J. Mycopathol, Res, **47(2)** : 169—174 (2009)

Studies on *Trichoderma* isolates from Mizoram and establishment of their antagonistic potential against some soil borne plant pathogens

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An attempt was made to isolate the antagonistic fungus. *Trichoderma* spp. from different crops, rhizosphere and to establish their antagonistic potential against *Rhizocotonia solani, Sclerotium rolfsii* and *Macrophomina phaseolina.* It was observed that only two species of *Trichoderma namely, T. harzianum* (one isolate) *and T. viride* (five isolates) were most prevalent in soil samples of Mizoram and the population density (c.f.u./g soil) varied between 3×10^3 to 9×10^3. *In vitro* antagonistic potential of *Trichoderma* isolates revealed that they attained S_1 stage at 4-5 days for *R. solani,* 6-8 days for S. *rolfsii* and for *M. phaseolina* within 4-6 days and the most efficient isolate being the ThM_1 and TvM_4, respectively in dual plate culture. The highest growth inhibition of all three pathogens were recorded with isolate TvM_5 (67.7% ; 57.8 and 72.2% for *S. rolfsii,* 66.7%; 72.2% and 80.0% *for R. solani* and 69.7% ; 75.2% and 82.0% for *M. phaseolina*) by their ability to produce volatile and non-volatile substances respectively. The antagonistic potential of the isolates were also correlated with their ability to produce chitinase and β-1, 3 glucanase and cellulase enzymes activity *in vitro.* The isolate TvM_3 and TvM_4 produced highest activity of chitinase, glucanase and cellulase enzymes followed by Th M_1 isolate in the basal medium as well as media amended with mycelial powder of M. *phaseolina and Pythium* sp, chitin and carboxy methyl cellulose.

Key words: Antagonistic potential, *Trichoderma* spp, *R. solani, S, rolfsii, M. phaseolina*, chitinase, β-1, 3 glucanase, cellulase enzymes

INTRODUCTION

Mizoram is one of the members of seven sister states, comprising hills and plateaus with dense forest with greater microbial biodiversity. *Trichoderma* spp. are found in almost all types of soil viz., cultivated soil, garden soil, fallow and pasture land and forest soil (Harman, 2000), and most commonly used fungal antagonist to suppress range of soil borne plant pathogens. The mechanisms by which strains of *Trichoderma* function are mycoparasitism, antibiosis, competition for nutrient or space. tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration of inorganic nutrients and inactivation of pathogens enzyme, etc. (Harman, 2000). Though there are voluminous literature on this novel fungus in India, but there is hardly any systematic study made on this fungus in North eastern states of India with particular reference to Mizoram state. Therefore, present investigation has been carried out to explore the native isolates of *Trichoderma* and to test their antagonistic potential against some soil borne plant pathogens and production of certain extra cellular hydrolytic enzymes.

MATERIALS AND METHODS

Different isolates of *Trichoderma* were isolated from rhizosphere soil of diverse crops from Mizoram state (tea. maize, cabbage, citrus and paddy) by soil dilution technique (Dhingra and Sinclair, 1995) and plated on modified *Trichoderma* selective medium (TSM) (Elad and Chet, 1983). The probable colonies

of *Trichoderma* were picked up, subcultured, purified and preserved in PDA slants at 4°C for subsequent use. The *Trichoderma* spp. were identified up to species level by slide culture technique following the taxonomic keys and monograph of Rifai. (1969).

In vitro antagonistic potential of *Trichoderma* (ThM_1, TvM_1, TvM_2, TvM_3, TvM_1 and TvM_5) was made by dual culture method against three soil borne plant pathogens *viz., R. solani, S. rolfsii* and *M. phaseolina* and rated as per modified Bell's scale–S_1 : antagonist completely overgrew the pathogen, S_2 : antagonist overgrew at least 2/3 growth of the pathogen, S_3 : antagonist colonized one half of the growth of the pathogen, S_4, antagonist and pathogen locked at the point of contact and S_5: pathogen starts overgrowing the antagonist.

