Antifungal Activity and Genetic Variability of *Trichoderma harzianum* Isolates

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Abstract

Trichoderma species are known to be effective antagonists of several plant pathogens. Thirteen isolates of *T. harzianum* were obtained from rhizosphere soil samples collected from field grown crops. They were tested for antagonistic capacity against soilborne pathogens viz, *Sclerotium rolfsii, Rhizoctonia solani* and *Fusarium oxysporum*. *T. harzianum* isolates effectively inhibited these pathogens (47–92%) in dual cultures. The most effective strain (Th 001) was mass cultured in Weindling medium at 25 C for 7 d. The metabolites produced in the medium were extracted with methanol and evaluated for antifungal activity. The crude metabolites at 2.5% completely inhibited all the pathogens. TLC of crude metabolites showed three antifungal compounds at R_f 0.17, 0.53 and 0.82. They were eluted individually and purified by preparative TLC and re-tested for antifungal activity. The evaluate from spot at R_f 0.53 (0.5%) showed complete inhibition of all the pathogens. To assess the genetic variability in *T. harzianum*, the isolates were analyzed by using random amplified polymorphic DNA markers. Isolates showed 78-80% similarity with standard culture of *T. harzianum* (MTCC 2050). In contrast, the isolates showed differences in the capacity to produce extracellular metabolites.

Key words: *T. harzianum*, antifungal activity, TLC, genetic variability, PCR- RAPD

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At present, around 30% of all plant species are affected by pathogens that cause disease and death of individual plants. Pesticides and organic compounds that are widely used to control plant pathogens do not degrade completely and leave toxic residues in food chain (Chet 1987; Lynch 1990). *Trichoderma* species have been increasingly used as biological control agents and a few of the isolates are commercially available (Henis 1984; Chet and Baker 1980; Chet and Inbar 1994). Results from different studies showed that several strains of *Trichoderma* exerted significant reducing effects on plant diseases caused by pathogens such as *R. solani, S. rolfsii, Pythium aphanidermatium, Fusarium oxysporum, F. culmorum* and *Gaeumannomyces graminis* under greenhouse and field conditions (Sivan and Chet 1993; Inbar et al 1994; Chet and Baker 1981). Isolates of *Trichoderma* spp. can produce lytic enzymes (Haran et al 1996) and antifungal antibiotics (Denis and Webster 1971a; Brewer et al 1987; Almassi et al 1991). It was reported that the production of metabolites from different *Trichoderma* strains depended on ecological factors, and that strains showed varying effects on pathogens (Henis 1984). Some of these metabolites have been isolated from sporulating or mycelial cultures. But repeated sub-cultures decreased the production of the peptide antibiotics by *Trichoderma* isolates (Dunlop et al 1989; Ghisalbetri and Sivasithamparam 1991). Identification of variability within *T. harzianum* isolates by morphological characters is very difficult. The random amplified polymorphic DNA (RAPD) procedure developed by Williams et al (1990) and Welsh and Mc Clelland (1990) that involves simultaneous amplification of several anonymous loci in the genome using primers of arbitrary sequence has been used for genetic, taxonomic and ecological studies of several fungi (Crowhurst et al 1995; Fungaro et al 1996; Manulis et al 1993; Zinno et al 1998; Abbasi et al 1999). In this study we describe on the antifungal activity of *T. harzianum* and genetic variability of native *Trichoderma* isolates obtained from rhizosphere regions of several crop species.

Materials and Methods

Isolation of *T. harzianum*. Seventy-four rhizosphere soil samples from field grown crops viz, castor, chilli, cotton, groundnut, grapes, redgram, tomato and sunflower in the state of Andhra Pradesh, India were collected. Each sample was mixed thoroughly and *T.*

harzianum was isolated using dilution plate and soil plate techniques on PDA and malt extract agar (MEA). *T. harzianum* colonies were identified according to the identification key based on branching of conidiophores, shape of phialides, emergence of phialides and spore characters (Gams and Bisset 1998). Pathogens viz, *S. rolfsii* and *F. oxysporum* were obtained from Directorate of Oil Seeds Research and *R.solani* from Directorate of Rice Research, Rajendranagar, Hyderabad, India. The antagonism between the isolates of *Trichoderma* and fungal pathogens was evaluated by the method of Dennis and Webster (1971b). The isolates were cultivated in petri dishes on PDA media for 7 d and then 5 mm discs were cut and transferred to another petri dish with PDA. Each plate received a disc of *T. harzianum* and another of a plant pathogen placed at 7 cm apart. Three replications were maintained for each experiment. The treatment without antagonist served as control. The plates were incubated at 25 C and percent inhibition was calculated after 7 d.

