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Inhibition and control effects of the ethyl acetate extract of *Trichoderma harzianum* fermented broth against *Botrytis cinerea*

Guoliang Yin[1], Weimin Wang[1], Sha Sha[1], Lei Liu[2] and Xiaoping Yu[1]*

[1]College of Life Sciences, China Jiliang University, Hangzhou 310018, China.
[2]Centre for Phytochemistry and Pharmacology, Southern Cross University, New South Wales 2480, Australia.

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Botrytis cinerea **is a widespread parasitic fungus that infects many crops and reduces their productivity.** ***Trichoderma harzianum*****, also a fungus, has been commercially used as a biofungicide to control B. cinerea. The spores of** ***T. harzianum*****, currently used in the application, have some drawbacks such as efficacy affected by the environmental condition and sensitivity to chemical fungicides. Instead of using the living microbe, the extract from** ***T. harzianum*** **fermented broth may be applied to control** ***B. cinerea*****. It was found that the extract could inhibit mycelial growth (EC_{50} = 13.6 mg/L), conidia germination (EC_{50} = 17.5 mg/L) and conidia production (EC_{50} = 23.6 mg/L) of** ***B. cinerea*****.** ***In vitro*****, the extract showed excellent control effects on tomato grey mould caused by B. cinerea and the protective effect (EC_{50}=99.6 mg/L) was better than the therapeutic effect (EC_{50}=135.8 mg/L). In pot experiments, the extract also showed persistent protective (EC_{50}=99.0 mg/L at day 7 and EC_{50} = 142.7 mg/L at day 15) and therapeutic (EC_{50} = 195.0 mg/L at day 7 and EC_{50} = 393.7 mg/L at day 15) effects over 15 days. This study showed that the extract was equivalent or superior to the commercial dicarboximide fungicide, procymidone. In conclusion, the ethyl acetate extract of** ***T. harzianum*** **fermented broth is effective in the treatment of tomato grey mould caused by** ***B. cinerea*****.**

Key words: *Botrytis cinerea*, fungicide, tomato grey mould, trichodermin, *Trichoderma harzianum.*

INTRODUCTION

Botrytis cinerea Pers. a prevalent phytopathogenic fungus produces grey mould disease in many crops such as tomato and cucumber and causes severe economic damage (Zhang et al., 2009) . Conventional dicarboximide fungicides such as iprodione and procymidone have been used to control *B. cinerea*. However the frequent use of these synthetic chemicals has led to fungicide resistance (Wang et al., 1986; Steel, 1996). The residues from conventional chemical fungicides have contaminated the environment and are hazard to public health (Muller et al., 2009; Chen et al., 2010). More effective and safer methods for managing *B. cinerea* are required.

*Corresponding author. E-mail: yxp@cjlu.edu.cn. Tel: +86-571-86836006. Fax: +86-571-8691440.

Biofungicide is an environmentally friendly alternative to chemical fungicide for controlling crop diseases. *Trichoderma harzianum*, a common fungus found in soil, is active against several phytopathogenic fungi including *B. cinerea* (Hjeljord et al., 2007). Some strains of *T. harzianum* have become commercially available as biological control agents for a number of crops such as strawberry, tomato, lettuce and outdoor ornamental plants. The spores of *T. harzianum* are currently applied to crops as the control and their efficacy is largely affected by weather conditions (Shtienberg and Elad, 1997), soil environment (Bae and Knudsen, 2005) and other chemical fungicides used on the corps (Lóránt et al., 2006).

To improve the application of *T. harzianum,* its bio-control mechanisms have been studied, which included antibiosis, mycoparasitism, competition for nutrients and space and induction of resistance in the plant (Ghisalberti

and Rowland, 1993; Schirmbock et al., 1994; Cruz et al., 1995; Harman, 2000; Benitez et al., 2004). It was found that *Trichoderma* species produce over 40 different secondary metabolites that may contribute to their mycoparasitic and antibiotic action (Sivasithamparam and Ghisalberti, 1998).

