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BIOCHEMICAL CHARACTERIZATION OF EFFICIENT *TRICHODERMA* SPECIES IN ASSOCIATION TO THEIR TOLERANCE EFFICACY TO FUNGICIDES

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ABSTRACT
Development of tolerance in efficient *Trichoderma* spp. isolates against fungicides as a part of the biocontrol strategy in plant disease management aimed to make biocontrol agent more effective in suppressing phytopathogens. Eight isolates belonging to Trichoderma harzianum, *T. viride, T. virens* and *T. asperellum* collected from rhizospheric soils of tomato, brinjal, sorghum, tobacco and groundnut were morphologically characterized revealing significant differences in terms of colony characters; sporulation; mycelial form; branching of conidiophores; phialospores color and shape. In vitro studies on effect of fungicides on radial growth of as well as tolerance induction in *T. viride, T. harzianum, T. virens* and *T. asperellum* subjected to increasing gradient of fungicides showed that the bioagents acquired tolerance to the higher doses of fungicides *viz*. Metalaxyl, Thiram, Mancozeb, Chlorothalonil, Copper oxychloride. While evaluating the ED50 values for fungicides, it indicated *T. viride, T. harzianum, T. virens* and *T. asperellum* could tolerate maximum to Metalaxyl followed by Metiram, Mancozeb, Thiram and Copper oxychloride even at higher concentration and not to Hexaconazole, Carbendazim, Propiconazole and Carbendazim (12%) + Mancozeb (63%). Protein profile of *T. viride, T. harzianum, T. virens* and *T. asperellum* isolates tolerant to higher concentration of fungicides analysed through SDS-PAGE indicated the increase in the protein content in form of more number of protein bands of higher molecular weight as compared to non-tolerant isolates.

INTRODUCTION

Application of biocontrol for the disease management has not taken off well due to susceptibility of bioagents to abiotic and pesticide induced stresses. Fungal bioagents are generally sensitive to pesticides, though in a pesticide free environment, they have been reported to be effective to the tune of 50-60% (Anon., 2010). *Trichoderma* spp. have proved effective and selective enough as biological control agents against a range of crop diseases due to their high reproductive capacity, ability to survive odd conditions, efficiency in the utilization of the nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms (Chet *et al.*, 1997).

Different species of *Trichoderma* have gained considerable importance either alone or in integration with lower dose of fungicides for the management of soil borne plant pathogens (Sharma and Mishra, 1995; Upadhyay and Mukhopadhyay, 1986). Several important plant diseases caused by *Pythium* spp., *Phytophthora* spp., *Rhizoctonia* spp., Fusarium spp. and *Sclerotium rolfsii*, etc. have been reported to be managed effectively by *T. viride, T. harzianum, T. hamatum* and *T. longibrachiatum* (Kumar *et al.*, 2012) Excellent results of integrated management have been attained with strains of T. *virens* and Metalaxyl against Pythium ultimum infecting cotton (Anahosur, 2001), *T. harzianum* and Captan against Verticillium dahliae infecting potato (Chet and Inbar, 1994), *T. virens* and Thiram against Rhizoctonia solani infecting tobacco and others (Chet *et al.*, 1997), Carbendazim and Trichoderma viride against Fusarium *oxysporum f.* sp. ciceri infecting chickpea (Patil *et al.*, 2015). Integration of bioagent with lower or sub-lethal doses of agrochemicals seems to be very promising and practical approach with a minimal interference to biological equilibrium (Papavizas *et al.*, 1982 and Cook and Baker, 1983). Hastie (1981) discussed the possible role of fungicides in formation of new fungal biotypes and opined that the fungicides may cause gene mutation, chromosome breakage, mitotic non-disjunction and mitotic recombination. The alterations like lysis of cell walls, extrusion of cytoplasmic material and irregular hyphal swelling, induced by this product may be due to the fact that it is an benzimidazole fungicide, that reduces sterol biosynthesis and inhibits protein synthesis, affecting cell membrane permeability. The molecular technique like Random Amplified Polymorphic DNA (RAPD) developed by Williams *et al.* (1990) has been used for genetic and taxonomic studies for several fungi including *Trichoderma* sp. (Muthumeenakshi and Mills, 1995; Dodd *et al.*, 2004) due to the complexity and closely related characters of the species showing morphological similarities. The Internal Transcribed Spacer (ITS) region of the ribosomal DNA is the most widely sequenced DNA region

in fungi. It has been most useful for molecular systemic study at species level, and even within species (Ospina-Giraldo *et al*., 1998; Kubieck *et al*., 2000; Kulling *et al*., 2002; Lee and Hseu, 2002).

Trichoderma spp. include a wide range of strains which differ in their adaptiveness, innocuousness and effectiveness as potential biocontrol agents. The success of this biocontrol agent also depends on the applicability of selected isolates to diûerent ecozones (Grondona *et al*., 1997). Furthermore, it is difficult to predict the degree of synergism and the behavior of a biocontrol agent in a natural pathosystem along with various biotic and abiotic stresses. It is, therefore, essential to evaluate *Trichoderma* isolates under a range of biotic and abiotic conditions. This will help in selecting the most eûcient and antagonistic strains for biocontrol ability. Fungicide use in agriculture is inevitable. Hence developing fungicide tolerant isolates for the disease management is a present day need.

