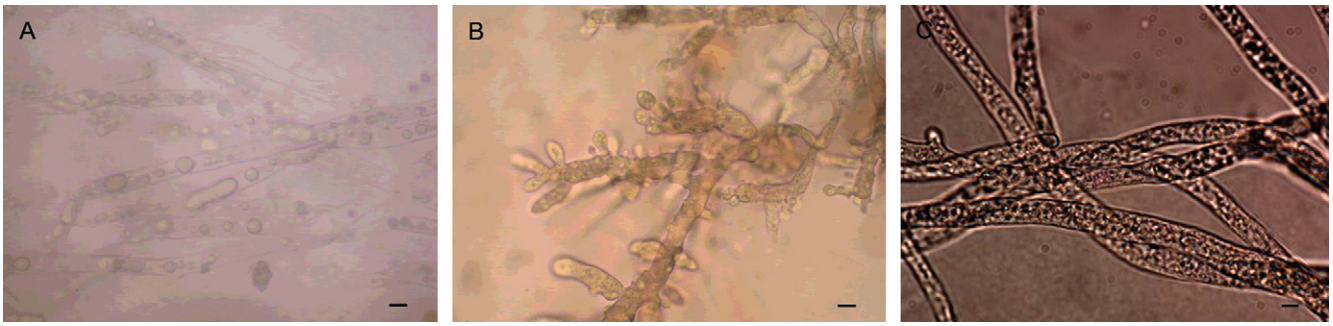

Fig. 1. Vacuolisation and distortion of *Phytophthora capsici* cells following treatment with Pst10 SLCF under light microscope. (A) Vacuolised cells after the treatment of mycelium disks in a 20-fold dilution of SLCF for 6–8 h, bar = 20 μm; (B) abnormal mycelium from a culture on PDA plate amended with 30-fold dilution of SLCF, bar = 20 μm; (C) normal mycelium from a culture on PDA plate, bar = 20 μm.

fold dilution of SLCF completely inhibited sporangium formation within 24 h. After 72 h, treated cultures had 8–10 sporangia whereas control cultures had more than 500 and many of the sporangia had released zoospores. Both sporangia and spores failed to germinate when incubated on PDA plates containing 20-fold dilution of SLCF of Pst10. In control plates, both sporangia and spores germinated and the mycelium fully covered the PDA plates.

3.3. Preliminary separation of antibiotics produced by Pst10

3.3.1. Extraction of LCF of Pst10 with organic solvents

Extraction of the liquid culture filtrates of Pst10 with various solvents showed that methanol, acetone, ethyl acetate, and chloroform extracts all had inhibitory activity, while the residue fractions of methanol or acetone and the aqueous fractions of ethyl acetate or chloroform did not have inhibitory activity (Table 2). On the contrary, the aqueous fraction, but not the *n*-hexane fraction, showed inhibitory activity. These data suggest that the antifungal substances which dissolved into the former four solvents but did not dissolve into *n*-hexane had a specific polarity. The organic extract fractions of methanol, ethyl acetate, and chloroform had similar inhibitory effects on the growth of *P. capsici* mycelium. Thus, the antifungal substances had no special requirements for extraction and ordinary solvents met the requirements, which is significant if antifungal substances are to be extracted in large scale and economical costs or operational flexibility must be taken into account.

3.3.2. Preliminary separation by thin-layer chromatography (TLC)

Fig. 2, shows that TLC of the methanol, acetone, ethyl acetate, and chloroform fractions resulted in four spots at the same positions on the plate. It is possible that each spot may contain one group of substance with the same or similar polarity. Bioassay results obtained from *in vitro* tests with *P. capsici* showed that the inhibitory activity of compounds recovered respectively from each spot of four in horizontal row B was the strongest with 100% inhibition of *P. capsici* mycelial growth, while compounds in spots in rows C and D exhibited only 40% and 50% inhibition, respectively. Compounds recovered from spots in row A did not have inhibitory activity against *P. capsici*. Additional studies on the purification of these active substances are under way at this time.