In our previous research (Shi et al., 2009), trichodermin, a secondary metabolite, was isolated from the fermented broth of *T. harzianum.* This compound showed antifungal activity against mycelial growth of 9 species of phyto-pathogenic fungi including *B. cinerea.* Since *T. harzianum* can naturally produce anti- microbial secondary meta-bolites (Verma et al., 2007) such as trichodermin, it is possible to use the extracted chemicals from *T. harzianum* fermented broth instead of its spores to control *B. cinerea*. This could be a solution to avoid the disadvantages of the application of living microbe and still keep the benefits of natural biofungicides to the environment. The aim of this research is to systematically investigate the capacity of the ethyl acetate extract of *T. harzianum* fermented broth for the control of tomato grey mould caused by *B. cinerea*.

MATERIALS AND METHODS

Strains of fungi

B. cinerea was obtained from the Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences. *T. harzianum* was isolated from *Llex cornuta*, which was collected from Tianmushan in Zhejiang province, China, through our previous research (Shi et al., 2009).

Instruments and reagents

Agilent 6890 N gas chromatograph system with a flame ionization detector (GC-FID) (Agilent Technologies, Inc.); secondary fermented system with a seeding tank (7 L) and fermentor (35 L) (Shanghai Lianhuan Bioengineering Equipments Co. Ltd.); trichodermin standard substance (Zhejiang Academy of Agricultural Sciences); 50% procymidone wettable powder (Sumitomo Chemical Co. Ltd.).

Preparation of *T. harzianum* and *B. cinerea*

T. harzianum was cultured on potato dextrose agar (PDA, potato 200 g/L, dextrose 20 g/L and agar 15 g/L) for 5 days. A 5 mm diameter agar plug was taken from the culture and inoculated into a conical flask (250 mL) with 50 mL of potato dextrose (PD). This solution was incubated at 28°C in a shaker (180 r/min) for 72 h. After incubation, the culture was transferred to the seeding tank of the secondary fermented system. The seeding tank contained a selected medium including potatoes 200 g/L, sugar 1 g /L, dextrose 10 g/L, sodium acetate 1.66 g/L, $MgSO_4 \cdot 7H_2O$ 5.29 g/L, peptone 0.5 g/L, manganese chloride 0.15 g/L, antifoam agent 0.05 g/L. In the seeding tank, the culture was incubated at 28°C, 180 r/min for 2 - 3 days. To produce sufficient fermented broth, the primary culture (500
- 1000 mL) was transferred from the seeding tank to the fermentor (35 L) of the secondary fermented system and incubated under the same conditions as above for 6d. *B. cinerea* was sub- cultured on PDA for 6d at 25°C.

Extraction of *T. harzianum* broth

To extract the secondary metabolites from *T .harzianum* fermented broth, the filtered liquid culture (4 L) was mixed with ethyl acetate (1 L) and shaken vigorously for 5 min. After settling for 1 h, the upper organic phase was removed. The above extraction procedure was repeated 5 times and the obtained organic extracts combined. This organic solution was concentrated to dryness using rotary evaporator.

Determination of trichodermin

Trichodermin in the concentrated extract was identified and quantified using GC-FID analysis. An Agilent HP-5 column was used and oven temperature was held at 100°C for 5 min following injection and programmed to 260°C at 10°C /min and held for 5 min. The temperature of injection port and detector was set at 250 and 270°C, respectively. The sample was injected in splitless mode. Nitrogen gas was used as the carrier, at a flow rate of 1.5 mL/min. Trichodermin reference solution was prepared by dissolving 1 mg of the standard in 5 mL of methanol. Trichodermin in the extract was identified by comparing the retention time with the reference standard and quantified using an external standard method.

Inhibition of mycelial growth

The suppression of fermented broth extract (FBE) against *B. cinerea* was measured using a growth rate method. Procymidone wettable powder dissolving with acetone was used as a positive control. Ethyl acetate and water were used as solvent controls (negative). A serial dilution of FBE and positive was mixed with the molten PDA in conical flasks. The PDA was autoclaved and then poured into Petri dishes. Once the agar had solidified, a 4 mm diameter agar plug with a 6-day-old *B. cinerea* culture was planted in the centre of each Petri dish. The plates were placed in a 25°C incubator. Mycelial growth was measured on two diametric lines when the diameter of undiluted FBE extract grew to 7 – 8 cm. Each experiment was carried out in triplicate. The inhibitory effect was calculated using following Equation:

$$IE(\%) = \frac{DC - DS}{DC - 4} \times 100\%$$

Where IE = inhibitory effect (%), DC = diameter of negative control (mm), DS = diameter of sample or positive control (mm).