Isolation of antifungal metabolites. The most effective *T. harzianum* isolate obtained from dual cultures were inoculated into 100 ml of Weindling broth medium in 250 ml conical flask (Weindling 1941) and incubated in an orbital shaker (120 rpm) incubator at 25 C for 7 d. The cultures were then individually filtered using Whatman No 1 paper under sterile conditions to get cell-free filtrates. The secondary metabolites were extracted from each culture filtrate with equal volume of methanol and concentrated to 20 ml (Geetha et al 2003). The crude extract was tested against plant pathogens by poison food technique (Nene and Thapliyal 1971) with three replications. Methanol served as control.

Identification of antifungal metabolites on TLC. Attempts were made to isolate the active principle of the secondary metabolite and for this, the dissolved crude residue was subjected to thin layer chromatography (TLC) analysis. Both analytical (0.25 mm layer thickness) and preparative (0.5 mm layer thickness) TLC were carried out on silica gel plates using chloroform-methanol (70:30) as solvent system and spots were visualized by exposing the plates to iodine vapours (Geetha et al 2003). From the preparative TLC plates, major bands at R_f 0.17, 0.53 and 0.82 were scrapped out and extracted individually with methanol. The purity of each of the eluates was tested on analytical TLC and the solvent was evaporated to dryness. The residue was dissolved in methanol and re-tested against soilborne pathogens by poison food technique.

Isolation of genomic DNA. Actively growing mycelial plugs were inoculated in 100 ml of potato dextrose-yeast broth for 2 d at 25 C with rotary shaking at 150 rpm. Mycelia were harvested by filtration through Whatman No 1 and washed with distilled water. The extraction of total genomic DNA of each isolate of *T. harzianum* along with standard culture was made as described by Raeder and Broda (1985). The standard culture of *T. harzianum* (MTCC 2050) was procured from Microbial type culture collection, Chandigarh, India. Ten primers, five each of OPA and OPB, as well as Taq DNA polymerase and the dNTPs were obtained from Operon Technologies, CA, USA.

RAPD analysis. The amplification reactions were performed in a volume of 25 µl containing 2.5 µl of 10 x Taq DNA polymerase buffer, 2.5 U Taq polymerase, 3.0 µl of 1.25 mM of each dNTPs, 15 ng of a single random primer and 20 ng of genomic DNA. The thermo–cycling profile consisted of initial denaturation at 94 C for 1 min followed by primer annealing at 36 C for 1 min, extension at 72 C for 2 min and a final extension at 72 C for 6 min on the thermocycler model PTC 100-60 (MJ Res. Inc., USA). To visualize the profile of thus amplified products, a 10 µl aliquot from each of the reaction products was electrophoresed in 1.4% agarose gel (w/v) in 1 x TBE, stained in ethidium bromide (1 µg/ml), and photographed using a UV transilluminator with the polaroid camera attachment. One kb DNA ladder (Stratagene, USA) was used as the molecular size marker. Each amplified product was scored as 1 or 0 depending on its presence or absence of bands. The genetic associations among isolates were evaluated by dice similarity coefficient. Similarity matrices were generated using 'SIMQUAL' sub-program of software NTSYS-PC and then used for cluster analysis of varieties using 'SAHN' program of NTSYS-PC. Dendrograms were then obtained using un-weighted pair-group method with arithmetic average (UPGMA) sub-program of NTSYS-PC software version 2.0 from Exeter software, NY.

Results and Discussion

Antagonistic activity of *T. harzianum* against soilborne pathogens. Thirteen isolates of *T. harzianum* were obtained in 74 soil samples from rhizosphere regions of several crop species. All the isolates of *T. harzianum* grew considerably faster on PDA than did the pathogens, in the same conditions of culture. There was an inhibition of the growth of three phytopathogenic fungi in the presence of most of the *T. harzianum* cultures studied. This assay showed variations in the level of inhibition of radial growth of the pathogens by the different isolates of *T. harzianum*. The highest inhibition was obtained with Th 001 isolate (83-92%) against *S. rolfsii, R. solani* and *F.oxysporum* (Table 1). Many studies have proved the antagonistic potential of *Trichoderma* spp. against several soilborne pathogens (Elad et al 1982).

