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In vitro Antagonistic Activity of *Trichoderma* Isolates against Phytopathogenic Fungi *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.)

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Fusarium wilt in tomato caused by *Fusarium oxysporum* is one of the intericate diseases. In this study, 12 native *Trichoderma* isolates (WT_1-WT_{12}) were isolated from different agricultural lands in Rayalaseema region of A.P, India and were tested for antagonistic activity against *F. oxysporum* using dual culture method. Among the 12 isolates maximum inhibition activity was shown by the isolate WT_2 (78.4%) compared to the control. The 12 isolates of *Trichoderma* were subjected to protein profiling by SDS-PAGE which showed that all the isolates varied in terms of production of proteins. Further all the *Trichoderma* isolates were screened for the production of different enzymes like lipase, protease and amylase. Quantitative assays were also conducted to estimate the amounts of cell wall degrading enzymes such as chitinase, β-1,3 glucanase and protease. The plate assay results were consistent with the results observed in the quantitative biochemical assays performed. Natural isolate variability in enzyme levels was significant. Isolate WT_2 was observed to be the most promising candidate in terms of levels of enzyme production as well as inhibition of *Fusarium oxysporum*. Studies on isolate variability could be rationally used to select isolates towards production of improved bio-fungicides.

Key words: *Trichoderma*, lytic enzymes, biocontrol, Tomato.

Tomato (*Lycopersicon esculentum* Mill.) is one of the most popular and important commercial vegetable crops grown throughout the world. It is rich in vitamins A, B and C. Many fungal and bacterial diseases can affect the yield and quality of the tomatoes. Among them *Fusarium oxysporum* f.sp. *lycopersici* is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves and low fruit yield. Increasing use of chemicals causes several negative effects such as development of pathogen resistance to the applied agents and their environmental impact. Therefore, environmental friendly alternative methods for control of plant diseases are need of the day. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods[1, 2].

Studies on the use of biological agents, such as antagonistic species from the genus *Trichoderma* were reported by several authors[3,4]. These species were well studied and have showed efficient biocontrol activity against different

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phytopathogens[5,6,7,8,9] including some phyloplane flora, such as *Botrytis (Pers. Ex Fr.), Cladosporium, Sclerotinia fuckeliana, Alternaria cerasi*[10]. Species of *Trichoderma* can produce extracellular enzymes and antifungal compounds and also act as competitors to fungal pathogens, induce resistance in plants and promote plant growth[11]. Cell wall- degrading enzymes such as chitinases, β -1, 3-glucanases, proteases and cellulases are involved in the antagonistic activity of biocontrol agents against phytopathogenic fungi[12, 13]. The role of the enzymes produced by the mycoparasitic fungi *Trichoderma* in biological control of fungal pathogens like *Botrytis* has been demonstrated[12, 14]. *T. harzianum* is one efficient biocontrol agent that is commercially produced to prevent development of several soil pathogenic fungi. The ability of *Trichoderma* to control *A. niger* varies considerably and it is possible to improve its biological control efficiency by the selection of isolates with high antagonistic potential and adapted to certain ecological or geographical areas[15].

The aim of the present investigation is to study protein banding patterns of native *Trichoderma* isolates employing SDS-PAGE (Sodium Do-decyl Sulphate Poly Acrylamide Gel Electrophoresis), estimate amounts of cell wall degrading enzymes and screen their *in vitro* antagonistic activity *against F. oxysporum*, a causative organism of fusarium wilt. Our observations provide information about the relationship between antagonistic capacity of *Trichoderma* isolates and production of cellwall degrading enzymes during antagonism.

MATERIALS AND METHODS

Collection and isolation of the fungi

Twelve native isolates of *Trichoderma* spp. were isolated by serial dilution method[16] from different rhizosphere soils of Rayalaseema region (Andhra Pradesh, India). The test pathogen, *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) 581 was obtained from IARI (Indian Institute of Agricultural Research), New Delhi. All microbes were maintained throughout the study by periodical transfers on PDA (Potato Dextrose Agar) medium under aseptic condition to keep the culture fresh and viable.