Influence of conidia production

After measuring diameters, the plates were incubated for 15 days after planting the pathogen plug. Each dish was drenched with 10 mL sterile distilled water and conidia were carefully freed from the culture surface. The number of conidia was determined by hemocytometer count.

Assessment of conidia germination

The conidia was suspended and diluted in 2% dextrose solution to 3 x 105 /mL. This solution (15 μL/well) was added to 96-well cell culture plate and mixed with a serial dilution of FBE (15 μL/well). The plate was incubated at 25°C in the dark for 5 h. Spores were

considered tobe germinated when the length of germ tube was equal to or more than that of the spore itself (Vicedo et al., 2006). The germination rate and germination inhibitory effect were calculated using the following equations:

$$GCR(\%) = \frac{GC}{TC} \times 100\%$$

$$IGC(\%) = \frac{CGCR - SGCR}{CGCR} \times 100\%$$

Where GCR=germination rate (%), GC=number of germinated conidia, TC= number of total conidia, IGC=germination inhibitory effect (%), SGCR = germination rate of the samples (%), CGCR = germination rate of the control (%).

The experiment was carried out in triplicate. Procymidone was used as positive control. Ethyl acetate and water were used as solvent controls (negative).The experiment was carried out in triplicate.

Suppression of *B. cinerea* on tomato *in vitro*

Healthy, fresh and uniform-sized tomatoes were selected in this trial. The surface of the tomatoes was initially wiped with 75% ethanol and dried. For the protective effect, the tomato surface was wiped with different concentrations of FBE first and incubated at 25°C for 24 h. After the incubation each tomato was pricked four times by a sterile needle and injected with spores of *B. cinerea* (1×107 /ml). For the therapeutic effect, FBE was wiped 24 h after the injection of spores. The suppression effect was evaluated by measuring the diameters of disease spots on tested tomato after incubating for 5 days. The inhibitory effect was calculated using following equation:

$$IE(\%) = \frac{DC - DS}{DC} \times 100\ \%$$

Where IE=inhibitory effect (%), DC=diameter of negative control (mm), DS=diameter of sample or positive control (mm). Procymidone was used as positive control. Ethyl acetate and water were used as solvent controls (negative).The experiment was carried out in triplicate.

Pot experiment

Tomato seedlings with 5 - 7 leaves and same growing conditions were used. For the protective effect, different concentrations of FBE were sprayed on the tomato seedlings. After the leaves dried, *B. cinerea* spores (4 - 5 × 106 /mL) in 2% sucrose solution with Tween 80 were sprayed on the tomato seedlings for 3 times with 24 h interval and kept wet for 24 h after the inoculation. For the therapeutic effect, the *B. cinerea* spores were sprayed on the seedlings and leaves were kept wet. Then different concentrations of FBE were sprayed on the tomato seedlings at 24 and 48 h after the inoculation. Each experiment consisted of 10 pots of plants and each pot has one seedling. Procymidone was used as positive control. Plants only sprayed with spores of *B. cinerea* were used as negative control. Each experiment was carried out in triplicate. At day 7, 10 and 15 after the inoculation, the tomato grey mould disease was graded according to the following criteria:

0 grade: no disease spot on leaf surface,
1 grade: area of disease spot is less than 5% of total leaf surface,
3 grade: area of disease spot is 6 - 10% of total leaf surface,
5 grade: area of disease spot is 11- 20% of total leaf surface,
7 grade: area of disease spot is 21- 40% of total leaf surface,
9 grade: area of disease spot is over 40% of total leaf surface.
The disease index, protective and therapeutic effects were then calculated according to the following equations:

$$DI = \frac{(ND_i \times DG_i)}{NS \times HG} \times 100$$

$$PPE(\%) = \frac{DIC - DIS}{DIC} \times 100\%$$

$$PTE(\%) = (1 - \frac{DICB \times DIS}{DIC \times DISB}) \times 100\%$$

Where DI = disease index, NDi = number of plants having disease at i grade, DGi = i or the grade of disease, NS = number of plants studied, HG = 9 (the highest grade of disease), PPE (%) = protective effect for the pot experiment, DIC = disease index after treated with negative control, DIS = disease index after treated with sample, PTE(%) = therapeutic effect for the pot experiment, DICB = disease index before treated with negative control, DISB = disease index before treated with sample.

