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Evaluation of suitable fungicide for integration with *Trichoderma* isolates for the control of tomato wilt

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Sixty five *Trichoderma* isolates were obtained from thirty soil samples and screened for their antagonistic activities against *Fusarium oxysporum* f.sp. *lycopersid,* the pathogen of tomato wilt. Out of the sixty five isolates ten showed class 1 type of antagonism in dual culture screening technique. The ten isolates were selected and further evaluated for production of volatile and non-volatile inhibitors against the pathogen. At 15% concentration of culture filtrate *T. viride* (TV19) showed maximum inhibition to pathogen's growth which was followed by the culture filtrate of *T.harzianum* (TH7). The same pattern of growth inhibition of the pathogen was observed when evaluated with volatile metabolites of 15-days old cultures of TV19 and TH7. Among the five fungicides tested against the pathogen and the two antagonist isolates, it was observed that Difenoconazole (Score) completely inhibited the growth of the pathogen while the two antagomsts were fairly tolerant to the fungicide up to 400 mg/ml concentration. It shows that the fungicide Difenoconazole can be integrated with the antagonists for control of tomato wilt.

Key words : *Trichoderma viride, T. harzianum, Fusarivm oxysporum* f.sp. *lycopersid,* fungicide, Difenoconazole.

INTRODUCTION

The Fusarium wilt of tomato *(Lycopersicon esculentum* Mill.) caused by *F.oxysporum* f.sh.*lycopersici* (Sacc.) Snyder and Hansen (*FOL*) is recognized as a devastating disease in major tomato growing regions worldwide (Beckman, 1987) and distributed in India in different regions in severe to moderate form (Kapoor, 1988). In Manipur the vegetable growers suffer more than 50% crop losses due to Fusarium wilt of tomato in heavily infested fields. Kapoor (1988) has reported that most of the common varieties of tomato are susceptible and fungicides are frequently used to control the wilt disease. However, the soil borne disease is very difficult and uneconomical to control with chemicals alone. In this context, biological control is an alternative and eco-friendly strategy for disease management. Mukhopadhyay (1987) has emphasized that biological control of plant pathogens can be successfully exploited within the framework of integrated pest management system. *Trichoderma* spp. are well known biocontrol agents that have gained considerable importance either alone or integrated with lower dose of fungicides for the management of soil borne plant pathogens. Sivan and Chet (1986) have reported successful reduction of fusarial wilt in many crops with application of different species of *Trichoderma.* Rini and Sulochana (2007) and Kapoor,(2008) have found *Trichoderma* spp. to be effective biocontrol agent against *F.oxysporum.* However it is also reported that all the isolates of *Trichoderma* species are not equally effective in the control of the pathogen *in vitro* (Bell *et al.*, 1982) and *in vivo* (Lewis and Papavizas, 1987). Therefore, a specific effective native *Trichoderma* isolate is to be identified for successful control of a particular pathogen. Since the biocontrol agents have to be applied in soil it becomes imperative to ascertain its tolerance to agrochemicals used in crop protection technology (Sharma and Mishra, 1995).

Keeping this in view and the growing importance of

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biological control agents, the present study has been carried out. The main objective is to study the biocontrol efficiency of native isolates of *Trichoderma* against *FOL* and to evaluate different fungicides at various concentrations to know the tolerance limit of antagonist *Trichoderma* isolates and the pathogen, *FOL*.

MATERIALS AND METHODS

Isolation of pathogen

The pathogen, *FOI* was recovered from tomato plants showing typical symptoms of wilt. *FOI* was isolated by placing infected roots (after surface disinfection with 1% sodium hypochlorite for 2 min) on *Fusarium* specific medium and incubating them at 25°C for 5 days. The pathogen was identified as *F.oxysporum* based on its morphological characters. The forma specialis of this pathogen was identified using pathogenicity tests.

Isolation of Trichoderma spp.

Sixty five isolates of *Trichoderma* were isolated from thirty soil samples collected from different ecological habitat of Manipur by dilution plate technique (Waksman and Fred, 1922) and plated on *Trichoderma* selective medium (TSM) (Elad *et ah,* 1981). The probable colonies of *Trichoderma* were picked up, subcultured, purified and preserved in Czapek's agar medium slant at 4°C for subsequent use. The *Trichoderma* spp. were identified up to species level following the taxonomic keys and monograph of Rifai (1969).

Dual culture technigue

Two mycelial discs (5 mm dia) removed from the margins of actively growing colonies of the test pathogen and biocontrol agent were placed 5 cm away from each other on opposite sides of 90 mm dia. Petriplate, containing about 20 ml of Czapeks agar medium. The paired cultures were incubated at 25±1°C for 5-7 days and then scored for degree of antagonism on a scale of class 1 to 5, class being highly antagonistic and class 5 being non antagonist as described by Bell *et al.* (1982).