Keeping in view the above issues, characterization of *Trichoderma* spp. isolates was undertaken based on morphological and molecular background in association to their sensitivity, growth inhibition and induction of tolerance towards fungicides and protein profiling through SDS-PAGE.

MATERIALS AND METHODS

Morphological Characterization

Soil samples were collected from rhizosphere of crops *viz*. Tomato, Brinjal, Sorghum, Tobacco, Groundnut grown in Anand region of middle Gujarat. The serial dilution plate technique was followed for isolation. Besides, Three local identified isolates *viz*. *T. viride, T. harzianum* and *T. virens* were also used in the study. Trichoderma spp. were isolated on Trichoderma specific selective medium with certain modifications (Elad *et al*., 1982). TSMC contained (gm/lit); $MgSO_4$.$7H_2O$-0.2; K_2HPO_4 -0.9; KCl-0.15; NH_4NO_3-1.0; Glucose-3.0, Chloramphenicol-0.25, Metalaxyl-0.3, Pentachloronitrobenzene-0.2, Rose Bengal-0.15, Captan-0.02 (post autoclaving), Agar-20. The isolated *Trichoderma* spp. maintained thereafter on the potato dextrose agar (PDA) medium without the antibiotic respectively. The morphological characterization and grouping of isolates was performed based on the key provided by Bissett (1991 a,b,c) as well as with literature *i.e. Trichoderma*: A Review of biology and systematics of the genus (Samuels, 1996), *Trichoderma*: a guide to identification and biology (Samuels, 2004) and Monographic contribution on *Trichoderma* pers. ex Fr. (Nagamani *et al*., 2002) which included the characters classified under colony, mycelia and spore patterns.

Sensitivity of *Trichoderma* spp. isolates to fungicides

Sensitivity on radial growth of efficient isolates of *Trichoderma* spp. viz. *T. viride, T. virens*, *T. harzianum* and *T. asperellum* to selected fungicides were evaluated using Poisoned food technique (Grover and Moore, 1961). The isolates of *T. viride*, *T. virens, T. harzianum, T. asperellum* were sub-cultured in a PDA medium containing progressively increasing three concentrations of the respective fungicides. The fungicides used in the study were Hexaconazole [(RS)-2-(2,4-dichlorophenyle)-1-(1H-1,2,4-triazol-1-yl) hexan-2-ol] (25, 50, 100 ppm), Carbendazim [1-H – Benzimidazol – 2 – ylcarbamic acid methyl ester] (100, 200, 250 ppm), Carbendazim (12%) + Mancozeb (63%) [1-H – Benzimidazol – 2 – ylcarbamic acid methyl ester + (Ethylenebisdithiocarbamato) manganese mixture with (ethylenebisdithiocarbamato) zinc] (25, 50, 100 ppm), Mancozeb [(Ethylenebis (dithiocarbamato)) manganese mixture with (ethylenebis (dithiocarbamato))] zinc (25, 50, 100 ppm), Copper oxychloride [Dicopper chloride trihydroxide] (200, 400, 600 ppm), Hexaconazole (5%) + Captan (70%) [2, 4 – triazole-1- ethanol + N- trichloromethylthio -4-cyclohexene -1, 2- dicaroximide] (50, 100, 200 ppm), Metalaxyl [Methyl N-(methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate] (500, 750, 1000 ppm), Thiram [bis(dimethyl thiocarbamoyl) disulphide] (25, 50, 100 ppm), Chlorothalonil [tetrachloro-isophthalonitrile] (50, 100, 200 ppm), Metiram [zinc ammoniate ethylene bis (dithiocarbamate)- poly (ethylenethiuram)] (100, 200, 300 ppm), Propiconazole [(+)-1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl methyl]-1H-1,2,4-triazole] (50, 100, 200 ppm) and their tolerance were tested. The fungicides were incorporated in sterilized PDA medium so as to give required concentrations and poured in petriplates keeping five replications of each treatment. First inoculation was made by taking 5 mm. mycelial disc of *T. viride, T. harzianum, T. virens* and *T. asperellum* multiplied on PDA for 72 hr. Subsequently, second, and third inoculation transfers were made in a similar way with the fungus obtained after 96 hr growth on successive concentrations. The plates were incubated at 28 ± 1ºC for 7 days and colony diameter and percent growth inhibition was measured (Vincent, 1947).

Statistical analysis

Data obtained during the investigation were subjected to statistical analysis by making use of analysis of variance technique (Steel and Torrie, 1980). The standard methods of analysis of variance for Completely Randomized Design was used in the experiments. The test of significance among the treatments was worked out by 'F' test. The appropriate standard error (S. Em. $\pm$) was computed in each case. For the treatments effects, which were found to be significant, the critical difference (CD) at 5 % level of probability was worked out to compare two treatment means.

ED50 values of fungicides

ED50 values of systemic and non-systemic fungicides were determined by plotting the chemical concentrations against percent inhibition on a log probit scale as described by Horsfall (1956).