Statistical analysis

The data significance test was performed at 5% level (Duncan's multiple range test) using SPSS.

RESULTS

Extract and trichodermin

The dry mass of one litre filtered *T. harzianum* fermented broth was 17.54 g, of which the dried ethyl acetate extract weighed 486.5 mg. According to GC-FID analysis, the concentration of trichodermin was 6.0 mg per litre of filter broth and 12.2 mg per gram of the dried ethyl acetate extract.

Inhibition of mycelial growth

The ethyl acetate extract of *T. harzianum* fermented broth (FBE) showed more inhibitory effect against the mycelial growth of B. cinerea at 129.0 and 64.9 mg/mL than the positive control, procymidone, at 250.0 mg/L (Table 1). Good dose responses were observed for the inhibitory effects of FBE. The EC50 of the inhibition of FBE against mycelial growth of *B. cinerea* was 13.6 mg/L and the EC90 was 72.0 mg/L.

Table 1. Inhibition of ethyl acetate extract of *T. harzianum* fermented broth (FBE) on mycelial growth of *B. cinerea.*

Sample		Diameter (cm)	Inhibition ratio*(%)
Water		8.2 ± 0.1	-
Ethyl acetate		7.9 ± 0.1	4.5 ± 1.7a
Procymidone (250 mg/L)		1.6 ± 0.1	85.2 ± 1.0e
FBE (mg/L)	129.7	0.7 ± 0.1	96.2 ± 1.2f
	64.9	1.5 ± 0.0	86.1 ± 0.6e
	32.4	2.3 ± 0.1	76.4 ± 0.6d
	16.2	3.3 ± 0.1	63.6 ± 1.3c
	8.1	6.0 ± 0.4	29.1 ± 5.6b

*Values with different letters in the same column are significantly different (P<0.05) according to Duncan's multiple range test.

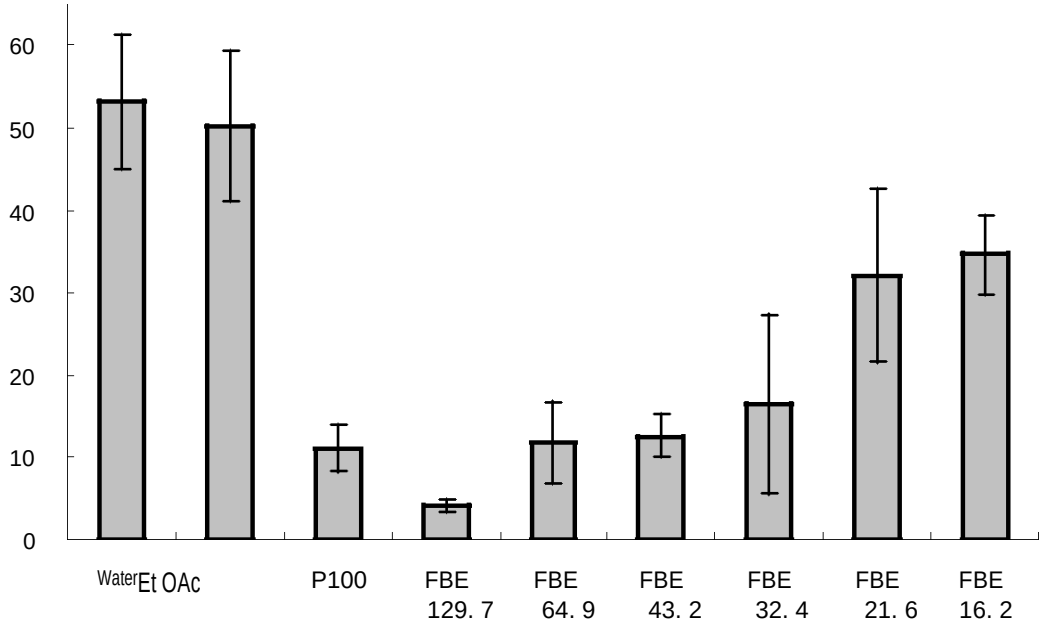

Figure 1. Influence of ethyl acetate extract of T. harzianum fermented broth (FBE) on conidia production of Botrytis cinerea (EtOAc represents ethyl acetate solvent control; P100 represents 100 mg/L procymidone).