Hyphal interactions

From the zone of interaction between the antagonist and *FOL* in dual culture plate, the mycelial mats were gently lifted with a needle and put in a drop of cotton blue on a microscopic slide, spread with needle and observed under microscope for hyphal interaction.

Effect of non-volatile metabolites

The effect of non-volatile metabolites of *Trichoderma* isolates on pathogen was studied following the method of Dennis and Webster (1971b). The different volumes of culture filtrate of antagonists were added to molten Czapek's agar media to obtain final concentration of 5, 10 and 15% (v/v). The amended medium was poured into Petriplate and inoculated with fresh pathogen mycelial plug. The Petriplates were incubated at 25±1°C for 3 days. Control plates were maintained without culture filtrate. Radial mycelial growth was recorded and inhibition (%) was calculated.

Effect of volatile metabolites

The effect of volatile metabolites of *Trichoderma* isolates on pathogen was studied by using the method employed by Dennis and Webster (1971a). Antagonists were grown on Petriplates containing Czapek's agar medium for 5, 10 and 15 days. The top of each Petriplate was replaced with the bottom of another Petriplate containing agar medium and inoculated centrally with a mycelia plug of the pathogen. Plates with agar medium without *Trichoderma* spp. at the lower lid and plates inoculated with mycelial disc of the pathogen on the upper lid were maintained as control. The pair of each two plates were taped together with cellophane adhesive tape. Radial mycelial growth of the pathogen was recorded after 72 hr of incubation at 25±l°C and per cent inhibition of mycelial growth was calculated.

Evaluation of fungicides

Five fungicides viz., carbendazim 50 WP (Derosal), captan 50 WP (Captaf), copper oxychloride 50 WP (Blitox), difenoconazole 25% EC (Score) and mancozeb 75% WP (Dhanuka) were tested at 5 different concentrations (50,100,200,300 and 400 mg/ml) against the pathogen and two highly antagonists *Trichoderma* isolates TV19 and TH7 *in vitro* by using Poisoned food technique (Fisher, 1969). 5 mm mycelial discs of pathogen and an-

tagonists removed from 3 days'old cultures were inoculated to Petriplates containing fungicide amended agar media. Control plates without any fungicide were also simultaneously inoculated for comparison. Colony diameter was recorded after 72 h of incubation at 25±1°C and per cent growth inhibition over control was calculated.

In all the experiments proper control sets and three replications were maintained. The per cent growth inhibition in all above experiments was calculated by the formula of Vincent (1947).

RESULTS AND DISCUSSION

Sixty five *Trichoderma* isolates could be obtained from 30 soil samples out of 35 samples examined during the study period. The isolates were distributed into 6 species of the genus, *Trichoderma viride, T. virens, T. hamatum, T. harzianum, T. koningii* and *T. longibrachiatum.* Table 1 showed distribution of 65 *Trichoderma* isolates among different antagonism classes as determined by their antagonistic activities against FOL in dual culture method. The antagonism tests revealed that approximately 15% of the isolates showed antagonism rating class 1, 48% had a rating of class 2 whereas 34% and 3% of the isolates had ratings of class 3 and 4 respectively. None of the isolates was found to show the class 5 type of antagonism against the pathogen *in dual* culture experiment. Ten isolations, 7 belonging to *T. viride* (TV1, TV7, TV11, TV16, TV18, TV19 and TV22), 1 each belonging to *T. koningii* (TK4), *T. harzianum* (TH7) and *T. longibrachiatum* (TL3) which showed class 1 antagonism, were selected for studying the mechanisms of their antagonistic activities against FOL. Askew and Laing (1994) recommended the dual culture method adopted by Bell *et al,* (1982) for screening and identifying aggressive strains *of Trichoderma.*

Table 1 : Distribution of *Trichoderma* isolates among different classes of antagonism against *F. oxysporum* f. sp. *lycopersici*

Trichoderma species	Antagonism class/ Number of isolates					Total
	1	2	3	4	5	
T. viride	7	12	6	1	-	26
T. virens	-	3	2	-	-	5
T. hamatum	-	3	8	1	-	12
T. koningii	1	6	1	-	-	8
T. harzianum	1	5	3	-	-	9
T. longibra chiatum	1	2	2	-	-	5
Total	10	31	22	2	-	65

