BIOLOGIA PLANTARUM 52 (3): 582-586, 2008

BRIEF COMMUNICATION

Control of fusarium wilt of *Solanum melongena* by *Trichoderma* spp.

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Abstract

Biological control of wilt of egg plant (*Solanum melongena* L.) caused by *Fusarium solani* was made with the application of five *Trichoderma* species, *T. harzianum, T. viride, T. lignorum, T. hamatum* and *T. reesei.* The effect of volatile and non-volatile antibiotics of *Trichoderma* origin on growth inhibition of the wilt pathogen was studied. *T. harzianum* showed maximum growth inhibition (86.44 %) of the pathogen through mycoparasitism. The non-volatiles produced by the *Trichoderma* species exhibited 100 % growth inhibition of the pathogen under *in vitro* condition. Production of siderophores and fungal cell wall degrading enzymes, chitinase and β-1,3-glucanase were found. Treatments with two most efficient *Trichoderma* species, *T. harzianum* and *T. viride* resulted in the decreasing population of *Fusarium solani* in soil thereby deterring disease incidence in field condition.

Additional key words: chitinase, egg plant, *Fusarium solani*, mycoparasitism, siderophore, β-1,3-glucanase.

Egg plant (*Solanum melongena* Linn) is a widely grown vegetable crop in Asian countries and *Fusarium solani* caused wilt of egg plant is a most serious disease. The possibility of applying the antagonistic fungi against the pathogen was first recognised by Weindling (1935). Of the various bio-agents, *Trichoderma* spp. have been known to suppress many soil borne fungi including *Fusarium* spp. by variety of mechanisms, such as competition for nutrients, production of inhibitory volatiles and non-volatiles, mycoparasitism involving the production of hydrolytic enzymes (Bruce *et al.* 1984, Desai and Schlosser 1999, Dhar *et al.* 2006) and production of siderophores (Rane *et al*. 2005, Dutta *et al*. 2006). The present communication has been designed to screen the most effective *Trichoderma* species among the five isolates as concern the growth of the *Fusarium solani,* wilt pathogen of egg plant both under *in vitro* and field conditions.

Fusarium solani (Mart.) Appel and Wollenw. was isolated from wilted egg plants and maintained in pure line on potato dextrose agar (PDA) slants at 4 °C till used. The identification of the pathogen was confirmed by Indian Agricultural Research Institute, New Delhi. *Trichoderma* spp. were isolated from rhizosphere soil of egg plants except *T. harzianum* and *T. hamatum* which were procured from IARI, New Delhi.

To study the competition and mycoparasitism between the pathogen and the antagonists, Royse and Ries (1978) method was followed. Mycelial disc (5 mm) of the antagonist and the pathogen were inoculated simultaneously 2 cm apart on the Petri dish. The plates were then incubated at 30 ± 1 °C for 7 d and the radial growth of both the pathogen and the antagonist was measured and percentage of inhibition of the pathogen was calculated (Table 1). Petri dish containing only the pathogen served as control.

Production of volatile antibiotics was studied according to Dennis and Webster (1971a,b). 15 cm^3 of PDA medium was poured both in the base and the lid of the Petri dish. The lid was inoculated with the pathogen and the base with the antagonist. The base and the lid was tightly joined and incubated at 30 ± 1 °C for 7 d. The percentage of pathogen growth inhibition was calculated.

Production of non-volatile antibiotic was estimated by placing an inoculum disc of antagonist centrally on a PDA plate covered by dialyser bag (*Sigma*, St. Louis, MO, USA). After 2 d of incubation at 30 ± 1 °C, the antagonists and the dialyser bag were removed. A 5 mm inoculum of the pathogen was then placed centrally on the same PDA plate and incubated for further 7 d. At the end of incubation, the growth rate of pathogen was recorded.