3.4. Effect of CPM on colonization of Pst10

CPM slightly increased colonization of the rhizosphere of chilli plants by Pst10. (Table 3) *P. striatisporum* colonies were not detected on agar medium containing acetamide from samples of potting soil or from CPM. However, the

Table 2
Toxicity of different fractions of liquid culture filtrate of Pst10 extracted with five organic solvents to *P. capsici* mycelia by bioassay on PDA added with concentrated organic or aqueous fractions[a]

Extraction solvents	Organic fraction[b]		Aqueous fraction[c]	
	Colony diameter (mm)	Inhibition (%)	Colony diameter (mm)	Inhibition (%)
Control	30 ± 0.8	—	30 ± 0.8	—
Methanol	17 ± 0.4	45 ± 1.5	31 ± 0.7	0 ± 0
Acetone	20 ± 0.6	34 ± 0.3	30 ± 0.2	0 ± 0
Ethyl acetate	17 ± 0.4	43 ± 0.9	30 ± 0.8	0 ± 0
Chloroform	18 ± 0.8	42 ± 1.4	31 ± 0.9	0 ± 0
n-Hexane	30 ± 0.7	0 ± 0	12 ± 0.5	60 ± 1.0

[a] The diameter growth of Pst10 was determined on PDA plate after incubation for 72 h in dark at 25–26 °C. Values were the means of five replicates and if followed by the same letter within each row did not differ significantly at $p = 0.05$. MINITAB statistical procedure was used for statistical treatment.
[b] Organic fraction or organic solvent extract of liquid culture filtrate of Pst10.
[c] Aqueous fraction or water phase extract of liquid culture filtrate of Pst10 (non-soluble residue in case of methanol and acetone).

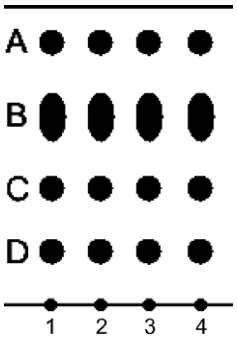

Fig. 2. Block diagram of thin layer chromatography (TLC) of GF_{254}. Preliminary separation of antifungal compounds extracted with four organic solvents from liquid culture of Pst10 grown in potato-dextrose broth by TLC using silica gel plate of GF_{254}.Vertical row 1, row 2, row 3 and row 4 indicate spots developed on TLC plate from samples from four organic fractions extracted with methanol, acetone, ethyl acetate and chloroform, respectively. These spots were observed under UV_{254}. Four spots in the same horizontal row with same R_f value belong to same substances; Four spots in the same vertical row with different R_f value belong to different substances. These spots were developed on GF254 plate owing to the polarity difference of each. Spots in horizontal rows A, B and C were bioactive and spots in horizontal row D were not bioactive. $R_{fA} = 0.85$; $R_{fB} = 0.64$; $R_{fC} = 0.35$ and $R_{fD} = 0.15$.

total number of Pst10 CFUs in the Pst10-CPM treatment was three times higher than that found in the Pst10 treatment alone after 10 days of planting and 17 times higher 20 days after planting. The proportion of Pst10 CFUs to the total number of fungi detected in the rhizosphere in the Pst10-CPM treatment was significantly higher than in the Pst10 treatment alone. At 40 days after planting, the number of Pst10 CFUs in the chilli rhizosphere in the Pst10-CPM treatment was 2.55×10^4 CFU/g dry soil; accounting for 53% of total number of fungi counted. Thus, Pst10 colonization of the root zone of chilli appears to be enhanced by organic fertilizers such as CPM.

3.5. Control of PRR of chilli by Pst10

The effect of Pst10 on the incidence of disease caused by *P. capsici* was measured in greenhouse experiments and the results are shown in Table 4. All of the plants in the non-infested soil remained healthy during these experiments. A total of 95% of the plants were infected within 7 days after planting in the infested soil. All of the plants were infected in the infested controls after 14 days. All the plants remained healthy for up to 7 days with the SLCF treatment and 80–92% of the plants were still healthy after 14 days. The percentage of healthy plants grown in soil infested with Pst10 conidia was 80% and 72% after 7 and 14 days after planting, respectively. The lowest incidence of PRR was in the treatment consisting of SLCF, conidial suspension and CPM. The impact of these treatments on the incidence of PRR needs to be investigated further to examine the duration of control of PRR to full maturity of chilli plants.

4. Discussion

An isolate of *P. striatisporum*, Pst10, was recovered from the rhizosphere of chilli peppers in Nanjing, China. This is the first report on the potential of this fungus as a biological control agent of soil-borne diseases caused by plant pathogens such as *Phytophthora* spp., *C. cucumerium*, and *Sclerotinia sclerotiorium*. The suppression of these pathogens including *P. capsici* may be due, at least partially, to the production of toxic metabolites by this biological control agent. Our results show that inhibition of *P. capsici* was through production of toxic metabolites by Pst10 and no mycoparasitism was observed. It appears that the inhibitory compounds secreted by Pst10 may have specific activity against several *Phytophthora* spp.