Inhibition of conidia production

Compared to procymidone (79.0% at 100.0 mg/L), the FBE showed similar level of inhibitory effect (92.3% at 129.7 mg/L and 77.6% at 64.9 mg/L) on the conidia production of B. cinerea (Figure 1). The EC50 for the FBE was 23.6 mg/L.

Inhibition of conidia germination

The FBE showed significantly higher inhibitory effect (100.0% at 48.7 mg/L) against the germination of *B. cinerea* conidia than the positive control, procymidone (77.4% at 500 mg/L, Table 2). Good dose responses were observed for the inhibitory effects of FBE.

Inhibition of *B. cinerea* on tomato *in vitro*

In the *in vitro* experiments, the FBE showed higher efficacy than procymidone (Table 3). The FBE showed a similar level of protective effect (69.9 and 51.7%) at 243.3 and 121.6 mg/L to procymidone (60.6%) at 500.0 mg/L.

Table 2. Inhibition of ethyl acetate extract of *T. harzianum* fermented broth (FBE) on conidia germination of *B. cinerea.*

Sample		Germination percentage	Inhibition ratio*(%)
Water		90.6 ± 1.6	-
Ethyl acetate		89.4 ± 0.9	1.4 ± 1.0a
Procymidone (500 mg/L)		20.5 ± 2.4	77.4 ± 2.7d
FBE (mg/L)	48.7	0.0 ± 0.0	100.0 ± 0.0g
	32.4	6.3 ± 1.2	93.1 ± 1.3f
	24.3	13.0 ± 1.1	85.7 ± 1.2e
	19.5	25.0 ± 1.5	72.4 ± 1.6d
	16.2	49.1 ± 1.7	45.8 ± 1.9c
	13.9	77.2 ± 1.5	14.9 ± 1.6b

*values with different letters in the same column are significantly different (P<0.05) according to Duncan's multiple range test.

For the therapeutic effects, the FBE showed a similar inhibitory effect (66.4%) at 243.3 mg/L to procymidone (67.8%) at 500 mg/L. At each concentration tested, the FBE showed better protective effect than the therapeutic effect. The EC50 of the FBE protective and therapeutic effects were 99.6 and 135.8 mg/L.

The pot experiment

In the pot experiment, the protective and therapeutic effects of the FBE against *B. cinerea* were similar or higher than procymidone at day 7, 10 and 15 (Tables 4 and 5). The protective effect of FBE was more persistent than procymidone over the 15 day period. The therapeutic effects of FBE and procymidone decreased from day 7 to 15.

DISCUSSION

In the present study, an ethyl acetate extract of *T. harzianum* fermented broth was evaluated for treating tomato grey mould caused by *B. cinerea.* The extract showed similar or higher inhibitory effects against mycelium growth, conidia production and conidia germination of *B. cinerea* compared to procymidone. Procymidone exhibited inhibition 77.4% at 500 mg/L against the conidia germination of *B. cinerea,* whereas the extract showed similar inhibition 72.4% but at 19.5 mg/L, one twenty-fifth of the concentration. This significantly high inhibitory efficiency is probably due to the trichodermin in the extract acting as an inhibitor of eukaryotic protein synthesis (Carter et al., 1976), as this process is vigorous during the stage of conidia germination.

The ethyl acetate extract had a lower inhibitory efficacy (EC50 = 13.6 mg/L) compared to that demonstrated by the pure compound trichodermin (EC50 = 3 mg/mL) in our previous study (Shi et al., 2009) against the mycelium growth of *B. cinerea*. At 50.0% inhibition, the concen-tration of trichodermin in the extract was 0.17 mg/mL which is approximately only 5.7% of the trichodermin required for the same activity with the pure compound. This suggests that other compound(s) in the extract either have direct activity or can enhance trichodermin's inhibi-tory effect against the mycelium growth of *B. cinerea*. Various fungicidal compounds such as nonanoic acid (Aneja et al., 2005), 6-pentyl- -pyrone (Cutler et al., 1986), 3-(2-hydroxypropyl)-4-(2-hexadienyl)-2(5H)- furanone (Ordentlich et al., 1992), and some peptaibols (Schirmbock et al., 1994) have been identified from *T. harzianum*.