Received 10 May 2007, *accepted* 5 April 2008.
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Low nutrient medium (LNM) omitting the iron was used for the production of siderophore by the antagonists and pathogen in liquid culture. The glassware were washed with HCl (6 M) to remove traces of iron and rinsed with distilled water. Double glass-distilled water was used for medium preparation. 50 cm^3 of the medium was dispensed in each conical flask (100 cm^3) and autoclaved. Each flasks were inoculated separately with 5 mm inoculum discs of the test fungi *F. solani*, *T. viride*, *T. lignorum* and *T. harzianum* from their actively growing cultures on PDA medium. After 10 d of incubation at 25 °C, the cultures were centrifuged at 10 000 *g* and the cell-free supernatants were assayed for siderophore production by using the standard protocols (Schwyn and Neilands 1987, Jalal and Helm 1990). $FeCl_3$ test was based on the method of Jalal and Helm (1990). To 0.5 cm^3 of cell-free supernatants 0.5 cm^3 of 2 % aqueous $FeCl_3$ solution was added. Appearance of reddish brown colour indicated the presence of siderophore. Chrome azurol sulphonate (CAS) assay and CAS agar plate test were done following the method of Schwyn and Neilands (1987). Spectrophotometric assay was made following the method of Mayer and Abdallah (1978). Cell-free culture supernatants were examined for their absorption maxima in *UV-Vis 160A* spectrophotometer (*Beckman*, Switzerland). A peak at or near 405 nm indicated the presence of siderophore. To determine the concentration of siderophore, cell-free supernatants of the cultures were assayed by measuring CAS activity (absorbance at 630 nm) following the method of Alexander and Zuberer (1991). The production of low molecular mass siderophores in cultures was determined by a method based on their high affinity for Fe(III) (Schwyn and Neilands (1987). When a strong chelator such as siderophore removes the iron from the dye, its colour turns from blue to purple or orange. When this CAS complex is incorporated into agar plates, halos around the colonies were formed indicating the production of siderophore.

Fusarium solani was grown in PDA medium at 30 °C for 15 d. The mycelia were then removed by filtration and frozen at -20 °C. Cell wall material was then prepared adopting the method of Bruce *et al*. (1995). Mycelia were ground in a morter and pestle before being exposed to ultrasonic disintegration (*Braun-sonic 1510*, Wertheim, Germany) for 3 min. Resulting mycelial pastes were then washed thrice (with repeated centrifugation at 500 *g*) with 0.1 M NaCl in 0.5 M acetate buffer at pH 5.5 (0.5 M anhydrous sodium acetate and 0.5 M acetic acid mixed at 6.2:1, respectively) followed by three washes with deionized distilled water. Samples were then lyophilized overnight before being ground to a fine powder in a mortar and pestle.

For the preparation of enzyme source, the methods of Bruce *et al*. (1995) and Dutta and Chatterjee (2004) were followed. *T. harzianum, T. viride* and *T. lignorum* isolates (50 cm^3) were cultured in Czapek dox broth (CDB) as basal medium, CDB + *F. solani* cell wall material (0.1 g), or CDB without sucrose + *F. solani*. All the flasks were incubated at 25 °C for 10 d. After incubation, fungal mycelium was removed by filtration and culture filtrates were sterilized by passing them through 0.45 µm membrane filters. Filtrates were then dialysed overnight (to remove residual sugars) in a continuous flowing cold water at 10 -12 °C using 2.4 nm pore size dialysis tubing prior to assay for β-1,3-gluca-nase and chitinase activity.

Enzymatic hydrolysis of laminarin (*Sigma*) was assayed by dinitrosalicylic acid method (Miller 1959). The reaction mixture (0.5 cm^3 of enzyme source, 0.2 cm^3 of citrate buffer, pH 4.8, 1.6 mg of soluble laminarin) was incubated at 40 °C for 60 min. Boiling stopped the reaction and the amount of reducing sugar was determined.

The assay for chitinase activity was based on the estimation of reducing sugars released during the hydrolysis of swollen chitin. The reaction mixture, containing 1 cm^3 of 0.5 % swollen chitin (suspended in 50 mM sodium acetate buffer at pH 5.21 containing 0.02 % sodium azide) and 1 cm^3 of enzyme source, was incubated for 1 h at 40 °C with shaking, and then centrifuged at 4 000 *g* for 5 min. The amount of reducing sugar released in the supernatant was determined by the method described by Miller (1959), using N-acetyl glucosamine as standard.