Multiple mechanisms have been implicated in disease suppression by some biological agents belonging to *Penicillium* spp. (De Cal et al., 1997a; Fang and Tsao, 1992; Nicoletti et al., 2004). *P. oxalicum* has been used effectively to control seedling blight of pea (Windels, 1981; Windels and Kommedahl, 1978, 1982) and tomato wilt (De Cal et al., 1997a,b, 1999). Mechanisms such as antagonism and induction of resistance by *P. oxalicum* may have been involved in disease suppression in these studies. *Penicillium funiculosum*, a biological control agent against Phytophthora root rots of azalea and citrus, has been shown to penetrate and degrade the cell walls of the pathogen and to produce antifungal substances (Fang and Tsao, 1989, 1992, 1995).

Our data shows that Pst10 produced antifungal chemicals in either liquid culture in PDB or solid fermentation on wheat bran. Production of antifungal substances continued to occur in liquid cultures for approximately 8–10 days when the inhibitory effects on mycelial growth of *P. capsici* reached a maximum. The reason that Pst10 had strong

Table 3
Effect of composted pig manure (CPM) on Pst10 colonization of the rhizosphere of chilli pepper[a]

Treatment	Pst10 colony[b](×10³ cfu/g dry soil)				Ratio of Pst10 to total fungi[c](%)			
	3d	10d	20d	40d	3d	10d	20d	40d
Pst10	0.54a	1.5ab	6.3b	7.6b	5.10a	10.40ab	33.60b	26.00b
Pst10 + CPM	0.86a	4.4b	107.0d	25.5c	10.50a	35.70b	76.00c	53.00bc

[a] Values were the means of three replicates and if followed by the same letter within each row did not differ significantly at $p = 0.05$. MINITAB procedure was used for statistical analysis.
[b] The population density of Pst10 was measured on selective medium containing acetamide at 2000 ppm as a sole nitrogen source.
[c] The number of total fungi was measured on Martin agar plates by standard soil dilution plating assay.

Table 4
Suppression of Phytophthora root rot (PRR) of chilli pepper caused by *Phytophthora capsici* with sterilized liquid culture filtrate (SLCF) and conidial suspension of Pst10 in pot tests[a]

Treatments	PRR incidence (%)	
	Day 7	Day 14
Control	95a	100a
SLCF[c]	0c	20bc
Conidia	20b	28b
SLCF[c] + Conidia	0c	15bc
SLCF[c] + Conidia + CPM[b]	0c	8c

[a] Means (average of two experiments) followed by same letter within each column do not differ significantly at $P = 0.05$.
[b] Composted pig manure (CPM) applied at 1% (mass/mass) rate.
[c] Thirty millimeters of SLCF applied before and 10 days after transplanting chilli peppers.

antifungal activity against *Phytophthora* spp., *C. cucumerium* and *S. sclerotiorium* but had no activity against the other plant pathogens tested is unknown and requires further investigation. Specific selectivity of antibiotic compounds may be a possible explanation. Several antifungal compounds have been purified from many *Penicillium* species. For example, mycophenolic acid, one of the oldest known fungitoxic compounds, was first characterized from *Penicillium brevicompactum* (Anderson, 1991), and later found in other *Penicillium* species (Lafont et al., 1979; Anderson et al., 1988; Pascual et al., 2000). Also production of patulin is widespread in the genus *Penicillium* (Pascual et al., 2000; Frisvad and Filtenborg, 1989). 3-*O*-Methylfunicone is another characterized metabolite with potent cytostatic properties (De Stefano et al., 1999; Stammati et al., 2002). *Penicillium verrucosum* is reported to produce a number of potent mycotoxins including ochratoxin A, verrucolon, citrinin, and the verrucins (Frisvad and Filtenborg, 1989; Larsen et al., 1999). Nicoletti et al. (2004) purified a compound, Sch 642305, from *Penicillium canescens* which completely inhibited the mycelial growth of *R. solani* and several other plant pathogenic fungi *in vitro*.