Protein Profiling of efficient fungicide tolerant as well as non tolerant isolates of *Trichoderma* spp. by using SDS- PAGE

Enzymatic variability in the protein profile of tolerant as well as non-tolerant isolates was studied with the help of "SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)" as described by Laemmli, (1970). Separating gel of 10 % strength and 5 % staking gel were prepared. Sodium dodecyl sulphate gel electophoresis (SDS-PAGE) was used to determine molecular size of various extracellular enzymes. For an accurate estimation of the molecular masses of *Trichoderma* spp. isolates enzymes, proteins were separated by SDS-PAGE as above using a Cleaver Scientific Protein Electrophoresis cell in larger gels.

Induction of higher tolerance in efficient isolates to higher concentration of selected fungicides

The efficient parent isolates of *Trichoderma* spp. found tolerant to sub-lethal doses of a fungicide were sub-cultured in PDA media containing progressively increasing concentrations for three number of generations (transfer) of fungicide so as to obtain more tolerant isolates. The fungicides with concentrations used in these experiments were Metalaxyl (1500, 2500, 5000 ppm), Thiram (150, 250, 400 ppm), Mancozeb (150, 250, 400 ppm), Chlorothalonil (300, 400, 500 ppm) and Copper oxychloride (750, 850, 1000 ppm). The parent isolate was exposed initially to first generation series of fungicide on potato dextrose agar (PDA), for the second generation series, the inoculants disks were taken from the culture that initially grew on first generation series and successively placed on PDA with concentration of fungicide resulting second generation series, likewise, it was followed for third generation series to induce higher tolerance in efficient *Trichoderma* isolates.

RESULTS AND DISCUSSION

Morphological Characterization

The growth patterns of eight *Trichoderma* isolates cultured on PDA was determined. Similarity a fine layer of 1 mm having 2 percent water agar plates with 0.5 percent of dextrose medium in plate inoculated with the bioagent were studied after four days of incubation at 28± 1° C which showed significant differences in nature of culture growth, sporulation patterns, phialides shape, conidiophores branching pattern, shape, conidia shape and ornamentation The Petridish was completely occupied with *Trichoderma* spp. culture growth within three to four days. The eight isolates of *Trichoderma* spp. produced green coloured loosely floccose to compactly tufted colonies and sporulated profusely producing masses of green spores in patches or in defined rings. The detail characteristics of the bioagents are given in table 1. These colonies were white in colour to begin with and turned to yellowish to greenish shades with or without concentric rings. The mycelia form was floccose to arachnoid and mostly mycelia appeared whitish in color. Conidiophores were regular to irregularly branched with conical to pyramidal in shape and compact to loose in pattern. Phialides were hyaline with globose to flask shaped forming large globoid masses. Phialospores were globose, ellipsoidal to subglobose with light green to dark green in colour (Fig. 1).

Table 1: Status of the Trichoderma spp. isolates used in study

Number	Isolate	Origin
1	*T. viride*	Local identified
2	*T. virens*	Local identified
3	*T. harzianum*	Local identified
4	*T. viride*	Brinjal rhizosphere
5	*T. asperellum*	Sorghum rhizosphere
6	*T. harzianum*	Tomato rhizosphere
7	*T. harzianum*	Tobacco rhizosphere
8	*T. viride*	Groundnut rhizosphere

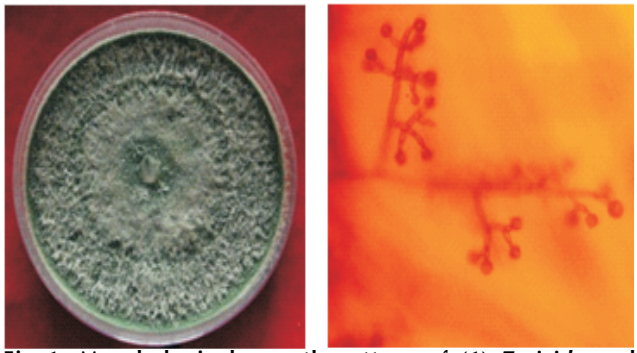


Fig 1: Morphological growth pattern of (1) *T.viride* and Microphotograph (2) *T.viride*

Sensitivity of isolates of *Trichoderma* spp. against fungicides

Effect of fungicides on Radial growth *in vitro*

The data on the effect of fungicides on radial growth (Table 2a) of *T. viride* at various three concentrations with different

Table 2a. Growth inhibition of *T. viride* against fungicides in vitro

Sr. No.	Fungicides	Concentration	Radial growth (cm)	Per cent Growth Inhibition (%)	Fungicide Mean (F)
1	Propiconazole	50	0.4	98.33	98.89
		100	0.45	98.66	
		200	0.2	99.66	
2	Hexaconazole	25	1.2	86	87.22
		50	1.1	87	
		100	1	88.67	
3	Copper oxychloride	200	8.4	1	10.56
		400	8.3	3	
		600	6	27.67	
4	Carbendazim -12% + Mancozeb -63%	25	0.6	97	98.44
		50	0.4	98.67	
		100	0.2	99.67	
5	Captan (70%) + Hexaconazole (5%)	50	5	42	54.67
		100	3.6	56.67	
		200	2.8	65.33	
6	Metiram	100	8.5	0	1.56
		200	8.2	1.67	
		300	8	3	
7	Chlorothalonil	50	4	52.67	60.56
		100	3.4	63	
		200	3	66	
8	Mancozeb	25	8.3	1.33	2.44
		50	8.2	1.67	
		100	7.8	4.33	
9	Thiram	25	8.4	1	2.44
		50	8.1	2	
		100	7.8	4.33	
10	Metalaxyl	500	8.5	0.67	1.44
		750	8.3	1.33	
		1000	8.1	2.33	
11	Carbendazim	100	0.5	96.67	98..44
		200	0.3	99	
		250	0.2	99.67	
12	Control	—-	—-	0	0
Concentration mean (C) at all three concentrations		44.45 (1st)	46.18 (2nd)	50.27 (3rd)	—-
		S.Em±	C.D. (0.05)		C.V. (%)
Fungicides (F)		0.703	1.986		—-
Concentration (C)		0.367	1.037		—-
Interaction (FxC)		1.217	3.439		4.49