Effect of volatile and non-volatile antibiotics produced by Trichoderma isolates

The antagonistic potential of *Trichoderma* isolate (six isolates) was evaluated against *R. solani, S. rolfsii* and *M. phaseolina* by their ability to produce volatile and non-volatile inhibitors following the methods of Dennis and Webster (1971 a,b). The antagonists were centrally inoculated into Petridishes containing PDA by placing 6 mm diam mycelial discs from young growing region of 4 days old culture of antagonist and incubated at 28 ±1°C for 4 days. Top of each Petridish was replaced with bottoms plate of PDA duly inoculated with mycelial disc (6 mm diam) of pathogen. The suitable control was also maintained with PDA medium without antagonist at the lower lid and upper lid with duly inoculated pathogen. Pairs of plates were sealed together with cellophane tape and incubated at ± 28 1°C for 7 days. Radial mycelial growth of pathogen was recorded and percentage inhibition of mycelial growth of pathogen by *Trichoderma* isolates was calculated according to the formula of Vincent (1947).

For the non-volatile substances, all *Trichoderma* isolates were grown for 10 days in 100 ml potato dextrose broth in 250 ml Erlenmeyer flasks with intermittent shaking. The culture filtrate was harvested by filtering through Whatman No. 42 filter paper. The culture filtrate was centrifuged at 5000 rpm for 10 min and sterilized by passing it through cellulose membrane Millipore filter paper (0.4 µm pores size). Batch of 10% and 20% concentration each of culture filterate in the PDA medium was prepared by adding required volume of culture filtrate in the molten PDA medium. The mycelial plug (6 mm diam.) from fresh culture of pathogens viz., *R. solani, S, rolfsii* and *M. phaseolina* were inoculated on PDA medium and incubated at 28 ± 1°C for 4 days. The suitable control was also maintained with no addition of culture filtrate in the PDA medium. The radial mycelial growth of the pathogens were recorded and percentage inhibition of respective pathogen by *Trichoderma* isolates was calculated as stated above.

Assay for extracellular enzyme activity

Six isolates of *Trichoderma* was separately inoculated into 100 ml broth media with five combinations viz., Czapek Dox broth (CDB) alone, CDB + chitin (10 g), CDB + mycelial powder of *M. phaseolina* (10 g), CDB + mycelial powder of *Phythim* sp. (10 g), and CDB + carboxy methyl cellulose (0.75 g) in Erlenmyer flasks and incubated at 28 ± 1°C for 7 days with intermittent shaking at 125 rpm twice a day. The culture filtrate of each isolate was harvested, filtered through the Whatman Filter Paper 42, centrifuged and assayed for cellulase, chitinase and β-1, 3 glucanase enzyme activity immediately.

Assay for β-1, 3 glucanase (E.C.3.2.1.58)

For assay of β-1, 3 glucanse enzyme, 0.5 ml laminarin, 1.0 ml of 0.05 M citrate buffer (pH 4.8) and 0.5 ml culture filtrate was mixed and incubated at 40°C for 60 min. An equal volume of dinitrosalicyclic acid reagent was added to the reaction mixture and warmed in boiling water for 15 min. The absorbance of reaction mixture was measured at 575 nm in a spectrophotometer and compared with standard graph drawn by following the same procedure but using different concentrations of glucose instead of culture filtrate. The quantity of reducing sugar was calculated from the glucose standards used in the assay and activity of β-1, 3 glucanase was expressed in nkat/ml. One nkat corresponds to the release of 1 n mol glucose equivalent per second.