Hyphal interaction studied at the contact of antagonist and pathogen revealed hyphal parasitism of antagonist on the test pathogen. Initially, the hypha of *T. viride* (TV19) ran parallel and got adpressed to the hypha of pathogen. *T. viride* hyphae when come in contact with FOL produced wavy coil like structure which helped in coiling around the pathogen. At the point of contact, sometimes, hooks-like structures were produced by *Trichoderma* hyphae, which entered into the pathogen. As a result of mycoparasitic activities , the host hyphae showed suppressed growth ,

Table 2: Effect of volatile metabolites of *Trichoderma* isolates on colony growth of *F. (oxysporum* f.sp. *lycopersici*

Trichoderma isolates	Age of antagonist (Days)					
	5		10		15	
	Radial Growth (cm)	Inhibition (%)	Radial Growth (cm)	Inhibition (%)	Radial Growth (cm	Inhibition (%)
TV1	3.27	4.66	3.07	9.71	2.97	14.41
TV7	2.93	14.58	2.83	16.76	2.70	22.19
TV11	3.07	10.50	2.80	17.65	2.67	23.05
TV16	3.10	9.62	2.78	18.24	2.73	21.33
TV18	3.13	8.75	3.03	10.88	2.83	18.44
TV19	2.53	26.24	2.33	31.47	2.10	39.48
TV22	3.17	7.58	3.07	9.71	3.0	13.54
TH7	3.07	10.50	2.63	22.65	2.50	27.95
TK4	2.87	16.33	2.83	16.76	2.77	20.17
TL3	3.03	11.66	3.0	11.76	2.63	24.21
Control	3.43	-	3.40	-	3.47	-
S Em±	0.06	-	0.07	-	0.08	-
CD at 5% 0.12		-	0.04	-	0.16	

Table 3 : Effect of non-volatile metabolites of *Trichoderma* isolates on *F. oxysporum* f.sp *lycopersici*

Trichoderma isolates	Concentration of culture filtrate (%)					
	5		10		15	
	Radial Growth (cm)	Inhibition (%)	Radial Growth (cm)	Inhibition (%)	Radial Growth (cm	Inhibition (%)
TV1	2.40	2.83	2.20	19.41	2.23	23.89
TV7	2.32	6.07	2.22	18.68	2.30	21.50
TV11	2.03	17.81	2.17	20.51	2.17	25.94
TV16	2.13	13.77	2.20	19.41	2.13	27.30
TV18	2.43	1.62	2.67	2.20	2.63	10.24
TV19	2.07	16.19	1.77	35.16	1.82	37.88
TV22	2.40	2.83	2.43	10.99	2.47	15.70
TH7	2.10	14.98	2.13	21.98	2.10	28.33
TK4	2.30	6.88	2.23	18.32	2.30	21.50
TL3	2.20	10.93	2.03	25.64	2.13	27.30
Control	2.47	-	2.73	-	2.93	-
S Em±	0.05	-	0.07	-	0.07	-
CD at 5%	0.17	-	0.14	-	0.16	-

excessive vacuolation and lysis of protoplasm. Antagonistic activity of 10 isolates of *Trichoderma* spp. against *F. oxysporum f.sp. ly'copersici* due to parasitism and antibiosis was reported by Padmodaya and Reddy (1996). Chattopadhyay and Kalpana Sastry (1997) studied the effect of naturally occurring antagonists on the reduction of wilt disease in safflower and showed mycoparasitism between *T. viride* and *F.oxysporum f.sp. lycopersici* . Kumar and Dubey (2001) observed coiling of antagonistic hyphae of *G.virens* and *T. harzianum* around the hyphae of pathogen *F. solani f.sp. pisi* and their lysis.

All the ten *Trichoderma* isolates proved effective in producing volatile metabolites against FOL at all the three stages of exposure and more particularly at 15 days of exposure. *T. viride* isolate (TV19) caused highest inhibition (39.48%) of mycelial growth followed by *T. harzianum* isolate (TH7) (27.95%) against FOL when 15-days-old culture of the antagonist was used. Very low volatile substances released by 5-days-old culture of *T. viride* isolate (TV1) inhibiting only 4.66% mycelial growth of the pathogen. An increase in inhibition of growth of *FOL* was evident with an increase in the age of *Trichoderma* isolates cultures (Table 2). Sawant and Mukhopadhyay (1990) reported that old cultures of *T.harzianum* had a greater inhibitory effect on the mycelial growth of *Pythium aphanidermatum* as compared to that of younger cultures. The greater inhibitory effects of older culture of *Trichoderma* spp. as compared to the younger culture against *Sclerotium rolfsii* Sacc. (Srinivasula *et al,* 2005) and *Rhizoctonia solani* Kuhn (Raju *et al,* 2008) have been reported. *Trichoderma* spp. are known to produce volatile (6-pentyl-α-pyrone) and non volatile (Trichodermin, Suzukacillin and Alamethicine) antibiotics. In the present investigation also some efficient *Trichoderma* spp. emitted intense coconut odour and secreted yellowish green metabolites in growth medium.