Table 2b. Growth inhibition of *T. harzianum* against fungicides *in vitro*

Sr. No.	Fungicides	Concentration	Radial growth (cm)	Per cent Growth Inhibition (%)	Fungicide Mean (F)
1	Propiconazole	50	0.2	98.67	99.11
		100	0.1	99.33	
		200	0.1	99.33	
2	Hexaconazole	25	1.1	84	86.56
		50	1	87	
		100	0.8	88.67	
3	Copper oxychloride	200	7.8	4.33	37.56
		400	5.1	45	
		600	1.9	65.33	
4	Carbendazim -12% + Mancozeb -63%	25	0.3	99	99.44
		50	0.2	99.67	
		100	0.2	99.67	
5	Captan (70%) + Hexaconazole (5%)	50	3.5	61.33	64
		100	3.4	63.33	
		200	2.9	67.33	
6	Metiram	100	8.5	0	0
		200	8.5	0	
		300	8.5	0	
7	Chlorothalonil	50	4.7	56.67	60.44
		100	3.5	61.33	
		200	3.4	63.33	
8	Mancozeb	25	8.5	0	0.22
		50	8.4	0.33	
		100	8.4	0.33	
9	Thiram	25	8.5	0	1.44
		50	8.5	0	
		100	7.8	4.33	
10	Metalaxyl	500	8.5	0	0
		750	8.5	0	
		1000	8.5	0	
11	Carbendazim	100	0.3	99	99..56
		200	0.2	99.67	
		250	0.2	100	
12	Control	—-	—-	0	0
Concentration mean (C) at all three concentrations		45.82 (1st)	50.52 (2nd)	53.24 (3rd)	—-
		S.Em±	C.D. (0.05)		C.V. (%)
Fungicides (F)		0.335	0.947		—-
Concentration (C)		0.175	0.494		—-
Interaction (FxC)		0.58	1.64		2.02

fungicides indicated that there was very little inhibition of radial growth *i.e.* 0.67 percent by Metalaxyl at 500 ppm. the bioagent could grow successively upto 1000 ppm. However, there was a increasing trend in the inhibition of growth with the increasing concentration with fungicides *i.e.* Captan (70%) + Hexaconazole (5%) and Chlorothalonil at 50, 100 and 200 ppm which was observed upto 65.33 and 66 percent respectively. A similar trend was observed in case of Copper oxy chloride but it was moderately inhibitory in comparison to Captan (70%) + Hexaconazole (5%) and Chlorothalonil which exhibited 27 percent inhibition upto 600 ppm concentration. Among other fungicides tested, Metiram, Mancozeb and Thiram showed very low inhibition *viz*. 3.0, 4.33 and 4.33 percent to the bioagent at 300, 100 and 100 ppm respectively. Hexaconazole and Carbendazim were next in order of the toxicity to the bioagent as both showed 88.66 and 99.66 percent growth inhibition at 100 and 250 ppm, respectively. Propiconazole and Carbendazim (12%) + Mancozeb (63%) were found most toxic which exhibited 99.66 and 99.66 percent growth inhibition at 200 and 100 ppm. The growth was recorded even at 1000 ppm in Metalaxyl whereas in Carbendazim (12%) + Mancozeb (63%), Propiconazole, Hexaconazole and Carbendazim, it stopped at 100, 200, 100 and 250 ppm, respectively, which means T. viride could tolerate almost 10 times the concentration of metalaxyl than Carbendazim (12%) + Mancozeb (63%) and Hexaconazole while 5 times that of the concentration of Propiconazole and Carbendazim (Fig. 2a and b).

The result of effect of various fungicides at different three concentrations on radial growth (Table 2b) of *T. harzianum* revealed no inhibition of radial growth by Metalaxyl at a concentration *viz*. 500 ppm. The bioagent could grow safely upto 1000 ppm. However, there was a increasing trend in the inhibition of growth with the increasing concentration with