Chitinase (E.C.3.2.1.1.4)

A mixture of 0.5 ml culture filtrate, 0.5 ml

suspension, 1.0 ml of McIlvaines buffer (pH 4,0) was incubated at 37°C for 2 hr in a water bath with constant shaking. The reaction was stopped by boiling 3 min in heated water bath, and then 3 ml of potanium ferricyanide reagent was added and warmed in boiling water for 15 min. The amount of N-acetyl glucosamine (NAG) released was estimated by measuring the absorbance of reaction mixture in a spectrophotometer at 420 nm. The amount of reducing sugar released was calculated from standard curves for NAG and chitinase enzyme activity was expressed in pkat (pmol/s) per mililitre.

Cellulase (E.C. 3.2.1.4)

The reaction mixture contained 1 ml cellulose (0.75%), 2 ml of 0.05 citrate buffer (pH 4.8) and 1 ml culture filtrate which was incubated for 30 min at 55°C in a water bath with constant shaking. The amount of glucose released in the reaction mixture was estimated by dinitrosalicyclic acid method and cellulase enzyme activity was expressed as release of 1 μ mol glucose/ml/min for one unit.

RESULTS AND DISCUSSION

The results presented in Table 1 revealed that 6 isolates of *Trichoderma* (*T. harzianum and T. viride*) was confirmed on the basis of morphological characteristics. The initial population of *Trichoderma* varied from 3×10^3 (*T. viride* from cabbage field of Serchhip) to 9×10^3 (*T. harzianum* from Tea field of Lunglei). Most of the isolates identified were *T. viride* with rough surface. Present findings are in accordance to taxonomic monograph of Rifai (1969) and predominance of *T. viride* in temperature regions (Papavizas, 1985).

Table 1. *Trichoderma* isolates from Mizoram

Location	cfu/plate	isolate code	Crop rhizosphere	Species confirmed
Lunglei	9×10^3	ThM_1	Tea	*T. harzianum*
Lunglei	8×10^3	TvM_1	Tea	*T. viride*
Aizwal	7×10^3	TvM_1	Maize	*T. viride*
Serchhip	3×10^3	TvM_1	Cabbage	*T. viride*
Serchhip	4×10^3	TvM_1	Citrus	*T. viride*
Phairuang (lunglei dist.)	5×10^3	TvM_1	Paddy	*T. viride*

The results on overall *in vitro* antagonistic potential of *Trichoderma* isolates from Mizoram state is presented in Table 2, The data indicated that their antagonistic potential differed with pathogen tested. All isolates of *Trichoderma* except TvM_2 showed high antagonistic potential against *R. solani*, attaining S_1 stage at 4 days. Similarly, ThM_1, TvM_1 and TvM_3

Table 2. *In vitro* antagonistic potential of *Trichoderma* isolates against three soil borne plant pathogens.

Isolate	Modified Bell's scale*		
	S. rolfsii	*R. solani*	*M. phaseolina*
ThM_1	$8S_1$	$4S_1$	$4S_1$
TvM_1	$8S_1$	$4S_1$	$4S_1$
TvM_2	$6S_5$	$5S_1$	$5S_1$
TvM_3	$7S_1$	$4S_1$	$4S_1$
TvM_4	$6S_1$	$4S_1$	$6S_1$
TvM_5	$6S_1$	$4S_1$	$5S_1$

* Means of five replications.

Table 3. Effect of volatile and non-volatile antibiotic(s) on growth of *Selecrotium rolfsii**

Isolate	Volatile antibiotics		Non-volatile antibiotics			
	Average colony diameter(mm)	Growth inhibition over control (%)	Radial growth (mm) in different concentration of cultural filtrate		Per cent inhibition over control	
			10%	20%	10%	20%
ThM_1	38.0	57.8	42.0	31.0	53.3	65.6
TvM_1	47.0	47.4	45.4	33.0	49.6	63.3
TvM_2	44.0	51.1	52.0	31.0	52.2	65.6
TvM_3	50.0	44.4	40.0	28.0	45.6	58.9
TvM_4	35.0	66.1	40.0	30.0	55.6	66.7
TvM_5	30.0	66.7	38.0	25.0	57.8	72.2
Control	90.0		90.0	90.0	—	—
SEd	3.65		3.08	3.16		
CD (0.05)	7.83		6.61	6.79		

* Mean of four replication.