The mechanisms of these compounds inhi- biting phytopathogenic fungi are different. For example, nonanoic acid is very inhibitory to spore germination and mycelial growth (Aneja et al., 2005), whereas peptaibols inhibited spore germination as well as hyphal elongation (Schirmbock et al., 1994). As the ethyl acetate extract of *T. harzianum* fermented broth contains many fungicidal compounds; these compounds could form an antifungal synergistic interaction that enhances the antifungal activity of the extract. Compared to a single or simple mixture of synthetic chemical fungicides, it is harder for *B. cinerea* to develop resistance to the extract as it contains a number of antifungal compounds with varied modes of action.

Our research showed that the ethyl acetate extract of *T. harzianum* fermented broth had better protective effects than therapeutic effects on both tomato fruits and seedlings. The higher protective effects may be because some compounds in the extract induce the plant defence response before the inoculation of *B. cinerea.* It has been reported that metabolites from *Trichoderma* spp. can

Table 3. Inhibition of ethyl acetate extract of *T. harzianum* fermented broth (FBE) against *B. cinerea* on tomato *in vitro.*

Sample		Protective effect* (%)		Therapeutic effect* (%)	
		Diameter of disease spot (mm), Day 5	Efficacy (%)	Diameter of disease spot (mm), Day 5	Efficacy (%)
Water		6.7 ± 0.7	-	6.5 ± 0.1	-
Ethyl acetate		6.6 ± 0.4	0.9 ± 6.7a	6.4 ± 0.2	0.8 ± 3.7a
Procymidone (500 mg/L)		2.6 ± 0.1	60.6 ± 2.0c	2.1 ± 0.2	67.8 ± 2.9c
FBE (mg/L)	973.0	0.3 ± 0.1	96.0 ± 0.9e	0.6 ± 0.1	91.0 ± 1.6d
	486.5	0.8 ± 0.1	88.0 ± 0.9e	1.3 ± 0.1	80.7 ± 1.3cd
	243.3	2.0 ± 0.1	69.9 ± 0.8d	2.2 ± 0. 2	66.4 ± 3.4c
	121.6	3.2 ± 0.2	51.7 ± 3.3c	3.8 ± 0.8	42.0 ± 12.4b
	60.8	3.9 ± 0.1	41.2 ± 1.3b	4.4 ± 0.1	32.2 ± 2.2b

*values with different letters in the same column are significantly different (P < 0.05) according to Duncan's multiple range test.

Table 4. Protective effect of ethyl acetate extract of *T. harzianum* fermented broth (FBE) against *B. cinerea* in pot experiment.

Sample		Day 7		Day 10		Day 15	
		Disease index	Efficacy*(%)	Disease index	Efficacy*(%)	Disease index	Efficacy*(%)
Water		21.7 ± 1.8	-	25.9 ± 2.7	-	22.6 ± 1.0	-
Ethyl acetate		21.3 ± 1.2	1.8 ± 5.7a	24.4 ± 0.7	5.8 ± 2.7a	23.0 ± 0.9	-
Procymidone (500 mg/L)		8.1 ± 0.6	62.7 ± 3.0bc	12.5 ± 1.7	51.7 ± 6.6b	11.1 ± 0.4	50.9 ± 1.8ab
FBE (mg/L)	778.4	5.9 ± 0.8	72.8 ± 3.6c	10.0 ± 1.6	61.4 ± 6.1b	8.5 ± 0.6	62.4 ± 2.7c
	389.2	7.5 ± 0.4	65.4 ± 1.7bc	11.0 ± 2.1	57.5 ± 8.0b	8.6 ± 0.4	62.0 ± 1.8c
	194.6	8.9 ± 0.7	59.0 ± 3.0bc	11.4 ± 1.1	56.0 ± 4.2b	9.3 ± 0.2	58.9 ± 0.9bc
	129.7	10.2 ± 1.8	53.0 ± 8.3b	12.1 ± 1.7	53.3 ± 6.4b	12.8 ± 0.9	43.4 ± 3.8a

*values with different letters in the same column are significantly different (P<0.05) according to Duncan's multiple range test.