Table 2c : Growth inhibition of *T. virens* against fungicides *in vitro*

Sr. No.	Fungicides	Concentration	Radial growth (cm)	Per cent Growth Inhibition (%)	Fungicide Mean (F)
1	Propiconazole	50	0.9	89	96.22
		100	0.2	99.67	
		200	0	100	
2	Hexaconazole	25	1.2	85.67	88.11
		50	1	88	
		100	0.7	90.67	
3	Copper oxychloride	200	8.3	1.33	13.33
		400	8.1	2.67	
		600	5.2	36	
4	Carbendazim (12%) +	25	0.3	99	99.33
	Mancozeb	50	0.25	99.33	
	-63%	100	0.2	99.67	
5	Captan (70%) +	50	3.8	77	83.67
	Hexaconazole (5%)	100	1.1	84.67	
		200	0.9	89.33	
6	Metiram	100	8.5	0	0.67
		200	8.4	0.33	
		300	8.2	1.67	
7	Chlorothalonil	50	6	27.67	35.17
		100	5.6	35.33	
		200	5.3	44.33	
8	Mancozeb	25	8.5	0	0.89
		50	8.3	1	
		100	8.2	1.67	
9	Thiram	25	8.1	2	3.67
		50	8.1	2	
		100	7.4	7	
10	Metalaxyl	500	8.2	1.67	4.44
		750	7.8	4.33	
		1000	7.3	7.33	
11	Carbendazim	100	0.3	99	99.33
		200	0.25	99.33	
		250	0.2	99.67	
12	Control	—-	—-	0	0
Concentration mean (C) at all three concentrations		44.27 (1st)	46.84 (2nd)	52.18 (3rd)	—-
		S.Em ±	C.D. (0.05)		C.V. (%)
Fungicides (F)		0.467	1.319		—-
Concentration (C)		0.244	0.689		—-
Interaction (FxC)		0.808	2.284		2.93

fungicides *i.e.* Captan (70%) + Hexaconazole (5%) and Chlorothalonil 50, 100 and 200 ppm which was observed upto 67.33 and 63.33 percent, respectively. A similar trend was observed in case of copper oxy chloride, but it was moderately inhibitory in comparison to Captan (70%) + Hexaconazole (5%) and Chlorothalonil at lower two dose i.e. 200 and 400 ppm while it exhibited similar percent growth inhibition upto 600 ppm concentration which was 63.33 percent. Among other fungicides tested, Metiram, Mancozeb and Thiram proved to be very compatible and safe with 0.0, 0.33 and 4.33 percent to the bioagent at 300, 100 and 100 ppm, respectively. Hexaconazole and Propiconazole were next in order of the toxicity to the bioagent as both showed 88.66 and 99.33 percent growth inhibition at 100 and 200 ppm respectively. Carbendazim and Carbendazim (12%) + Mancozeb (63%) were found most toxic which exhibited 100.0 and 99.66 percent growth inhibition at 250 and 100 ppm.

Among various fungicides tested at different three concentrations (Table 2c) on radial growth of *T. virens*, the results depicted less inhibition of radial growth 0.0 percent by Metiram at 100 ppm. The bioagent could grow safely upto 300 ppm. However, there was a increasing trend in the inhibition of growth with the increasing concentration with fungicides *i.e.* Captan (70%) + Hexaconazole (5%) and Chlorothalonil at 50, 100 and 200 ppm which was observed upto 89.33 and 44.33 percent, respectively. A similar trend was observed in case of Copper oxychloride, but it was moderately inhibitory in comparison to Captan (70%) + Hexaconazole (5%) and Chlorothalonil upto 600 ppm concentration gave only 36.0 percent growth inhibition. Among other fungicides tested, Metalaxyl, Mancozeb and Thiram proved to be very compatible and safe at 1000, 100 and 100 ppm here in 7.33, 1.66 and 7.0 percent growth inhibition was obtained. Hexaconazole was next in order of

Table 2d : Growth inhibition of *T. asperellum* against fungicides *in vitro*

Sr. No.	Fungicides	Concentration	Radial growth (cm)	Per cent Growth Inhibition (%)	Fungicide Mean (F)
1	Propiconazole	50	1.1	86.33	95.11
		100	0.2	99.67	
		200	0	100	
2	Hexaconazole	25	1.2	86.67	88.78
		50	0.8	88.67	
		100	0.7	91	
3	Copper oxychloride	200	8.5	0	0.78
		400	8.4	0.67	
		600	8.2	1.67	
4	Carbendazim -12% + Mancozeb -63%	25	0.2	99.67	99.78
		50	0.2	99.67	
		100	0	100	
5	Captan (70%) + Hexaconazole (5%)	50	4.3	50.33	63.11
		100	3.1	66.33	
		200	2.8	72.67	
6	Metiram	100	8.5	0	0.44
		200	8.45	0.33	
		300	8.3	1	
7	Chlorothalonil	50	3.6	60.33	64.11
		100	3.4	63	
		200	3	69	
8	Mancozeb	25	8.5	0	0.44
		50	8.45	0.33	
		100	8.4	0.67	
9	Thiram	25	8.45	0.33	1.67
		50	8.4	0.67	
		100	7.9	4	
10	Metalaxyl	500	8.5	0	0.56
		750	8.4	0.67	
		1000	8.3	1	
11	Carbendazim	100	0.3	99	99.33
		200	0.25	99.33	
		250	0.2	99.67	
12	Control	—-	—-	0	0
Concentration mean (C) at all three concentrations		44.18 (1^{st}) S.Em ±	47.21 (2^{nd}) C.D. (0.05)	48.87 (3^{rd})	—- C.V. (%)
Fungicides (F)		0.361	1.02		—-
Concentration (C)		0.188	0.532		—-
Interaction (FxC)		0.625	1.766		2.32

the toxicity to the bioagent as it showed 90.66 percent growth inhibition at 100 ppm respectively. Propiconazole, Carbendazim and Carbendazim (12%) + Mancozeb (63%) were found most toxic which exhibited 100.0, 99.66 and 99.66 percent growth inhibition at 200, 250 and 100 ppm.