Table 4. Effect of volatile and non-volatile antibiotic(s) on growth of *Rhizoctonia solani**

Isolate	Volatile antibiotics		Non-volatile antibiotics			
	Average colony diameter(mm)	Growth inhibition over control (%)	Radial growth (mm) in different concentration of cultural filtrate		Per cent inhibition over control	
			10%	20%	10%	20%
ThM_1	33.0	63.3	42.0	30.5	53.3	66.1
TvM_1	45.0	50.0	44.5	33.5	50.7	43.7
TvM_2	48.0	46.7	55.5	42.1	38.3	53.2
TvM_3	40.0	55.6	50.0	35.0	44.4	61.1
TvM_4	32.0	64.4	26.0	16.0	71.1	82.2
TvM_5	30.0	66.7	25.0	18.0	72.2	80.0
Control	90.0	–	90	90	–	–
SEd	3.06		4.11	2.61		
CD (0.05)	6.56		8.83	6.61		

* Means of four replications.

Table 5. Effect of volatile and non-volatile antibiotic(s) on growth of *Macrophomina phaseolina**

Isolate	Volatile antibiotics		Non-volatile antibiotics			
	Average colony diameter(mm)	Growth inhibition over control (%)	Radial growth (mm) in different concentration of cultural filtrate		Per cent inhibition over control	
			10%	20%	10%	20%
ThM_1	40.0	55.6	40.0	26.5	55.6	70.6
TvM_1	47.0	47.8	50.0	37.5	44.4	58.3
TvM_2	40.0	45.6	55.0	40.0	38.9	55.6
TvM_3	50.0	44.4	48.0	37.0	46.7	58.9
TvM_4	30.0	66.7	25.0	18.0	72.2	80.0
TvM_5	30.0	69.7	25.0	16.0	75.2	82.2
Control	90.0	—	90.0	90.0	—	—
SEd	2.77		3.70	4.07		
CD (0.05)	5.95		8.10	8.73		

* Means of four replications.

Table 6. Chitinase enzyme activity of *Trichoderma* isolates from Mizoram (pkat/ml)*

Isolate	CDB	S + M.P.	S+Chitin	S+ CMC	S-P.U.
ThM_1	40.2	121.5	111.7	97.8	115.6
TvM_1	2.5	45.4	68.4	51.0	79.6
TvM_2	24.0	69.0	62.0	55.0	82.0
TvM_3	45.5	140.2	123.5	109.5	123.6
TvM_4	42.0	132.8	114.9	102.2	117.8
TvM_5	20.5	63.2	58.4	51.6	59.6
SEd	3.36	4.60	4.10	7.65	4.79
CD (0.05)	7.33	10.02	8.9	10.46	10.44

* Means of four replications.