Table 1. Antagonistic activity of *T. harzianum* against soilborne pathogens in dual culture

Isolates	Crop	*S. rolfsii*		*R. solani*		*F.oxysporum*	
		Mycelial growth (mm)	INH (%)	Mycelial growth (mm)	% INH	Mycelial growth (mm)	% INH
Th 001	Grape	15	83	7	92	18	80
Th 002	Cotton	48	47	34	62	46	49
Th 003	Groundnut	42	53	42	53	42	53
Th 004	Red gram	34	62	40	55	40	55
Th 005	Chilli	34	62	42	53	46	49
Th 006	Sunflower	46	49	45	50	42	53
Th 007	Cotton	40	55	46	49	28	69
Th 008	Chilli	38	58	41	54	32	64
Th 009	Castor	25	72	28	69	27	70
Th 010	Castor	46	49	40	55	45	50
Th 011	Chilli	28	69	34	62	28	69
Th 012	Groundnut	34	62	48	47	35	61
Th 013	Tomato	38	58	45	50	48	47
Control		90	-	90	-	90	-
C. D (P> 0.05)		3.1	-	5.6	-	5.5	-
CV (%)		2.5	-	4.1	-	3.8	-

INH= Inhibition over control

Antifungal compounds. Metabolites of *T. harzianum* produced in culture in our study inhibited the growth of all the three soilborne plant pathogens tested invitro. The crude antifungal compounds extracted from *T. harzianum* culture filtrate at 2.5% completely inhibited all the pathogens (Table 2). *Trichoderma* species are known to produce a number of antibiotics, such as trichodermin, trichodermol, harzianum A and harzianolide (Simon and Sivasithamparam 1988; Dennis and Webster 1971a). *Trichoderma* species produce both volatile and non-volatile metabolites that adversely affect growth of different fungi (Bruce et al 1984; Corley et al 1994; Horvath et al 1995; Moses et al 1975). Dennis and Webster (1971b) found that some *Trichoderma* isolates produced volatile components, which were inhibitory to the growth of other fungi. Kucuk and Kuvanc (2003) confirmed the fact that several isolates were highly antagonistic to *R. solani*. Whipps (1988) considered *T. harzianum* to be a promising organism, particularly for use against *R. solani.* Grondona et al (1997) reported on the antifungal activity of *T. harzianum* against soilborne pathogens with 50% inhibition on all pathogens. The isolates of *T. harzianum* identified in our study also retarded the growth and sporulation of soilborne pathogens. Its rapid growth gives *Trichoderma* an important advantage in the competition for space and nutrients with plant pathogenic fungi. Acetaldehyde was identified tentatively as one of the metabolite of *T. viride* inhibitory to other fungi. Kucuk and Kivanc (2003) found that culture filtrates of *T. harzianum* were effective against soilborne pathogens. *T. harzianum* is known to produce antifungal compounds viz, azaphilone and butenolide that show marked *invitro* inhibition of *R. solani* (Vinale et al 2006). In our investigation three bands with R_f 0.17, 0.53 and 0.82 were separated as individual secondary metabolites by preparative TLC. The individual metabolite (0.5%) eluated from spot at R_f 0.53 on TLC completely inhibited growth in all three pathogens (Table 3). The remaining two compounds showed low inhibition levels. Geetha et al (2003) identified several secondary metabolites produced by *Trichoderma* spp. that were inhibitory to mosquito larvae on TLC and purified them by preparative TLC. Our study demonstrated the potential of antifungal metabolites produced by *Trichoderma* isolates from rhizosphere of field crops in the control of *S. rolfsii*, *R. solani* and *F.oxysporum*.

Table 2. Crude antifungal compounds against soilborne pathogens

Conc. (%)	*S. rolfsii*		*R. solani*		*F.oxysporum*	
0.5	70	22	58	35	62	31
1.0	48	47	32	64	48	46
1.5	26	71	20	78	36	60
2.0	14	84	10	89	22	75
2.5	0	100	0	100	0	100
Control	90	-	90	-	90	-
C. D (P> 0.05)	4.2	-	6.2	-	3.1	-
CV (%)	1.6	-	4.6	-	2.4	-

Table 3. Individual antifungal compounds against soilborne pathogens

Antifungal compound and Conc. (%)	*S. rolfsii*		*R. solani*		*F.oxysporum*	
	Mycelial growth (mm)	% INH	Mycelial growth (mm)	% INH	Mycelial growth (mm)	% INH
Antifungal compound (R_f 0.17)						
0.1	90	0	90	0	90	0
0.2	82	9	86	4	80	11
0.3	74	18	78	13	72	20
0.4	68	24	72	20	61	32
0.5	54	51	65	28	48	47
Antifungal compound (R_f 0.53)						
0.1	54	40	48	47	58	35
0.2	42	53	32	64	40	55
0.3	28	69	15	83	28	69
0.4	12	87	8	91	19	79
0.5	0	100	0	100	0	100
Antifungal compound (R_f 0.82)						
0.1	78	13	72	20	84	7
0.2	72	20	64	29	73	19
0.3	59	34	49	45	56	38
0.4	42	53	38	58	48	47
0.5	35	61	25	72	35	61
Control	90	-	90	-	90	-
C. D (P> 0.05)	5.8	-	4.8	-	3.2	-
CV (%)	4.2	-	4.4	-	2.6	-

INH= Inhibition over control

RAPD polymorphism. Of the 10 primers tried, six primers (OPA 02, OPA 03, OPA 05, OPA 08, OPB 01 and OPB 02) failed to effect any amplification in the isolates tested, while four other primers (OPA 04, OPB 08, OPB 09 and OPB 11) showed amplification. Primer OPB 11 showed the highest amplification in all the isolates compared to others. A total of 230 loci were produced with the four primers used. The polymorphism between the isolates varied from 37 to 100 per cent. All the strains produced 1 to 11 bands (Fig 1).