Table 5. Therapeutic effect of ethyl acetate extract of *T. harzianum* fermented broth (FBE) against *B. cinerea* in pot experiment.

Sample		Disease index before treated	Day 7		Day 10		Day 15	
			Disease index	Efficacy*(%)	Disease index	Efficacy*(%)	Disease index	Efficacy*(%)
Water		10.1	24.4 ± 0.5	-	25.4 ± 1.0	-	20.4 ± 1.0	-
Ethyl acetate		9.9	25.0 ± 0.4	-	23.9 ± 0.8	2.8 ± 4.2a	20.5 ± 0.3	10.7 ± 7.2a
Procymidone (500 mg/L)		9.7	11.5 ± 0.8	50.9 ± 3.2ab	12.5 ± 0.6	48.8 ± 2.3b	11.7 ± 0.8	48.5 ± 3.0b
FBE (mg/L)	778.4	9.5	8.7 ± 0.9	62.1 ± 4.0b	9.0 ± 0.6	62.3 ± 2.3c	9.3 ± 0.3	57.7 ± 4.7b
	389.2	9.8	11.5 ± 0.9	51.4 ± 3.6ab	12.8 ± 1.0	48.1 ± 3.9b	11.9 ± 1.0	47.2 ± 8.4b
	194.6	9.7	11.7 ± 1.1	50.1 ± 4.8ab	13.0 ± 0.5	46.7 ± 2.1b	13.0 ± 1.0	42.9 ± 3.1b
	129.7	10.1	12.6 ± 1.2	48.4 ± 4.8a	13.9 ± 0.4	45.3 ± 1.6b	13.6 ± 1.1	42.6 ± 3.3b

*values with different letters in the same column are significantly different (P<0.05) according to Duncan's multiple range test.

induce plant resistance by eliciting the synthesis of phytoalexins and pathogenesis -related proteins (Mercedes Dana et al., 2001; Harman et al., 2004). The plant defence system could be systemically boosted by metabolites produced by *T. harzianum* (Meyer et al., 1998; Yedidia et al., 1999). More recently, (Gallou et al., 2009)

reported that *T. harzianum* can induce the expression of defence response genes in potato roots. The plant defence inducers from the ethyl acetate extract of *T. harzianum* fermented broth should be further explored.

In conclusion, the ethyl acetate extract of *T. harzianum* fermented broth is equivalent or better than the dicarboximide fungicide, procymidone, for treating tomato grey mould caused by *B. cinerea*. Compared to synthetic chemical fungicides, the extract as a natural product is readily biodegradable and not likely to cause fungicide resistance due to its diverse modes of action. This extract has great potential to be developed as a natural fungicide for the control of many phytopathogenic fungi including *B. cinerea.*

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REFERENCES

Aneja M, Gianfagna TJ, Hebbar PK (2005). *Trichoderma harzianum* produces nonanoic acid, an inhibitor of spore germination and mycelial growth of two cacao pathogens. Physiol. Mol. Plant Pathol., 67(6): 304-307.

Bae YS, Knudsen GR (2005). Soil microbial biomass influence on growth and biocontrol efficacy of *Trichoderma harzianum*. Biol. Control, 32(2): 236-242.

Benitez T, Rincon AM, Limon MC, Codon AC (2004). Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol., 7(4): 249-260.

Carter CJ, Cannon M, Smith KE (1976). Inhibition of protein synthesis in reticulocyte lysates by trichodermin. Biochem. J. 154(1): 171-178.

Chen L, Li XS, Wang ZQ, Pan CP, Jin RC (2010). Residue dynamics of procymidone in leeks and soil in greenhouses by smoke generator application. Ecotoxicol. Environ. Saf., 73(1): 73-77.

Cutler HG, Cox RH, Crumley FG, Cole PD (1986). 6-Pentyl-a-pyrone from *Trichoderma harzianum*: Its Plant Growth Inhibitory and Antimicrobial Properties. Agric. Biol. Chem. 50(11): 2943-2945.

de la Cruz J, Pintor-Toro JA, Benítez T, Llobell A (1995). Purification and characterization of an endo-beta-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J. Bacteriol., 177(7): 1864-1871.

de las Mercedes Dana M, Limón M, Mejías R, Mach RL, Benítez T, Pintor-Toro JA, Kubicek CP (2001). Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*. Curr. Genet., 38: 335-342.