against *M. phaseolina,* TvM_2, TvM_4 and TvM_5 against *S. rolfsii*, were most effective in their mycoparasitic action attaining S_1 atage at 4 days and 6 days after inoculation, respectively. Our present finding revealed that there was strong selectivity of these antagonistic fungi towards the respective pathogen. A similar result was also found by earlier researchers (Papavizas, 1985 ; Pan and Bhagat, 2007). All the isolates of *Trichoderma* significantly inhibited the growth of *S. rolfsii, R solani* and *M. phaseolina* by producing volatile and non-volatile substances (Tables 3-5). The highest growth inhibition of *S. rolfsii* was observed with TvM_5 (66.7% ; 57.2%) isolate by producing volatile and non-volatile antibiotics at 10% and 20% concentrations, respectively. The next best isolates were TvM_4 (66.1% ; 55.6% and 66.7%). ThM_1 (57.8% 53.3% and 65.6%). TvM_2 (51.1% ; 52.2% and 65.6%) and least effect was recorded with TvM_3 (44.4% ; 45.6 and 58.9%) by inhibiting the mycelial growth of *S. rolfsii* by producing certain volatile and non-volatile metabolites, respectively. Similar results were found with *R. solani*, TvM_5 isolate being the most effective in suppression of mycelial growth of pathogen by 66.7% ; 72.2% and 80.0% with the effect of volatile and non-volatile inhibitors, respectively. The next best isolates were TvM_4 and ThM_1 and lowest suppression of pathogen was recorded with TvM_1 isolate. The highest mycelial growth inhibition of *M. phaseolina* was found with isolate TvM_5 inhibiting 69.7%, 72.5% and 82.2% growth inhibition respectively over control, followed by TvM_4 (44.7%; 72.2 and 80.0%) and ThM_1 (55.6% ; 55.6%, 70.6%) by producing volatile and non-volatile substances at 10 and 20% concentration. The lowest growth inhibition of *M. phaseolina* was recorded with TvM_2 inhibiting only 45.6% ; 38.9% and 55.6% mycelial growth with the effect of its volatile and non-volatile substances.

Table 7. β-1, 3 glucanase enzyme activity of *Trichoderma* isolates from Mizoram nkt/ml)*

Isolate	CDB	S+M.P.	S+Chitin	S+CMC	S+P.U
ThM_1	42.5	55.7	30.8	48.5	83.8
TvM_1	23.5	37.8	15.8	38.0	75.0
TvM_2	25.0	39.0	17.2	31.5	80.0
TvM_3	48.1	65.8	31.2	49.2	86.2
TvM_4	44.2	61.2	35.0	53.0	89.0
TvM_5	29.8	46.0	12.5	29.5	68.7
SEd	3.71	3.75	3.26	2.82	4.18
CD (0.05)	8.08	8.18	7.11	6.16	9.12

* Means of four replications.

Table 8. Cellulase enzyme activity of *Trichoderma* isolates from Mizoram (nkat/ml)*

Isolate	CDB	S+M.P.	S+Chitin	S+CMC	S+P.U
ThM_1	32.5	65.7	37.2	55.5	85.9
TvM_1	28.4	52.2	28.8	41.5	44.5
TvM_2	29.0	55.4	31.5	44.9	49.2
TvM_3	33.7	68.9	42.3	60.2	88.9
TvM_4	36.0	76.4	45.8	66.7	97.5
TvM_5	21.0	38.5	26.0	39.0	44.0
SEd	3.05	3.62	3.12	3.3	4.55
CD (0.05)	6.65	7.88	6.8	7.26	9.93

* Means of four replications.

It appeared from the data in Table 6 that all isolates showed significantly higher chitinase enyzme activity with supplement of different carbon sources as substrates in the basal media. The highest chitinase enzyme activity was recorded with TvM_3 (140.2 U) in CDB + mycelial powder of *M. phaseolina,* followed

by TvM_4 (132.8 U) and ThM_1 (121.5 U). The next highest chitanase activity was expressed in the *Pythium* sp. (59.6 – 123.6 U) and chitin (58.4 – 123.5 U) amended medium whereas lowest chitinase enzyme (51.6 – 109.5 U) activity was recorded with CMC amended media and comparatively very low enzyme activity was observed with Czapek dox broth media. The partial substitution of sucrose by same carbon sources in the basal medium has profound effect as the β-1, 3 glucanase and cellulase enzyme activity as compared to main media (CDB). Highest β-1, 3 glucanase enzyme activity was recorded with TvM_4 (89.0 U), followed by TvM_3 (86.2 U). ThM_1 (83.8 U), TvM_2 (80.0 U), TvM_1 (70.0 U) and least enzyme activity was found in TvM_5 (68.7 U) isolate in the media amended with mycelial powder of *Pythium* sp. Among all carbon sources as amendment of media, mycelial powder of *Pythium* supported higher β-1, 3 glucanase enzyme activity than any other amendment, followed by substitution with mycelial powder of *M. phaseolina,* carboxy methyl cellulose and least enzyme activity was recorded with chitin amendment. Similar trend was also recorded with cellulase enzyme activity of *Trichoderma* isolates, where highest cellulase enzyme activity was recorded in mycelial powder of *Pythium* as carbon source ranging from 44.0 U (TvM_5) to 97.5 U (TvM_4) followed by CDB + *M. phaseolina* ranging from 38.5 U (TvM_5) to 76.4 U (T_vM_4), CBD + CMC ranging from 39.0 U to 66.7 U and least activity was observed with chitin (26.0 U – 45.8 U).

