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IN VITRO STUDIES ON THE FUNGICIDAL EFFECT ON *TRICHODERMA* SPECIES IN TEA PLANTATION

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Abstract

Tolerance of *Trichoderma* species collected from tea plantation to fungicides was evaluated *in vitro* using poisoned food method. Fungicides like Carbendazim 50 WP, Copperoxychloride 50 WP, Hexaconazole 5 EC, and Propiconazole 25 EC were used at two lower and two upper of recommended doses. The growth of *T. harzianum* was mostly inhibited by the Carbendazim 50 WP and Propiconazole 25 EC with the recommended doses; while *T. viride* could grow easily with the said doses. Both species of *Trichoderma* grew easily in medium containing Copperoxychloride even at highest doses. Hexaconazole 5 EC proved to be highly toxic with no growth of *Trichoderma harzianum* in treatments containing 40.0 and 42.05 ppm. At highest concentration (42.5 ppm) of Hexaconazole 5 EC, *T. viride* grew after 72 hours of incubation. It was 14.44% over the control on 4th day of plating.

Keywords: *In vitro*, fungicidal effect, trichoderma species, tea.

Introduction

Trichoderma spp. are free-living fungi that are common in soil and root ecosystems. They are highly interactive in root, soil and foliar environments. They produce or release a variety of compounds that induce localized or systemic resistance responses in plants. *Trichoderma* strains have long been recognized as biological agents, for the control of plant disease and for their ability to increase root growth and development, crop productivity, resistance to abiotic stresses, and uptake and use of nutrients. *Trichoderma* spp. have been found as effective biocontrol agent of many soil borne plant pathogenic fungi such as *Fusarium, Sclerotium, Rhizoctonia* etc. (Chet and Inbar, 1994). The genus *Trichoderma* is not only one of the most common, isolated from various habitats soil fungi but also known to be secreting to the environment various secondary metabolites of a wide spectrum of effects on various fungal groups. Bissett (1984) characterized the genus *Trichoderma* as "rapidly growing colonies bearing tufted or pustulate, repeatedly branched conidiophores with lageniform phialids and hyaline or green conidia borne in slimy heads".

Metal containing pollutant are increasingly being released into the soil from industrial waste water as well as from wastes derived from chemical fertilizers and pesticides as agricultural applications (Ting and Choong, 2009; Errasquim

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and Vazquez, 2003). Evidence suggested that *Trichoderma* spp. exhibited considerable tolerance against metals and accumulates high amounts of the metals from pollutant habitants (Anand *et al*., 2006; Errasquin and Vazquez, 2003). *Trichoderma* species have gained considerable importance as a successful biocontrol agent for the control of soil borne diseases (Chet and Elad, 1982). It has been exceptionally good model with which to study biocontrol because it is ubiquitous, easy to isolate and culture, grow rapidly on many substrates, affect a wide range of plant pathogens, is rarely pathogenic on higher plants, acts as a mycoparasite, competes well for food and site, produces antibiotics and has an enzyme system capable of attacking a wide range of plant pathogens. The species of *Trichoderma* are known to suppress infection of root by soil borne pathogens like *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium* species and *Pythium* species on various crops (Ehteshamul Haque *et al*., 1990; Benitez *et al*., 2004; Adekunle *et al*., 2001; Lutchmeah and Cooke, 1985; Howell, 1982). Species of *Trichoderma* also have growth promoting capabilities that may or may not be integral to biological control (Benitez *et al*., 2004; Dubey *et al*., 2007; Yedidia *et al*., 1999).