Potential antagonists *T. harzianum* and *T. viride*, were finally subjected to the field trials for the two consecutive years (2001 and 2002) in order to assess their efficacy in controlling the infection of egg plants caused by *F. solani*. Different biocontrol preparations were made from the cultures of the antagonists. 250 cm^3 of CBD medium was taken in each flask, sterilized and inoculated with respective antagonists and incubated at 30 ± 1 °C for 15 d. After incubation, the mycelial mats were harvested, the culture filtrates were collected and applied to the soil at an interval of 15 d. The mycelial mats were also applied to the soil by mixing with organic wastes like wheat bran and saw dust. Results were taken by counting the total number of plants and the number of infected plants and the percentage reduction of infection was recorded.

Among all the antagonists used, *Trichoderma harzianum* was the most potent one to control the growth of the pathogen with 86.44 % efficiency followed by *T. viride* and *T. lignorum* which showed 83.11 and 81.33 % growth inhibition of the pathogen (Table 1). The assay of volatile and non-volatile antibiotics suggested that the three species of *Trichoderma* are capable of producing volatile and non-volatile antibiotics under *in vitro* condition and that they show adverse effects on growth of the pathogen. The non-volatiles was found to act better than volatiles.

The production of siderophore, an iron chelating compound was confirmed in all the fungi tested by different positive tests like $FeCl_3$ test, CAS assay, CAS agar plate test and spectrophotometric assay. In CAS agar plate test, different sizes and colours of zones of halos

were observed when different *Trichoderma* spp. and *F. solani* were subjected to grow on CAS or on CAS + LNM medium (low nutrient medium omitting the iron). The activity was further verified by measuring the A_{630} values of the individual fungus. In this case the CAS activity in the cell-free supernatant of fungal culture remained in the order of *T. lignorum* > *T. harzianum* > *T. viride* > *F. solani* respectively, where *T. lignorum* significantly being the highest producer (Table 2).

Mycoparasitic action of *Trichoderma* spp. on fungal pathogens was the result of their ability to produce hydrolytic enzymes, β-1,3-glucanase and chitinase. For all *Trichoderma* spp. tested, similar pattern of their production was recorded in the media types used. Addition of cell wall material of the pathogen *F. solani* to the CDB resulted in a decrease in the enzyme activity in all *Trichoderma* spp. However, when *F. solani* cell wall material was supplied as the sole sugar source in CDB, all the species showed higher β-1,3-glucanase and chitinase activity. Among the *Trichoderma* spp., *T. harzianum* exhibit significantly higher β-1,3-glucanase activity than the others.

Table 1. Effect of *Trichoderma* spp. on growth of *Fusarium solani* applied in different modes. Means of five replicates (CD at 5 % for mycoparasitism 2.13, volatile antibiotics 3.15, non-volatiles 1.09).

Antagonists	Mode of action	Colony diameter [cm]	Growth inhibition [%]
T. harzianum	mycoparasitism	1.30	86.44 ± 0.74
	volatiles	1.96	78.22 ± 1.25
	non-volatiles	0	100.00 ± 0
T. viride	mycoparasitism	1.60	83.11 ± 1.81
	volatiles	2.60	71.11 ± 0.51
	non-volatiles	0	100.00 ± 0
T. lignorum	mycoparasitism	1.70	81.33 ± 0.42
	volatiles	3.00	65.77 ± 1.28
	non-volatiles	0	97.78 ± 1.36
T. hamatum	mycoparasitism	2.20	75.33 ± 1.13
	volatiles	3.95	55.92 ± 0.42
	non-volatiles	0	97.78 ± 1.83
T. reesi	mycoparasitism	2.30	75.33 ± 0.95
	volatiles	4.00	44.40 ± 0.66
	non-volatiles	0.85	91.11 ± 0.87

Table 2. Siderophore production by the test fungi. Means of five replicates.

Fungi	Siderophore production [mg g^{-1}(f.m.)]
T. viride	0.34 ± 0.008
T. lignorum	0.51 ± 0.011
T. harzianum	0.42 ± 0.007
F. solani	0.08 ± 0.008

Control of *F. solani* causing wilt of egg plant was conducted in the fields for two consecutive years throughout the developmental stages starting from the young seedling stage of the plants with the application of the biocontrol agents (Table 4). Of the two antagonists, excellent performance was obtained with *T. harzianum* showing maximum control of infection, 40.82 % in 2001 and 24.60 % in 2002 as against 35.92 % in 2001, and 21.56 % in 2002 with *T. viride*.