Antagonistic activity

The dual culture technique described by Morton and Strouble[17] was used to test the antagonistic ability of *Trichoderma* against *F. oxysporum*. The test fungus and twelve *Trichoderma* isolates were grown on PDA for a week at 28° C in an incubator. Disk of 5 mm of the target fungus *F. oxysporum* cut from the periphery was transferred to the petriplates containing PDA. *Trichoderma* isolate was also transferred aseptically to the same plate. Each plate received two disks, one with *Trichoderma* mycelium and another with *F. oxysporum* placed at a distance of 7 cm away from each other. The experiment was conducted in five replications for each antagonist. The plates were incubated at 28° C and observed after six days for growth of antagonist and test fungi. Index of antagonism as per cent growth inhibition of *F. oxysporum* was determined by the method of Watanabe[18].

SDS- PAGE sample preparation

For total protein analysis, isolates were grown in 250 ml Erlenmeyer flasks containing 100 ml of PDB. Actively growing hyphal tips of 4-day-old isolates were cut with a 5 mm diameter cork borer and transferred into flasks. Each inoculated flask was incubated at 28° C for 9 days, and the resulting mycelial mass was harvested by filtering through a double layered sterile muslin cloth before being washed twice with 100 ml of distilled water. The mycelia from each isolate were placed into individual plastic bags and excess liquid was drained out, before labeling and storing at -20° C until for further use. Frozen mycelia were removed and immediately ground to powder by using a mortar and pestle that had been sterilized by swabbing with ethanol and samples were stored at -20° C.

Electrophoresis

SDS-PAGE was carried out by using the protocol of Laemmli[19] with 5 % acrylamide stacking and 12 % acrylamide separating gels. The gels were stained with Coomassie brilliant blue to visualize proteins.

Disc preparation for inoculation on different enzyme analysis

Seven to ten day old cultures on PDA slants were used for preparing spore suspension in 0.02% Tween 80 solution at $1x10^6$ spores/ml. 200 µl of the spore suspension was plated on PDA

medium and incubated for 3 days at 28° C. At the end of 3 days, 5 mm mycelial disc with agar was retrieved with help of cork borer and placed in middle of fresh test substrate plates (3 replicates/ isolate were maintained) and incubated at 28° C for 8 days. The control plates did not have the substrate on them; they just had minimal media solidified on 2 % agar. Enzyme activities were calculated as an index of the total diameter of the colony + halo divided by the diameter of the colony[20]. Enzymatic index value >1.0 indicates enzyme activity.

Protease plate assay

1 % Gelatin extract in minimal media (0.003 % NaCl, 0.03 % $MgSO_4$ and 0.015 % K_3PO_4) in conjunction with 2 % agar (for solidifying), pH adjusted to 7.0 before autoclaving, was used for *in vitro* estimation of protease activity. The plates were inoculated with 5 mm agar disc with mycelia and incubated at 28°C for 8 days. At the end of the incubation period, the culture plates were flooded with a solution of 15 % Mercuric chloride in 2 N HCl. A distinct transparent zone of clearance could be seen around the colony while the rest of the plate appeared translucent white in color.

Amylase plate assay

Starch Agar (30 g/ 1000 ml), containing starch as the sole carbon source was used as substrate with pH 7.4 at 28 °C[21]. About 20ml media was poured in 9 cm Petri plates. The plates were inoculated with 5 mm agar disc with mycelia and incubated at 28 °C for 8 days. Colony growth of the fungus implies that starch has been degraded and utilized. At the end of the incubation period, the culture plates were flooded with Lugol's Iodine solution (1 g Iodine and 2 g Potassium Iodide in 300 ml distilled water). A yellow colored halo zone could be seen around the colony in an otherwise blue medium indicating starch hydrolytic activity of the amylases.

Lipase plate assay

1 % Tributyrin extract in minimal media in conjunction with 2 % agar (for solidifying), pH adjusted to 7.0 before autoclaving, was used for *in vitro* estimation of lipase. The plates were inoculated with 5 mm agar disc with mycelia and incubated at 28°C for 8 days. At the end of the incubation period, formation of lipolytic enzymes by a colony was seen as either a visible or as a clearing of such a precipitate around the colony.