Michael et al. (2005) obtained seven chemicals from an Australian isolate of *P. striatisporum* that exhibited selective antifungal activity against *C. albicans* but not *S. cerevisiae*, in which calbistrin E, a member of a rare class of fungal metabolites, striatisporin A and striatisporolide A were new triene and butenolide acids. Similarly, the SLCF of Pst10 showed selective antifungal activity against *C. albicans* but not against *S. cerevisiae*. We found three active compounds through preliminary separation by TLC. Subsequently, we obtained four different fractions by HPLC in which each one had antifungal activity, separately. Two of the four fractions were purified and relevant studies on structure analysis and identification are in progress.

The ability of introduced antagonistic fungi to establish and proliferate in the soil is an important factor in successful biological control (Lewis and Papavizas, 1984; Fang and Tsao, 1995). The populations of Pst10 increased in the rhizosphere of chilli peppers when this biological control agent was co-applied with an organic fertilizer, CPM. This increase in populations of Pst10 may be as a result of the availability of organic nutrients at the initiation of colonization of the chilli rhizosphere by Pst10. Similarly, utilization of wheat bran as a food base has been reported by other workers (Fang and Tsao, 1995; Sivan et al., 1984; Smith et al., 1990). CPM may have favored colonization, enhanced and stabilized the performance of Pst10 in chilli rhizosphere and reduced the disease severity of Phytophthora root rot.

Additional studies on effects of Pst10 and its metabolites on the microorganism community in plant rhizosphere are planned. An evaluation of environmental safety also needs further investigation.

Acknowledgments

We are deeply grateful to Lazarovits George and Abbasi Pervaiz, Southern Crop Protection and Food Research Centre, Agriculture and Agriculture-Food Canada, for their warmhearted assistance and supervision in the preparation of the manuscript. The paper was supported by the projects numbered as 2006AA10A211, nyhyzx07-054 and Jiangsu Technology Support program numbered as BE2006304.

References

Anderson, B., 1991. Consistent production of phenolic compounds by *Penicillium brevicompactum* for chemotaxonomic characterization. Antonic van Leeuwenhock 60, 115–123.

Anderson, H.A., Bracewell, J.M., Fraser, A.R., Jones, D., Robertson, G.W., Russell, J.D., 1988. 5-Hydroxymaltol and mycophenolic acid, secondary metabolites from *Penicillium echinulatum*. Transactions of the British Mycological Society 91, 649–651.

Dai, X.-Y., Guan, G.-L., 1999. Study on two strains of antagonistic bacteria producing antagonistic proteins to *Phytophthora capsici*. Chinese Journal of Biological Control 15, 81–84.

De Cal, A., Sagasta, E.M., Melgarejo, P., 1988. Antifungal substance produced by *Penicillium frequents* and their relationship to the biocontrol of *Monilinia laxa*. Phytopathology 78, 888–893.

De Cal, A., Sagasta, E.M., Melgarejo, P., 1990. Biological control of peach twig blight (*Monilinia laxa*) with *Penicillium frequentans*. Plant Pathology 39, 612–618.

De Cal, A., Pascual, S., Melgarejo, P., 1997a. Involvement of resistance induction by *Penicillium oxalicum* in the biocontrol of tomato wilt. Plant Pathology 46, 72–79.

De Cal, A., Pascual, S., Melgarejo, P., 1997b. Rapid laboratory method for assessing the biological control potential of *Penicillium oxalicum* against Fusarium wilt of tomato. Plant Pathology 46, 699–707.

De Cal, A., Garcia-Lepe, R., Pascual, S., Melgarejo, P., 1999. Effects of timing and method of application of *Penicillium oxalicum* on efficacy and duration of control of Fusarium wilt of tomato. Plant Pathology 48, 260–266.

De Stefano, S., Nicoletti, R., Milone, A., Zambardino, S., 1999. 3-*O*-Methylfunicone, a fungitoxic metabolite produced by the fungus *Penicillium pinophilum*. Phytochemistry 52, 1399–1401.

Ezziyyani, M., Requena, M.E., Egea-Gilabert, C., Candela, M.E., 2007. Biological control of Phytophthora root rot of pepper using *Trichoderma harzianum* and *Streptomyces rochei* in combination. Journal of Phytopathology 155, 342–349.

Fang, J.G., Tsao, P.H., 1989. *Penicillium funiculosum* as a biological control agent for Phytophthora root rot of azalea. Phytopathology (Abstract) 79, 1159.

Fang, J.G., Tsao, P.H., 1992. Mechanisms of biological control of Phytophthora root rot by *Penicillium funiculosum*. Phytopathology (Abstract) 82, 1119.