Gallou A, Cranenbrouck S, Declerck S (2009). *Trichoderma harzianum* elicits defence response genes in roots of potato plantlets challenged by *Rhizoctonia solani*. Eur. J. Plant Pathol., 124(2): 219-230.

Ghisalberti EL, Rowland CY (1993). Antifungal Metabolites from *Trichoderma harzianum*. J. Nat. Prod., 56(10): 1799-1804.

Harman GE (2000). Myths and Dogmas of Biocontrol Changes in Perceptions Derived from Research on *Trichoderma harzinum* T-22. Plant Dis., 84(4): 377-393.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004). *Trichoderma* species--opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol., 2(1): 43-56.

Hjeljord LG, Stensvand A, Tronsmo A (2007). Antagonism of Nutrient - Activated Conidia of *Trichoderma harzianum* (atroviride) P1 Against Botrytis cinerea. Phytopathology, 91(12): 1172-1180.

Lóránt Hatvani, László Manczinger, László Kredics, András Szekeres, Zsuzsanna Antal, Csaba Vágvölgyi (2006). Production of *Trichoderma* strains with pesticide-polyresistance by mutagenesis and protoplast fusion. Antonie van Leeuwenhoek., 89: 387-393.

Meyer GD, Bigirimana J, Elad Y, ofte MH (1998). Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of Botrytis cinerea. Eur. J. Plant Pathol., 104: 279-286.

Muller AK, Bosgra S, Boon PE, van der Voet H, Nielsen E, Ladefoged O (2009). Probabilistic cumulative risk assessment of anti-androgenic pesticides in food. Food Chem. Toxicol. 47(12): 2951-2962.

Ordentlich A, Wiesman Z, Gottlieb HE, Cojocaru M, Chet I (1992). Inhibitory furanone produced by the biocontrol agent *Trichoderma harzianum*. Phytochemistry, 31(2): 485-486.

Schirmbock M, Lorito M, Wang YL, Hayes CK, Arisan-Atac I, Scala F, Harman GE, Kubicek CP (1994). Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl. Environ. Microbiol., 60(12): 4364-4370.

Shi YJ, Shentu XP, Yu XP (2009). Identification of an endophytic fungus isolated from Llex cornuta and the biocontrol effects of its secondary metabolite. Acta Phytopathologica sinica, 4(39): 362-367.

Shtienberg D, Elad Y (1997). Incorporation of Weather Forecasting in Integrated, Biological-Chemical Management of Botrytis cinerea. Phytopathology, 87(3): 332-340.

Sivasithamparam K, Ghisalberti EL (1998). Secondary metabolism in *Trichoderma* and *Gliocladium*.In: G. E. Harman and C. P. Kubicek(eds) *Trichoderma* and *Gliocladium*. 1. London, Taylor and Francis Ltd: 139-191.

Steel CC (1996). Catalase activity and sensitivity to the fungicides iprodione and fludioxonil in Botrytis cinerea. . Lett. Appl. Microbiol., 22(5): 335-338.

Verma M, Brar SK, Tyagi RD, Surampalli RY, Val o JR (2007). Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. Biochem. Eng. J., 37(1): 1-20.

Vicedo B, Leyva M, Flors V, Finiti I, Amo G, Walters D, Real M, García-Agustín P, González-Bosch C (2006). Control of the phytopathogen *Botrytis cinerea* using adipic acid monoethyl ester. Arch. Microbiol., 184(5): 316-326.

Wang ZN, Coley-Smith JR, Wareing PW (1986). Dicarboximide resistance in *Botrytis cinerea* in protected lettuce. Plant Pathol., 35(4): 427-433.

Yedidia I, Benhamou N, Chet I (1999). Induction of Defense Responses in Cucumber Plants (*Cucumis sativus* L.) by the Biocontrol Agent *Trichoderma harzianum*. Appl. Environ. Microbiol., 65(3): 1061-1070.

Zhang CQ, Hu JL, Wei FL, Zhu GN (2009). Evolution of resistance to different classes of fungicides in *Botrytis cinerea* from greenhouse vegetables in eastern China. Phytoparasitica, 37(4): 351-359.