Spectrophotometric Estimations

Protease

Proteolytic activity was assayed by a modified method of Kunitz[22]. 100 µl enzyme samples were incubated with 400 µl of 0.5 % (w/v) gelatin in 50 mM Tris- HCl buffer, pH 10.0 at 50°C for 20 minutes. The enzyme reaction was terminated by addition of 500 µl 10 % (w/v) trichloroacetic acid and kept at room temperature for 10 minutes. The reaction mixture was centrifuged at 10,000 g for 10 minutes at 4 °C and the absorbance measured against water as blank at 280 nm. One unit of proteases was defined as the amount of enzyme releasing the equivalent of 1 µM of tyrosine per minute under the defined assay conditions.

Chitinase

Chitinase assay was done by the method of Yanai[23]. 500 µl of culture supernatant was incubated with 300 µl of 10 % (w/v) colloidal chitin (sigma) and 200 µl of 0.2 M acetate buffer (pH 4) at 37 °C for 2 hrs. The reaction product N-acetyl glucosamine was determined by using para-dimethyl-Amino benzaldehyde reagent[24]. Absorbance at 585 nm was taken against water as blank. Sampling was done every two days till tenth day of culture incubation. One unit of chitinase activity was defined by the amount of enzyme that produced 1 µM of N- acetyl glucosamine per minute under the above conditions.

β- 1, 3- Glucanase

β-1,3-glucanase was assayed similarly by incubating 1 ml 0.2 % laminarin (w/v) in 50 mM sodium acetate buffer (pH - 4.8) with 1 ml enzyme solution at 50 °C for 1 hr and by determining the reducing sugars with DNS[25]. Absorbance at 540 nm was taken against water as blank. The amount of reducing sugars released was calculated from standard curve for glucose. One unit of β-1,3 glucanase activity was defined as the amount of enzyme that catalyzed the release of 1µmol of glucose equivalents per min.

RESULTS

***In vitro* growth inhibition of *F.oxysporum* by Trichoderma isolates**

Percent growth inhibition of pathogen (*F. oxysporum*) was significantly higher in WT_2 (78.4 %) antagonist followed by WT_{10} (71.4 %), WT_4 (71.3 %) and WT_7 (70.06 %) at 6 DAI (Days After

Initiation) (Table 1). The isolates other than WT_2, WT_{10}, WT_4 and WT_7 inhibited the pathogen from 54.1 to 68.5 percent, respectively. Thus, it was observed that WT_2 antagonist (i.e., interaction between WT_2 and pathogen *F.oxysporum*) showed a better growth of inhibition of test fungus *F. oxysporum* compared to other bio-control agents (Fig. 1).

Table 1. *In vitro* screening of *Trichoderma* isolates against *F. oxysporum*

S. No	Isolates	Radial growth of *Fusarium oxysporum*(mm)*	Percent inhibitionover control
1	WT_1	25.00	65.2
2	WT_2	15.55	78.4
3	WT_3	33.00	54.1
4	WT_4	20.66	71.3
5	WT_5	24.33	66.2
6	WT_6	26.66	62.9
7	WT_7	21.55	70.0
8	WT_8	27.33	62.0
9	WT_9	30.33	57.8
10	WT_{10}	20.55	71.4
11	WT_{11}	24.00	66.6
12	WT_{12}	22.66	68.5
13	Control (*F. oxysporum*)	72.00	—-
14	S.Em.	0.47	
15	CD (p=0.05)	1.16	

Table 2. Enzymatic Index Values of extra-cellular enzymes produced by *Trichoderma.* isolates

S.No	Enzymes			
	Isolate	Protease	Lipase	Amylase
1	WT_1	0.00	1.93	2.10
2	WT_2	2.61	2.02	3.29
3	WT_3	1.77	0.00	2.82
4	WT_4	2.32	1.88	2.64
5	WT_5	2.22	1.73	2.19
6	WT_6	2.00	0.00	1.86
7	WT_7	2.43	1.88	1.98
8	WT_8	1.62	0.00	3.36
9	WT_9	1.82	0.00	2.42
10	WT_{10}	1.46	1.92	2.33
11	WT_{11}	1.79	0.00	1.92
12	WT_{12}	1.32	0.00	0.00
13	Control (*F. oxysporum)*	1.24	1.62	1.86

Enzymatic Index Value = (total diameter of the colony + halo) / diameter of the colony

Fig. 1. Antagonism between *Trichoderma* isolates and *F. oxysporum* at 6 DAI. Antagonists Petri dish (WT1toWT12) have *F. oxysporum* pathogen at the top and *Trichoderma* isolates at the bottom

Protein polymorphism

SDS-PAGE analysis (Figure not shown) reveals that each of the *Trichoderma* isolates studied was unique in protein banding patterns. The differences in proteins among the isolates were expressed in terms of relative mobility (Rm) values at different loci. Two protein bands of molecular weight 29 kDa and 22 kDa were seen in most of the isolates which might be responsible for the antagonistic activity of *Trichoderma* isolates (Figure not shown). Average Group Linkage Method of the SDS-PAGE data (Fig. 2) grouped the *Trichoderma* isolates into one main cluster.