Fang, J.G., Tsao, P.H., 1995. Efficacy of *Penicillium funiculosum* as a biological agent against Phytophthora root rots of azalea and citrus. Phytopathology 85, 871–878.

Frisvad, J.C., Filtenborg, O., 1989. *Terverticillate penicillia*: chemotaxonomy and mycotoxin production. Mycologia 81, 837–861.

Harrison, Y.A., Stewart, A., 1988. Selection of fungal antagonists for biological control of onion white rots in New Zealand. New Zealand Journal of Experimental Agriculture 16, 249–256.

Hee, J., Jung, Y.L., In, S.H., Bong, S.Y., Beom, S.K., Byung, K.H., 2006. Isolation and antifungal and antioomycete activities of staurosporine from *Streptomyces roseoflavus* strain LS-A24. Journal of Agricultural Food and Chemistry 54, 3041–3046.

Jung, H.-K., Kim, S.-D., 2003. Purification and characteriztion of an antifungal antibiotic from *Bacillus megaterium* KL 39, a biocontrol agent of red-papper Phytophtora blight disease. Korean Journal of Microbiology and Biotechnology 31, 235–241.

Jung, H.-K., Kim, S.-D., 2004. Selection and antagonistic mechanism of *Pseudomonas fluorescens* 4059 against phytophthora blight disease. Korean Journal of Microbiology and Biotechnology 32, 312–316.

Jung, H.K., Kim, S.-D., 2005. An antifungal antibiotic purified from *Bacillus megaterium* KL39, a biocontrol agent of red-pepper Phytophthora-blight disease. Journal of Microbiology and Biotechnology 15, 1001–1010.

Kelly, J.M., Hynes, M.J., 1985. Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO Journal 4, 475–479.

Kharbanda, P.D., Dahiya, J.S., 1990. A metabolite of *Penicillium verrucosum* inhibitory to growth of *Leptosphaeria maculans* and *Rhizoctonia solani*. Canadian Journal of Plant Pathology 12, 335.

Kim, H.Y., Choi, G.J., Lee, H.B., Lee, S.W., Lim, H.K., Jang, K.S., Son, S.W., Lee, S.O., Cho, K.Y., Sung, N.D., Kim, J.-C., 2007. Some fungal endophytes from vegetable crops and their anti-oomycete activities against tomato late blight. Letters in Applied Microbiology 44, 332–337.

Lafont, P., Debeaupuis, J.P., Gaillardin, M., Payen, J., 1979. Production of mycophenolic acid by *Penicillium roqueforti* strains. Applied and Environmental Microbiology 37, 365–368.

Larena, I., Melgarejo, P., De Cal, A., 2002. Production, survival, and evaluation of solid-substrate inoculate of *Penicillium oxalicum*, a biocontrol agent against Fusarium wilt of tomato. Phytopathology 92, 863–869.

Larsen, T.O., Franzyk, H., Jensen, S.R., 1999. UV-gudied isolation of verrucins A and B, novel quinazolines from *Penicillium verrucosum* structurally related to anacine from *Penicillium aurantiogriseum*. Journal of Natural Products 62, 1578–1580.

Lee, E.T., Lim, S.K., Nam, D.H., Khang, Y.H., Kim, S.D., 2003a. Pyoverdin 2112 of *Pseudomonas fluorescens* 2112 inhibits *Phytophthora capsici*, a red-pepper blight-causing fungus. Journal of Microbiology and Biotechnology 13, 415–421.

Lee, J.Y., Hwang, B.K., 2002. Diversity of antifungal actinomycetes in various vegetative soils of Korea. Canadian Journal of Microbiology 48, 407–417.

Lee, J.Y., Moon, S.S., Hwang, B.K., 2003b. Isolation and antifungal and antiomycete activities of aerugine produced by *Pseudomonas fluorescens* strain MM-B16. Applied and Environmental Microbiology 69, 2023–2031.

Lee, J.Y., Moon, S.S., Hwang, B.K., 2003c. Isolation and *in vitro* and *in vivo* activity against *Phytophthora capsici* and *Volletoteichum orbiculare* of phenazine-1-carboxylic acid from *Pseudomonas aeruginosa* strain GC-B26. Pest Management Science 59, 872–882.

Lewis, J.A., Papavizas, G.C., 1984. A new approach to stimulate population proliferation of *Trichoderma* species and other potential biocontrol fungi introduced into natural soils. Phytopathology 74, 1240–1244.