Among various fungicides tested at three concentrations (Table 2d) on radial growth of *T. asperellum*, the results showed less inhibition of radial growth *i.e.* 0.0 percent by Metalaxyl at 500 ppm. The bioagent could grow safely upto 1000 ppm. However, there was a increasing trend in the inhibition of growth with the increasing concentration of fungicides *i.e.* Captan (70%) + Hexaconazole (5%) and Chlorothalonil at 50, 100 and 200 ppm which was observed upto 72.66 and 69.0 percent, respectively. Among other fungicides tested, Metiram, Mancozeb, Copper oxy chloride and Thiram proved to be compatible and safe with 1.0, 0.66, 1.66 and 4.0 percent growth inhibition to the bioagent at 300, 100, 600 and 100 ppm, respectively. Hexaconazole was next in order of the toxicity to the bioagent as it showed 91.0 percent growth inhibition at 100 ppm. Propiconazole, Carbendazim and Carbendazim (12%) + Mancozeb (63%) were found most toxic which exhibited 100.0, 99.66 and 100.0 percent growth inhibition at 200,250 and 100 ppm, respectively. The compatibility effect of Metalaxyl, Mancozeb, Chlorothalonil and Copper oxychloride on growth and biological activity of *T. viride, T.virens* and *T. harzianum* (Saju *et al.*, 2005; Madhusudan *et al.*, 2010) have been described. sMore specifically, all the antagonists were highly sensitive to Carbendazim, Hexaconazole, Propiconazole for mycelial radial growth as stated by other authors (Singh *et al.*, 2010; Sarkar *et al.*, 2010).

ED50 values of fungicides against efficient isolates of

Table 3 : ED50 values of fungicides against *T. virens, T. asperellum, T. viride* and *T. harzianum*

Fungicide	ED50 for Radial growth (ppm)			
Propiconazole	28	31	25	25
Hexaconazole	15	16	15	16
Copper oxychloride	548	825	675	364
Carbendazim (12%) + Mancozeb (63%)	13	14	15	13
Captan (70%) + Hexaconazole (5%)	16	23	33	22
Metiram	425	436	485	515
Chlorothalonil	205	44	42	39
Mancozeb	162	172	164	182
Thiram	248	362	348	360
Metalaxyl	2875	3478	3235	3745
Carbendazim	54	54	54	54
Control	0	0	0	0

Fig. 2a : Sensitivity of different *Trichoderma* spp. against Metalaxyl at three concentrations

***Trichoderma* spp.**

ED50 values for radial growth indicated maximum tolerance of *T. viride* for Metalaxyl at 3235 ppm. It was followed by Copper oxychloride (675 ppm) and Metiram (485 ppm) Whereas, ED50 values of Thiram, Mancozeb and Carbendazim were recorded as 348, 164 and 54 ppm. The value for Chlorothalonil against T. viride has been found to be 42 ppm which was next in toxicity order as compared to Captan (70%) + Hexaconazole (5%) and Propiconazole *i.e.* 33 and 25 ppm for growth of *T. viride* while Hexaconazole and Carbendazim (12%) + Mancozeb (63%) were found highly toxic for radial growth each with 15 ppm.

ED50 values for radial growth indicated maximum tolerance of T. harzianum for the Metalaxyl at 3745 ppm. It was followed by Metiram (515 ppm), Copper oxychloride (374 ppm) and Thiram (360 ppm) Whereas, ED50 values of Mancozeb and Carbendazim were recorded as 182 and 54 ppm, respectively. The value for Chlorothalonil against *T. harzianum* has been reported to be 39 ppm which was next in toxicity order as compared to Propiconazole, Captan (70%) + Hexaconazole (5%) and Hexaconazole *i.e.* 25, 22 and 16 ppm respectively for growth of *T. harzianum,* while Carbendazim (12%) + Mancozeb (63%) at 13 ppm was found highly toxic for radial growth.

ED50 values for radial growth indicated maximum tolerance of *T. virens* for the Metalaxyl at 2875 ppm. It was followed by Copper oxychloride (548 ppm) and Metiram (425 ppm). Whereas, ED50 values of Thiram and Chlorothalonil were recorded as 248 and 205 ppm, respectively. The value for Mancozeb and Carbendazim against *T. virens* has been reported to be 162 and 54 ppm respectively which was next in toxicity order as compared to Propiconazole, Captan (70%) + Hexaconazole (5%) and Hexaconazole *i.e.* 28, 16 and 15 ppm for growth of *T. virens* while Carbendazim (12%) + Mancozeb (63%) was found highly toxic for radial growth viz. 13 ppm.