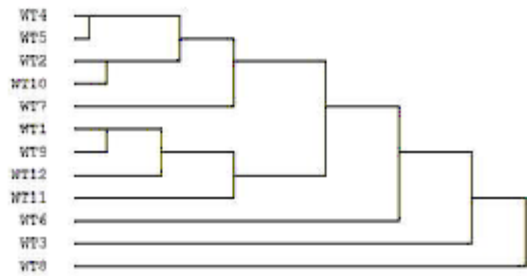

Fig. 2. Dendrogram showing relationship between different isolates of *Trichoderma* based on extracellular protein profile

This cluster is subdivided into two sub-clusters where the first sub-cluster corresponds to the isolates WT_4, WT_5, WT_2, W_{10} and WT_7. The second sub-cluster corresponds to the isolates WT_1, WT_9, WT_{12} and WT_{11}. The other isolates (WT_6, WT_3 and WT_8) did not group with either of these clusters and each formed separate branches.

In-vitro enzyme activity

Twelve *Trichoderma* isolates, tested for antagonism with fungal pathogen, produced and secreted substantial amounts of various cell wall degrading enzymes (Table 2). It was observed that the enzymatic index values of in-vitro protease activity exhibited in the range 2.61-1.32, the highest producer being isolate WT_2 (2.61) followed by isolate WT_7 (2.43). Isolate WT_{12} exhibited minimal activity with enzymatic index value of 1.32. No protease activity was noticed in case of WT_1. The isolates exhibited lipase activity with index values ranging from 2.02-1.73 (Table 3), the most potent secretor being isolate WT_2. No lipase activity was observed in isolates WT_3, WT_6, WT_8, WT_9, WT_{11} and WT_{12}. Isolates WT_4 and WT_7 showed similar activity with an index of 1.88 (Table 3). The isolates exhibited amylase index values in the range 3.36-1.86, the highest producer being WT_8 with enzymatic index value of 3.36, followed by WT_2 and WT_3 with enzymatic index value of 3.29 and 2.82 respectively. Isolate WT_{12} exhibited no amylase activity. Enzymatic index values observed with pathogen are lower than most of the *Trichoderma* isolates (Fig. 3).

Table 3. In-vitro Specific activity of cellwall degrading enzymes of *F.oxysporum* and *Trichoderma* isolates with at 6 days after inoculation (DAI).

S. No	Isolates	Enzymes		
		Protease (U/ml)	β-1,3-glucanase (U/ml)	Chitinase (U/ml)
1	WT_1	2.76	1.42	0.61
2	WT_2	5.11	3.72	2.76
3	WT_3	1.89	1.12	0.89
4	WT_4	4.72	3.12	1.96
5	WT_5	3.27	1.53	0.42
6	WT_6	2.11	2.10	0.89
7	WT_7	2.76	2.76	1.42
8	WT_8	3.79	1.48	0.32
9	WT_9	3.23	1.23	0.52
10	WT_{10}	6.24	2.93	1.83
11	WT_{11}	1.62	1.81	0.68
12	WT_{12}	3.78	2.22	0.72
13	Control *(F. oxysporum)*	0.72	0.15	0.21
	S.Em.	0.0111	0.0221	0.0099
	CD(p=0.05)	0.0332	0.0668	0.0299