Potential antagonism of *Trichoderma* as evidenced by the results is due to competition, antibiosis and mycoparasitism. Mycoparasitism of plant pathogenic fungi by *Trichoderma* isolates has been well documented. The mechanism of hyperparasitism includes different kinds of interactions like coiling of hyphae around the pathogen, penetration, production of haustoria and lysis of hyphae. Mycoparasitic fungi recognize these cell wall components and hydrolyse them and utilize as substrate (Harman *et al*. 2004). Specific strains of *Trichoderma* colonize and penetrate plant root tissues and initiate a series of morphological and biochemical changes in the plant, considered to be a part of the plant defense responses, which at the end leads to induced systemic resistance (ISR) in the entire plant (Ozbay and Newman 2004).

Antibiotics secreted by antagonistic agents may be volatile or non-volatile and *Trichoderma* spp produce both the types as evidenced from the results. They exert strong inhibitory action by the production of compounds like isopropylalcohol, caryophyllene, pentadecane, suzukacillin, demadin, trichodermin, viridepyronone (Krisosherekova and Mishchenk 1990, Wheatly *et al*.

Table 3. Changes in activities of β-1,3 glucanase and chitinase induced by T*richoderma* spp. in different media (CDB - Czapek's Dox Broth, *F.s.*- cell wall material of *F .solani,* S - sucrose). Means values of five replicates (CD at 5% for β-1,3-glucanase 5.20, for chitinase 7.39).

Trichoderma spp.	β-1,3-glucanase [U mg^{-1}(protein) min^{-1}]			Chitinase [U mg^{-1}(protein) min^{-1}]		
	CDB	CDB + *F.s*	CDB - S + *F.s*	CDB	CDB + *F.s*	CDB - S+ *F.s*
T. harzianum	1090.0 ± 4.5	844.0 ± 4.0	1616 ± 5.1	142.4 ± 1.1	96.6 ± 0.7	172.4 ± 1.1
T. viride	977.2 ± 1.9	748.0 ± 12.1	1268 ± 5.8	99.2 ± 2.9	62.4 ± 1.1	162.4 ± 1.1
T. lignorum	824.0 ± 2.4	568.0 ± 5.8	1116 ± 6.8	78.4 ± 0.7	38.6 ± 0.6	123.4 ± 1.9

Table 4. Field trials with screened potential antagonists on the reduction of incidence of wilt of egg plant caused by *F. solani.* Means of five replicates (CD at 5 % for 2001 6.73, for 2002 9.75).

Year	Treatment	Total number of plants	Number of infected plant	Infection [%]	Reduction of infection [%]
2001	control	70	23	32.85	
	T. harzianum	36	7	19.44	40.82 ± 3.73
	T. viride	38	8	21.05	35.92 ± 5.35
2002	control	98	20	20.40	
	T. harzianum	52	8	15.38	24.60 ± 6.74
	T. viride	50	8	16.00	21.56 ± 3.25

1997, Bruce *et al*. 2000, Evidente *et al*. 2003, Chakraborty *et al*. 2004).

The potent antagonistic effect of *Trichoderma* is contributed due to its ability of competition with the host fungi for the same nutritional status. In natural environment, microorganisms have to compete with others to acquire the iron with the aid of their siderophores and different species of *Trichoderma* and *F. solani* exhibited high siderophore production (Chakraborty 2005, Dutta *et al*. 2006). One of the essential characteristics of *Trichoderma* spp. acting as mycoparasite of fungal plant pathogens is their ability to excrete hydrolytic enzymes. Laminarinase activity in all the antagonists remains much higher in the CDB containing *Fusarium* cell wall material without sucrose than when sucrose is supplemented. The laminarinase activity in CDB + pathogen cell wall is lower than the activity in the pure CDB which is indicative of binding of the enzyme to the cell wall substrate prior to extraction of the filtrate for assay (Chakraborty 2005).

It is evident from the results that careful consideration must be given to the culture condition if this mode of antagonism is to be used to assess the biological control capabilities of isolates against pathogenic fungi. It may be advantageous to select an isolate that shows high enzyme activity in presence of cell wall material from a specific pathogen responsible for a disease to be protected. Conversely if the biological control practice is intended to be applied against a wide range of pathogenic fungi, it would be appropriate to identify a *Trichoderma* isolate that constitutively possesses an expression of producing high amounts of lytic enzymes.

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