Culture or cell free-filtrates of all *Trichoderma* isolates were suppressive to the radial growth of *FOL* (Table 3). TV19 showed highest mycelial growth inhibition (37.88%) against the pathogen through production of non-volatile metabolites at 15% concentration. This was followed by TH7 (28.33%). With an increase in the concentration of the culture filtrate of the *Trichoderma* isolates, a corresponding increase in percent inhibition of the mycelial growth of FOL was noticed. Similar results were noted with an increase in concentrations of the culture filtrate of *Trichoderma* spp. the greater inhibition of the mycelial growth of *R. solani* (Khan and Sinha, 2007). The antifungal effect of the culture filtrate is attributed because of the viridin as s fungistatic metabolite produced in the culture by *Trichoderma* (Chet *et al,* 1977).

The results (Table 4) showed that difenoconazole totally inhibited the growth of *FOL* at all the concentration tested. It inhibited the growth of TV19

Table 4: Effect of different concentrations of fungicides on the radial growth of *Fusarium oxysporum* f.sp. *lycopersici* and two highly antagonist *Trichoderma* spp

Fungicide	Cone (µg/ml)	*FOL*		TV19		TH7	
		Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)
Captaf (Captan)	50	1.45	57.35	3.50	61.11	3.20	64.44
	100	1.17	65.59	1.13	87.44	2.90	67.78
	200	1.06	68.82	1.10	87.78	2.50	72.22
	300	1.03	69.71	1.03	88.56	1.75	80.56
	400	1.00	70.59	0.73	91.89	1.00	88.89
Dhanuka (Mancozeb)	50	2.10	38.24	3.00	66.67	2.90	67.78
	100	1.80	47.06	2.70	70.00	2.40	73.33
	200	1.50	55.88	2.40	73.33	1.60	82.22
	300	1.43	57.94	2.00	77.78	1.30	85.56
	400	1.36	60.00	2.30	74.44	1.10	87.78
Blitox (Copper oxychloride)	50	2.93	13.82	5.50	38.89	4.70	47.78
	100	2.73	19.71	5.00	44.44	4.00	55.56
	200	2.50	26.47	4.30	52.22	3.50	61.11
	300	2.33	31.47	3.90	56.67	3.00	66.67
	400	2.17	36.18	3.50	61.11	2.60	71.11
Score (Difenoconazole)	50	0	100	4.30	52.22	2.40	73.33
	100	0	100	2.50	72.22	1.75	80.56
	200	0	100	1.73	80.78	1.63	81.89
	300	0	100	1.36	84.89	1.45	83.89
	400	0	100	1.06	88.22	1.17	87.00
Derosal (Carbendazim)	50	0	100	0	100	0	100
	100	0	100	0	100	0	100
	200	0	100	0	100	0	100
	300	0	100	0	100	0	100
	400	0	100	0	100	0	100
Control	-	3.4	-	9.0	-	9.0	-
S Em±	-	0.13	-	0.28	-	0.20	-
CD. at 5%	-	0.14	-	0.18	-	0.14	-

ranging from 52.22 to 88.22% and TH7 73.33 to 87% indicating that it was less inhibitory to the antagonists tested. Thus TV19 showed 48% and TH7 26.67% tolerance to difenoconazole at 50 µg/ml after 3 days of incubation. Similarly, Khosla and Gupta (2008) stated that difenoconazole (0.1%) although had inhibitory effect on *T. viride*, yet it did not completely check the growth of it. Therefore, difenoconozole was highly suitable for integration with antagonists for the control of tomato wilt as it totally inhibited the mycelial growth of the pathogen but not as inhibitory to the antagonists. Other fungicides captan, mancozeb and copper oxychloride were moderately inhibitory to the pathogen and the antagonists. Therefore, these fungicides could also be integrated with the antagonists with some limitations. Khalko *et al.*, (2006) also reported that mancozeb, captan and blitox were not lethal to the *T. viride, T. harzianum* and *G. virens*. Carbendazim showed 100% growth inhibition of the pathogen as well as the antagonists at all the concentration tested. So,

carbendazim should not be used for integration with the antagonists. Pandey and Upadhyay (1998) reported that *T. harzianum* and *T. viride* were highly sensitive to carbendazim. Similarly Khalko *et al,* (2006) and Jha *et al,* (2008) reported complete inhibition of *Trichoderma* spp. by carbendazim at 50 µg/ml.

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