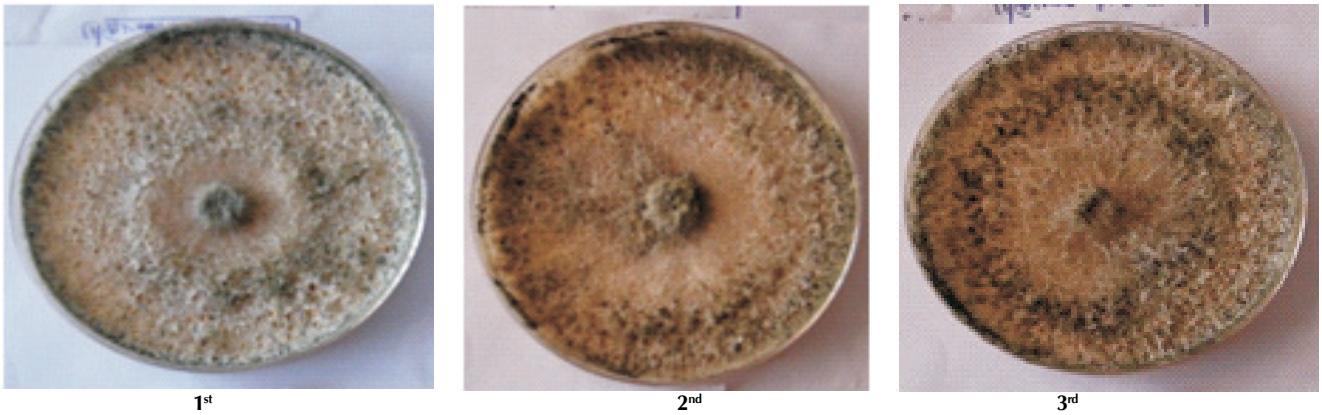
ED50 values for radial growth indicated maximum tolerance of *T. asperellum* for the Metalaxyl at 3478 ppm. it was followed by Copper oxychloride (825 ppm) and Metiram (436 ppm) Whereas, ED50 values of Thiram and Mancozeb were recorded as 362 and 172 ppm, respectively. The value for Carbendazim and Chlorothalonil against *T. asperellum* has been reported to be 54 and 44 ppm which was next in toxicity order as compared to Propiconazole, Captan (70%) + Hexaconazole (5%) and Hexaconazole *i.e.* 31, 23 and 16 ppm respectively for growth of *T. asperellum* while Carbendazim (12%) + Mancozeb (63%) was found highly toxic for radial growth viz. 14 ppm (Table 3). According to the literature *T. virens, T. viride* and *T. harzianum* (Sharma *et al.*, 2001) are compatible with Metalaxyl and showed higher tolerance in *T. harzianum* with ED50 value as 1050μg/ml and very high sensitivity towards Thiram with ED50 value as 25 μg/ml.

Protein Profiling of efficient fungicide tolerant isolates of *Trichoderma* spp.

T. viride, T. harzianum, T. virens and *T. asperellum* were tested against different fungicides and moderate to higher level tolerance was observed and effect of induced tolerance on protein behavior of bioagents were further characterized by SDS-PAGE method. Increase in protein content in form of banding pattern of *T. viride, T. harzianum, T. virens* and *T. asperellum* exposed to higher concentration of fungicides was observed through SDS-PAGE (Fig.3).

T. viride exposed at 1000 ppm of Metalaxyl and Copper oxychloride recorded a maximum of seven protein bands having 10 KDa to 159.48 and 10 KDa to 154.93 KDa molecular weight. Out of these, four protein bands were found to possess molecular weight between 112.64 KDa to 159.48 and 35 KDa to 154.93 KDa which were more as compared to control having two protein bands of low molecular weight of 13.72 and 81.24 KDa. *T. viride* exposed at 100 ppm of Mancozeb recorded six protein bands having 10 KDa to 164.17 KDa molecular weight. Out of these, five protein bands were found to possess molecular weight between 33 KDa to 164.17 KDa. Minimum banding pattern was observed in *T. viride* exposed at 100 ppm of Thiram which recorded four protein bands having 10.02 KDa to 148.34 KDa molecular weight.

T. virens exposed at 1000 ppm of Metalaxyl recorded a maximum of eight protein bands having 10.17 KDa to 161.87 KDa molecular weight, followed by Mancozeb and Copper oxychloride which recorded seven protein bands having 10.17 KDa to 124.67 KDa and 10.17 KDa to 121.11 KDa molecular weight. Out of these, seven protein bands were found to possess molecular weight between 13.10 KDa to 161.87 KDa in protein banding among Metalaxyl exposed *T. virens*, while six protein bands were found to possess molecular weight



1st 2nd 3rd

Fig. 2b: Induction of higher tolerance in *T. viride* to Metalaxyl at three generations

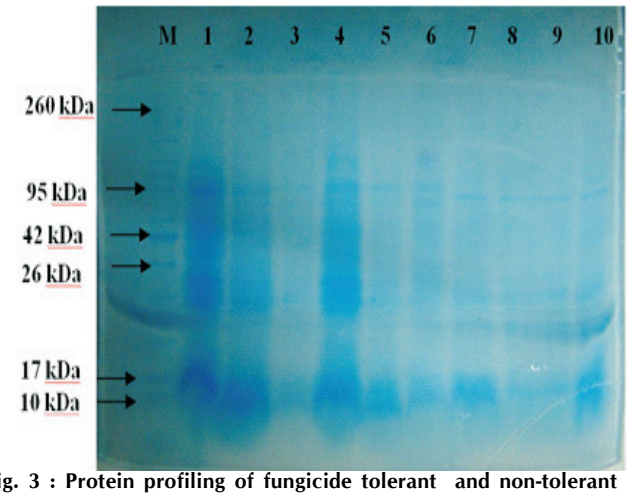


Fig. 3 : Protein profiling of fungicide tolerant and non-tolerant efficient isolates of *Trichoderma* spp.