The antagonism by *Trichoderma* spp. against many soil borne plant pathogens has been established (Harman, 2000 ; Pan and Bhagat, 2007 ; Pan et al., 2001). Strong antagonism by *Trichoderma* spp. against a range of soil brone plant pathogens has been reported (Pan et al., 2001). Although the results of *in vitro* studies reflecting the antagonistic potential of the microorganisms are not always related to the degree of antagonism observed in the field yet such studies are important for screening the antagonists effective against soil brone pathogens. In the present experiment strong selectivity of the isolates of *Trichoderma* in their antagonistic efficiency towards a particular pathogen was observed. Bell *et al.* (1982) have screened antagonistic potential of 77 isolates of *T. harzianum* against 6 plant pathogens and recorded significant differences between pathogen—antagonist interactions. Sarmah and Mukhopadhyay (1999) have showed that while some isolates were highly antagonistic to some pathogens yet there is clear variability in degree of antagonism. Practically, strain specificity against a particular pathogen is one of the major deterrent factors to commercial use of the antagonist. Selective activity of both volatile and non-volatile substances released by *Trichoderma* isolates has also been noticed against the pathogen. Antibiosis mediated by specific and non-specific metabolites of *T.* virens *as* the principal mechanism in biocontrol of cotton seedlings induced by *R. solani* has been reported (Howell *et al.*, 1993). *Trichoderma* spp. antagonistic to a range of fungi have been reported to produce volatile and non-volatile antibiotics (Pan and Bhagat, 2007)

It is well known fact that *Trichoderma* spp. have the potential to produce cell wall degrading enzymes by using the materials that are present in the growth medium (Harman, 2000). Production of hydrolytic enzymes such as β-1,3 glucanase, chitinase, cellulase and proteinase increased significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or purified host fungal cell walls (Carsolio *et al.*, 1994 ; Cruz *et al.*, 1995). Ulhoa and Peberdy (1991) have found that products of chitin degradation also regulate the chitinase synthesis in *T. harzianum*. Kumar and Gupta (1999) have reported that cell wall of *M. phaseolina* and *S. rolfsii* is known to have glucan and chitin that should have resulted in the induction of glucanse and chitinase in mycelial mat amended media. High β-1,3 glucanase and chitinase activities are detected in dual culture when *T. harzianum* parasitized *R. solani* and *S. rolfsii* compared with low levels of substrates or in absence of pathogen (Elad *et al.,* 1983). In present investigation, it is found that chitinase β-1, 3 glucanase and cellulase enzyme activities are increased with the substitution of specific carbon source at 1% concentration. These findings are in accordance with Ulhoa and Peberdy (1991) where they have suggested that chitinase activity is substrate's concentration dependent above 0.5% (w/v) chitin. There was further synthesis of the chitinase in the growth medium by *T. harzianum* is increased up to 1% concentration, whereas β-1,3 glucanase enzyme production increase up to 1% concenration of laminarin but decrease at higher concentrations. This may be due to the fact that at higher concentration of sugar activity of this enzyme is inhibited.

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(Accepted for publication August 03, 2009)