Use of fungicides for the control of soil borne diseases is costly and also produces environment and health hazards to men and also adversely affects the beneficial microorganisms in soil (Dluzniewska, 2003). This has diverted the attention of plant pathologist towards alternate methods for the control of plant diseases. The combined use of biocontrol agents and chemical pesticides has attracted much attention in order to obtain synergistic or additive effects in the control of soil borne diseases (Locke *et al*., 1985). Reduced amount of fungicide can stress and weaken the pathogen and render its propagules more susceptible to subsequent attack by the antagonist (Hjeljord and Tronsmo, 1998). Srinivas and Ramakrishnan (2002) have reported that integration of biocontrol agents and commonly used fungicides showed positive association by reducing the seed infection compared to fungicide and the fungal antagonists individually. In tea plantation, different groups of fungicides like Carbendazim, Copperoxychloride, Copper oxide, Copper hydroxide, Hexaconazole, Propiconazole etc. are used for controlling different tea diseases (Huq *et al*., 2007). In tea plantation, most of the diseases are centralized in mid canopy region and infect stems and branches. Fungicides are used directly towards stem and branches. There is a great chance to drop down the fungicides in the soil. Besides, due to frequent rainfall fungicides washout in the soil. From these points of view, soil microorganisms may be affected. In tea cultivation, there is no information regarding the impact of fungicides on the biocontrol agent persist in tea soil. In the point of view, the present study was undertaken to screen *Trichoderma* species *viz*., *T. harzianum* and *T. viride* for tolerance to fungicides.

Materials and Method

The experiment was carried out at the Plant Pathology laboratory of Bangladesh Tea Research Institute, Srimangal during 2009. Potato Dextrose Agar (PDA) medium was prepared in the laboratory. Medium and necessary glassware were sterilized in autoclave (Islam *et al*., 2008).

Soils were collected from 15 different marks of tea areas under main farm of Bangladesh Tea Research Institute at 0-9 inches. All collected samples were mixed thoroughly to make a composite sample. One gram (dry weight basis) soil was mixed into 9 ml of sterile distilled water. Then 1 ml of suspension was taken into another tube containing 9 ml of sterile distilled water to make 1:10 solution. This serial dilution technique was continued up to 1: 10,000. From the final dilution (1: 10,000), 1 ml suspension was transferred to each of the three petridishes. 20 ml of melted agar medium was poured in each plate and mixed with the suspension by giving a gentle whirling motion to the plate and allowed them to incubate in room temperature (Islam *et al.,* 2008). *Trichoderma harzianum* and *T. viride* were identified by using CMI sheet. Sub culturing was performed and the culture of *Trichoderma* in pure form was maintained.

Tolerance to fungicides of *Trichoderma* species was evaluated using poisoned food method (Nene and Thapliyal, 1982). Fungicides viz. Carbendazim 50 WP (325, 350, 375, 400 and 450 ppm), Copperoxychloride 50 WP (750, 1000, 1250, 1500 and 1750 ppm), Hexaconazole 5 EC (32.5, 35.0, 37.5, 40.0 and 42.5 ppm) and Propiconazole 25 EC (162.5, 175.0, 187.5, 200.0 and 212.5 ppm) were added to PDA medium. The concentrations of fungicides were selected as two lower and two upper doses of recommended dose (Huq *et al.,* 2007). PDA medium without fungicides served as control. A 5mm inoculum disc of *Trichoderma* species was cut from the margin of actively growing colony and placed in centre of each Petri plate. Petri plates were incubated at room temperature. Five Petri plates were used for each treatment. The experiment was laid out in a Complete Randomized Design with four replications. Radial growth of *Trichoderma* species was observed daily.

Data subject to analysis of variance by MSTAT computer program. Data were transformed to minimize the dependence of mean over variance. Mean separation was done by Duncun's Multiple Range Test (Kader *et al.,* 2000).