between 13.10 KDa to 124.67 KDa and 13.10 KDa to 121.11 KDa which were more as compared to control having two protein bands of low molecular weight of 13.10 and 45.96 KDa. Minimum banding pattern was observed in *T. virens* exposed at 100 ppm of Thiram which recorded three protein bands having 38.0 KDa to 96.05 KDa molecular weight.

T. harzianum exposed at 1000 ppm of Metalaxyl recorded a maximum of six protein bands having 33.84 KDa to 121.11 molecular weight. Out of these, five protein bands were found to possess molecular weight between 48.21 KDa to 121.11 KDa which was more as compared to control having two protein bands of low molecular weight of 13.10 and 46.32 KDa. *T. harzianum* exposed at 100 ppm of Mancozeb recorded five protein bands having 13.10 KDa to 86.75 KDa molecular weight, followed by Metiram (300 ppm) which recorded four protein bands having 13.10 KDa to 88.79 KDa molecular weight. Minimum banding pattern was observed in *T. harzianum* exposed at 100 ppm of Thiram which recorded three protein bands having 13.02 KDa to 74.18 KDa molecular weight.

T. asperellum exposed at 1000 ppm of Metalaxyl recorded a maximum of six protein bands having 12.80 KDa to 91.59 KDa molecular weight. Out of these, four protein bands were found to possess molecular weight between 21.34 KDa to 91.59 KDa which was more as compared to control having two protein bands of low molecular weight of 13.10 and 34.51 KDa. *T. asperellum* exposed at 600 ppm of Copper oxychloride recorded five protein bands having 13.10 KDa to 91.59 KDa molecular weight. Minimum banding pattern was observed in *T. asperellum* exposed at 100 ppm of Thiram and 100 ppm of Mancozeb which recorded three protein bands having 13.10 KDa to 88.62 KDa and 13.10 KDa to 91.59 KDa molecular weight respectively. The results are in accordance to earlier study by Bhagat and Pan (2010) which evaluated effect of different substrate as carbon sources on enzyme activities of *Trichoderma* spp. through SDS-PAGE and suggested that all isolates synthesized the *â* -1,3 glucanase, cellulase and chitinase enzymes.

CONCLUSION

The study showed that the mycelial radial growth of Trichoderma isolates had a broad range of sensitivity to all the tested fungicides. More specifically, all the antagonists were highly sensitive to Carbendazim, Hexaconazole, Propiconazole for mycelial radial growth. The compatibility effect of Metalaxyl, Mancozeb, Chlorothalonil and Copper oxychloride on growth and biological activity of *T. viride, T.virens and T. harzianum*. Moderate sensitivity was observed among fungicides *viz*. Chlorothalonil, Mancozeb and Carbendazim with ED50 values as 625, 231 and 65 *µ*g/ml but no information is available for T. asperellum in context to Metalaxyl, Mancozeb, Chlorothalonil and Copper oxychloride. The results showed that *T. viride, T. harzianum, T. virens* and *T. asperellum* gained moderate to high growth with higher tolerance against selected five fungicides *viz*. Metalaxyl, Mancozeb, Thiram, Chlorothalonil and Copper oxychloride at higher concentrations which could be safely used in integrated disease management practices. Protein profile of *T. viride, T. harzianum, T. virens* and *T. asperellum* isolates exposed to higher concentration of fungicides isolates were analysed by SDS-PAGE which showed maximum protein banding pattern against Metalaxyl exposed at very higher concentration of 1000 ppm followed by Mancozeb (100 ppm) and Copper oxychloride (600 ppm) while lowest number of protein bands were observed with Thiram at concentration of 100 ppm. The results evaluated effect of different substrate as

carbon sources on enzyme activities of Trichoderma spp. through SDS-PAGE and suggested that all isolates synthesized the *â*-1,3 glucanase, cellulase and chitinase enzymes. The molecular weight of these proteins ranged from 28 to 73 kDa. The genetic distinctness of eight isolates belonging to *T. harzianum, T. viride, T. virens* and *T. asperellum* as well as high degree of intra and interspecific variation existing among isolates authenticated their higher degree of polymorphism. The dendrogram pattern obtained through RAPD was almost indistinguishable from the phylogenetic tree obtained from ITS 1 and ITS 2 sequences. It is obvious from the present study that genetic diversity analysis through RAPD and ITS based markers as well as analysis of ORFs of isolates depicted a good number of conserved transcribing regions present in rDNA which encodes for large number of amino acids with protein mass of higher molecular weight. All results obtained lays a concrete platform of positive correlation with the tolerance efficacy of *Trichoderma* isolates to fungicides. Thus an integrated approach of morphological, biochemical and molecular markers can be employed to identify a potent strain of *Trichoderma* in terms of its genetic profile and its further use through induction of tolerance against fungicides in plant disease management.

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