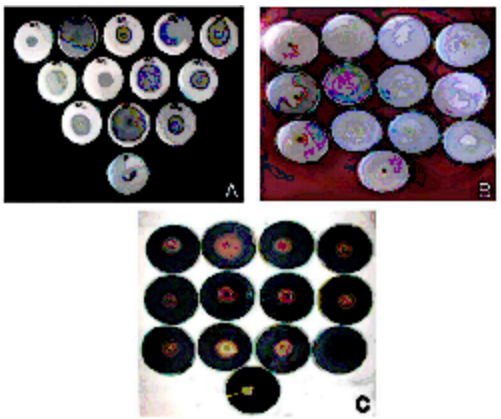

Fig. 3. Potency of production of various lytic enzymes by *Trichoderma* isolates (WT1-WT12) based on halo formation and a comparison with pathogen, *Fusarium oxysprum.* A. Protease B. Lipase C. Amylase (plates containing WT1 to WT12 isolates were arranged in numerical order from left to right, the single plate located in last row contained *F. oxysprum*)

Enzyme assays

Studies on protease activity among twelve *Trichoderma* isolates demonstrated highest activity by WT_{10} which recorded 6.24 U/ml of enzyme production where the lower limit of protease activity was observed in case of isolate WT_{11} with 1.62 U/ml protein of enzyme. β-1,3-glucanase production ranged from 3.72 -1.12 U/ml, where isolate WT_2 was found to be the highest producer. Among the isolates screened for antagonism the upper and lower limits of chitinase production were found to be 2.76 U/ml and 0.32 U/ml. The best secretor of chitinase enzyme was noticed to be WT_2 (2.76 U/ml) followed by WT_4 (1.96 U/ml) and WT_{10} (1.83 U/ml). Pathogen produced 0.21 U/ml, 0.15 U/ml and 0.72 U/ml of chitinase, β-1,3glucanase and protease respectively. The above results (Table 3) reveals WT_2 and WT_{10} isolates as potent antagonists with high percent inhibition (78.4 % and 71.4% respectively) in dual culture exhibiting high levels of cell wall degrading enzymes

DISCUSSION

Several studies have reported antagonism of *Trichoderma* species against various pathogens[5,26,27,28,29]. As dual culture method is widely used in antagonistic studies[26,30,31], the present investigation, was carried out to screen twelve strains of *Trichoderma* species against *F. oxysporum* by this method. The degree of inhibition varied from one strain to another. Maximum inhibition of the pathogen (78.4 %) was observed with WT_2. Formation of inhibition zone at the contact between *Trichoderma* and *F.oxysporum* in dual cultures could be explained on the basis of production of volatile and nonvolatile metabolites as well as the production of extracellular hydrolytic enzymes by *Trichoderma*[28].

In the present investigation, SDS-PAGE analysis of proteins of *Trichoderma* isolates revealed the presence of two protein bands (22 kDa and 29 kDa) common in almost all the isolates. A protein of molecular weight 22 kDa as established by SDS-PAGE was obtained from a commercial preparation of *Trichoderma viride* which is having an endo – 1,4 β – xylanase activity [32]. Similarly a 29 kDa protein band was obtained by SDS-PAGE from purified β–1,3-glucanase enzyme of *Trichoderma harzianum* in sequential steps by gel filtration, hydrophobic interaction and ion exchange chromatography[33]. The antagonistic activity of *Trichoderma* isolates may be due to the presence of protein bands 22 and 29 kDa which encode for the enzymes endo – 1,4 β – xylanase and β – (1,3) glucanase since most of the fungal cell wall constitute chitin and β – (1,3) glucan.

The pooled data revealed that the extracellular enzymes produced by *Trichoderma* strains may be correlated with the antagonism. *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1, 3-glucanases and proteases[34]. In the present study, WT_2 was observed to be the efficient producer of chitinases (2.76 U/ml), β-1, 3-glucanases (3.72 U/ml) and proteases (5.11 U/ml). This might be one of the reasons for its biocontrol potentiality. As fungal cell wall also contains lipids and proteins[35], the antagonistic fungi synthesize proteases which might act on the host cell wall. Involvement of proteases in biocontrol processes has already been reported[14]. The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathogenic fungi[36]. *Trichoderma* spp. attach to the host hyphae by coiling, hooks or apressorium-like structures and penetrate the host cell walls by secreting hydrolytic enzymes such as a basic proteinase[37], β-1,3-glucanase and chitinase[16]. Production of extracellular β-1,3-glucanases, chitinases and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls[37,38]. These observations, together with the fact that chitin, β-(1,3) glucan and protein are the main structural components of most fungal cell walls[39], are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens[5].

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