Results and Discussion

T. harzianum showed growth on medium containing Carbendazim 50 WP @ only 650 ppm. The growth started after 48 hours of incubation and continued gradually up to 144 hours of incubation. No growth was observed when

Carbendazim was used @ 700, 750, 800 and 850 ppm (Table 1). On 144 hours after incubation, plates were 100% filled by the agent on medium containing no fungicide, while only 33.33% growth was observed with 650 ppm of Carbendazim. This species of *Trichoderma* grew easily in medium containing Copperoxychloride 50 WP @ 750, 1000 and 1250 ppm and plates were filled on 5th day of incubation (Table 2). Growth of *T. harzianum* was significantly reduced where Copperoxychloride was used @ 1500 and 1750. Hexaconazole 5 EC proved to be highly toxic with no growth of *T. harzianum* was observed in treatments containing 40.0 and 42.5 ppm (Table 3). A similar degree of growth was observed at 32.5 and 35.0 ppm of Hexaconazole 5 EC on 6^{th} day of incubation. Propiconazole 25 EC was found to be more effective since it completely inhibited the growth of *T. harzianum* at 187.5, 200.0 and 212.5 ppm concentrations (Table 4). A growth of 91.11 and 86.66% over control was observed at 162.5 and 175.0 ppm respectively on 5^{th} day when 100% percent growth was observed with control plates.

There was a significant ($p= 0.05$) negative correlation between the growth of *T. viride* and the concentration of Carbendazim even after 144 hours of incubation. Growth in 750, 800 and 850 ppm treatments started after 48 hours of incubation (Table 1). Overall growth of *T. viride* was slow as compared to control up to 5^{th} day. Growth of *T. viride* showed little negative correlation with the concentration of Copperoxychloride 50 WP, plates were filled after 144 hours in 750, 1000 and 1250 ppm treatments, respectively (Table 2). A decline and statistically identical growth with 25.55 and 23.33% was observed at 1500 and 1750 ppm treatments respectively over the control. Hexaconazole 5 EC at 32.5, 35.0, 37.5 and 40.5 ppm was not able to suppress the growth of *T. viride.*even after 24 hours of incubation. Plate was found to be filled @ 32.5 ppm treatment after 120 hours of incubation (Table 3). The agent was observed to start growth @ 42.5 ppm concentration after 72 hours of inoculation and it was 14.44% over the control after 144 hours of inoculation. Propiconazole 25 EC @ 200.0 and 212.5 ppm was observed to inhibit the growth of *T. viride* up to 48 hours of incubation. Growth was started from 72 and 96 hours of incubation with 200.0 and 212.5 ppm concentrations respectively (Table 4). A gentle decline in growth of *T. viride* was observed over control after 144 hours of incubation. However, Carbendazim was found to be more effective since it completely inhibited the growth of *T. harzianum* even @ 650 ppm where very minute mycelial growth was observed after 2 days of incubation. All other fungicides showed no complete inhibition of the growth of *T. harzianum* and *T. viride* after 144 hours of incubation. From the result it is revealed that, Carbendazim 50 WP and Propiconazole 25 EC with their recommended doses completely inhibit the growth of *T. harzianum*; while *T. viride* can grow gradually.

Table 1. Effect of Carbendazim 50 WP on *In vitro* growth of *T. harzianum* and *T. viride.*

Concentration (ppm)	Colony diameter (cm) after different time intervals of plating											
	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs		144 hrs	
	T. h	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*
00	1.8	1.5	3.7	3.6	5.4	4.8	6.9	6.7	8.3	8.2	9.0 a	9.0 a
650	0.0	0.8	0.8	1.1	1.2	2.5	2.7	3.6	2.7	4.2	3.0 b	5.8 b
700	0.0	0.5	0.0	1.0	0.0	2.3	0.0	3.4	0.0	4.0	0.0 c	5.6 b
750	0.0	0.0	0.0	0.9	0.0	2.1	0.0	3.4	0.0	3.8	0.0 c	5.0 c
800	0.0	0.0	0.0	0.5	0.0	1.9	0.0	2.6	0.0	3.4	0.0 c	3.6 d
850	0.0	0.0	0.0	0.0	0.0	0.8	0.0	1.2	0.0	2.0	0.0 c	3.1 d

Means within the column with same letter (s) did not differ significantly by Duncun's Multiple Range Test (DMRT) at p= 0.05.