Liu, R., Lu, Z.-J., 2003. Inhibition of *Trichoderma harzianum* T2 against the soil-borne fungal diseases of capsicum. Journal of Zhongkai Agrotechnical College 16, 6–11.

Lin, X.-C., Niu, Z.-G., Dong, M.-Z., 2003. Evaluation on association effects of biological bacteria and matalaxyl on chilli pepper blight. Shandong Agricultural Science, 35–36.

Martin, J.P., 1950. Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. Soil Science 69, 215–232.

Melouk, H.A., Akem, C.N., 1987. Inhibition of growth of *Sclerotsinia minor* and other pathogens by citrinin in the filtrate of *Penicillium citrinum*. Mycopathologia 100, 91–96.

Michael, S., Robert, J.C., Ernest, L., Shaun, T., Jennifer, H.G., 2005. Calbistrin E and two other new metabolites from an Australian isolate of *Penicillium striatisporum*. Journal of Natural Products 68, 581–584.

Morino, T., Nakamura, K., Nishikawa, K., Shimada, K., Nakagawa, T., Saito, S., 1994. Application: JP patent, JP6172374.

Nicoletti, R., Stefano, M.D., Stefano, S.D., Trincone, A., Marziano, F., 2004. Antagonism against *Rhizoctonia solani* and fungitoxic metabolite production by some *Penicillium* isolates. Mycopathologia 158, 465–474.

Paul, D., Sarma, Y., 2006. Antagonistic effects of metabolites of *Pseudomonas fluorescens* strains on the different growth phases of *Phytophthora capsici*, foot rot pathogen of black pepper (*Piper nigrum*L.). Archives of Phytopathology and Plant Protection 39, 311–314.

Pascual, S., Melgareji, P., Magan, N., 2000. Accumulation of compatible solutes in *Penicillium frequentans* grown at reduced water activity and biocontrol of *Monilinia laxa*. Biocontrol Science and Technology 10, 71–80.

Proksa, B., Adamcova, J., Fuska, J., 1992. 2-Methylsorbic acid, an antifungal metabolite of *P. vermiculatum*. Applied Microbiology and Biotechnology 37, 443–445.

Qiu, S.-X., He, H., Ruan, H.-C., Guan, X., Hu, F.-P., 2004. Biological control of pepper Phytophthora blight by endophytic TB2 (*Bacillus* sp.). Acta Phytopathologica Sinica 34, 173–179.

Sabuquillo, P., De Cal, A., Melgarejo, P., 2006. Biocontrol of tomato wilt by *Penicillium oxalicum* formulations in different crop conditions. Biological Control 37, 256–265.

Sivan, A., Elad, Y., Chet, I., 1984. Biological control effects of a new isolate of *Trichoderma harzianum* on *Pythium aphaniedermatum*. Phytopathology 74, 498–501.

Smith, V.L., Wilcox, W.F., Harman, G.E., 1990. Potential for biological control of Phytophthora root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. Phytopathology 80, 880–885.

Stammati, A., Nicoletti, R., Stefano, D.S., Zampaglioni, F., Zucco, F., 2002. Cytostatic properties of a novel compound derived from *Penicillium pinophilumr*: an *in vitro* study. Alternatives to Laboratory Animals 30, 1–7.

Stolk, A.C., 1969. Four new species of *Penicillium*. Antonie van Leeuwenhoek 35, 261–274.

Sztejnberg, A., Ohr, H.D., Tsao, P.H., 1988. Screening *Aspergillus* and *Penicillium* spp. for potential biocontrol of *Phytophthora gummosis* of citrus. Phytopathology (Abstract) 78, 1557.

Windels, C.E., 1981. Growth of *Penicillium oxalicum* as a biological seed treatment on pea seed in soil. Disease Control and Pest Management 71, 929–933.

Windels, C.E., Kommedahl, T., 1978. Factors affecting *Penicillium oxalicum* as a seed protectant against seedling blight of pea. Disease Control and Pest Management 68, 1656–1661.

Windels, C.E., Kommedahl, T., 1982. Pea cultivar effect on seed treatment with *Penicillium oxalicum* in the field. Ecological Epidemiology 72, 541–543.

Zhu, Z.-Y., Zhou, X.-G., Song, R.-H., Lu, J.-P., Wang, S.-J., 1995. Capsicum Phytophthora Blight with Biological-"fangyi—I". Acta Agriculture Shanghai 11, 64–68.

Zuo, X.-X., 2002. Chinese Chilli. Chinese Agricultural Publishing House, Beijing, p. 17.