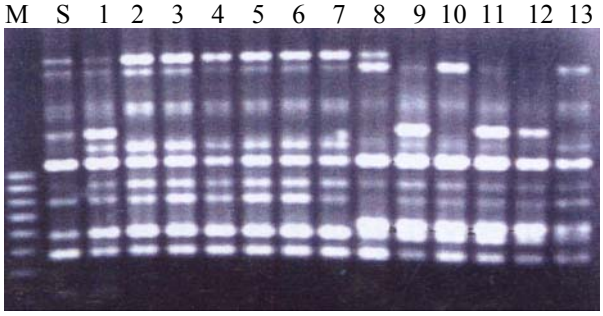

Fig 1. RAPD patterns of *T. harzianum* isolates with OPB 11 primer

M- 1kb ladder, S- Standard culture of *T. harzinum* (MTCC 2050), 1- Th 001, 2- Th 002, 3- Th 003, 4- Th 004, 5- Th 005, 6- Th 006, 7- Th 007, 8- Th 008, 9- Th 009, 10- Th 010, 11- Th 011, 12- Th 012, 13- Th 013.

Genetic similarity among the *T. harzianum* isolates. Among the 13 isolates, only one isolate, Th 012 showed a negligible divergence from others including standard culture. The isolates grouped together with its standard culture of *Trichoderma* with 80 per cent similarity. Six isolates (Th 002 to Th 007) and 2 isolates (Th 009 and Th 011) showed 100 per cent similarity (Fig 2). In contrast, the isolates showed differences in the capacity to produce extracellular metabolites. Muthumeenakshi et al (1998) characterized genetically 15 aggressive strains of *T. harzianum* from mushroom compost in the United States and England using RAPD, The strains of *T. harzianum* assigned to group 4 that presented a high degree of homogeneity. They compared with molecular data of group 4 with group 2 that caused epidemic green mold in industrial mushrooms in England. This study had indicated that the isolates of *T. harzianum* group 4 were different from that of group 2. Goes et al (2002) used RAPD to examine the genetic variability among the 14 isolates of *Trichoderma*. Gomez et al (1997) analyzed the RAPD profiles of strains of *T. harzianum* and classified them in different groups according to their capacity for control of plant pathogenic fungi. Schlick et al (1994) used RAPD to analyze the strains of *T. harzianum* and mutants induced by gamma radiation that originated from a wild isolate. With RAPD, it was possible to differentiate all these mutant strains for at least one primer. Therefore, it was concluded that the

method was valuable for identification and fast differentiation of strains. In our study also RAPD helped to confirm the identity of isolates as *T. harzianum*.

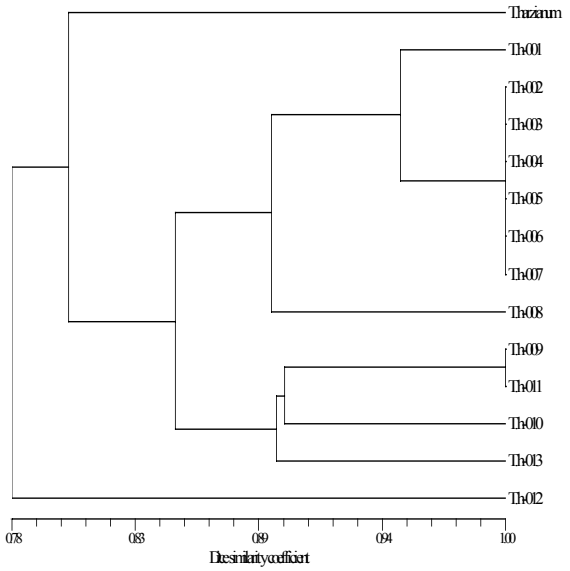

Fig 2. UPGMA dendogram based on the DICE similarity index illustrating the genetic relationships among *T. harzianum* isolates

We have demonstrated that secondary metabolites of *T. harzianum* isolate (Th 001) possessed a high level of antagonistic effect on soilborne pathogens. This isolate is a potential candidate for further mass production and use in biological control of these ubiquitous pathogens.

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