Table 2. Effect of Copperoxychloride 50 WP on *In vitro* growth of *T. harzianum* and *T. viride.*

Concentration (ppm)	Colony diameter (cm) after different time intervals of plating											
	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs		144 hrs	
	T. h	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*
00	1.6	1.6	4.2	3.8	7.8	4.9	8.2	6.8	9.0	8.2	9.0 a	9.0 a
750	1.2	1.4	2.6	2.0	5.0	3.9	7.5	5.8	8.6	7.8	9.0 a	9.0 a
1000	1.0	1.0	2.4	1.8	4.9	3.7	7.1	5.6	8.2	7.6	9.0 a	9.0 a
1250	0.8	0.7	2.1	1.6	4.3	3.0	6.2	4.2	7.8	7.2	8.6 b	9.0 a
1500	0.0	0.5	1.1	0.9	1.9	1.3	2.4	1.9	2.9	2.1	3.1 c	2.3 b
1750	0.0	0.4	0.6	0.8	1.1	1.1	1.4	1.8	1.8	2.0	2.0 d	2.1 b

Means within the column with same letter (s) did not differ significantly by Duncun's Multiple Range Test (DMRT) at p= 0.05.

Table 3. Effect of Hexaconazole 5 EC on *In vitro* growth of *T. harzianum* and *T. viride.*

Concentration (ppm)	Colony diameter (cm) after different time intervals of plating											
	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs		144 hrs	
	T. h	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*
00	2.3	2.3	4.8	5.2	6.3	7.7	8.8	9.0	9.0	9.0	9.0 a	9.0 a
32.5	1.5	1.6	2.6	4.7	6.1	6.3	7.2	8.6	8.2	9.0	9.0 a	9.0 a
35.0	1.0	0.9	1.4	3.9	5.3	5.9	6.1	7.1	7.4	8.2	9.0 a	8.5 a
37.5	0.6	0.6	1.3	3.5	2.9	3.0	4.2	5.7	6.5	6.3	8.0 b	6.9 b
40.0	0.0	0.1	0.0	0.9	0.0	1.3	0.0	2.3	0.0	4.6	0.0 c	5.6 c
42.5	0.0	0.0	0.0	0.0	0.0	0.3	0.0	1.3	0.0	1.8	0.0 c	2.3 d

Means within the column with same letter (s) did not differ significantly by Duncun's Multiple Range Test (p= 0.05).

Table 4. Effect of Propiconazole 25 EC on *In vitro* growth of *T. harzianum* and *T. viride.*

Concentration (ppm)	Colony diameter (cm) after different time intervals of plating											
	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs		144 hrs	
	T. h	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*	*T. h*	*T. v*
00	1.6	1.8	3.7	4.2	5.2	7.8	8.6	9.0	9.0	9.0	9.0 a	9.0 a
162.5	0.9	0.8	1.2	4.0	4.1	5.9	6.0	7.8	8.2	8.8	9.0 a	8.8 a
175.0	0.2	0.7	1.0	3.8	3.8	5.6	5.0	6.6	7.8	7.2	8.6 a	7.3 b
187.5	0.0	0.5	0.0	3.3	0.0	5.0	0.0	5.4	0.0	6.1	0.0 b	6.2 c
200.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	2.2	0.0	5.6	0.0 b	6.0 c
212.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.6	0.0 b	1.8 d

Means within the column with same letter (s) did not differ significantly by Duncun's Multiple Range Test (p= 0.05)

Most of the time, fungicides produce undesirable effects on non-targeting organisms, so the use of microorganisms that antagonize plant pathogenic fungi should be risk free (Benitez *et al.*, 2004). Between the two *Trichoderma* species *viz*., *T. harzianum* and *T. viride*, the growth of *T. harzianum* was mostly inhibited by the fungicides except Copperoxychloride; the most effective fungicide was Carbendazim and Propiconazole to suppress the growth of these biocontrol agents with the recommended doses which was supported by Khan and Shazad (2007). The result of the present screening would help in the selection of biocontrol agents, which can be used, with reduced dose of selected fungicides for the control of plant pathogenic fungi.

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