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Variability in antagonistic activity and root colonizing behaviour of *Trichoderma* isolates

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Abstract

Antagonistic activity and root colonizing behaviour of 10 *Trichoderma* isolates collected from different agro-ecological zone of West Bengal were evaluated. Of these, *T. viride* from Bishnupur (red and lateritic zone) and *T. roseum* of Alipurduar (*terai* zone) were most prominent showing superior antagonistic effects and fast growth; both overgrew *Rhizoctonia solani* after 3 days of incubation in dual culture. Production of volatile and non-volatile substances was highest for *T. viride* of Falakata (*terai*) origin as evidenced by the greatest mycelial growth inhibition in *R. solani*, followed by *T. roseum* from Alipurduar. The highest growth stimulation of the antagonist by both exudates and extract of Bengal gram (*Cicer arietinum* Linn.) roots was found in *T. harzianum* of Kalyani (new alluvial zone) source. This isolate, however, was not only antagonistic to the pathogen, but also colonized the rhizosphere and maintained high population growth (224 x 10^6 cfu g^{-1} of soil at 30 days after sowing). Conversely, very low rhizosphere colonizing ability was found in *T. roseum* collected from Bardhaman's old alluvial zone. Colonization of non-rhizospheric soil by *Trichoderma* isolate was very low compared to that of rhizosphere (81.33 x 10^6 cfu g^{-1} of soil at 20 days after sowing for *T. virens* from Kalimpong hill zone). Significantly, the isolates showing high antagonism were not always highly rhizosphere competent.

Keywords: Antagonistic activity, *Cicer arietinum,* Root exudates, Rhizosphere, Non-rhizosphere colonization.

Introduction

Trichoderma, a typical mycoparasite found in forest humus and orchard soils, is effective against a wide range of soil-borne plant pathogens. In general, the efficacy of *Trichoderma* seed treatment is dependent on their biocontrol qualities and the ability to multiply rapidly in the rhizosphere. Papavizas (1985) suggested that if the antagonist inoculums multiplied around the site of application, it probably suppressed the pathogens causing seed rot and seedling diseases. However, to deter the pathogens from causing root diseases, multiplication along the root surfaces away from the cotyledon attachment is critical. Although *Trichoderma* has been isolated from roots of diverse plants and that numerous attempts have been made to use them for biocontrol of root diseases, quantitative studies on survival, establishment, and proliferation in crop rhizosphere are scarce. Hence, an attempt was made to evaluate the antagonistic potential and rhizosphere competence of promising *Trichoderma* isolates collected from different agroecological zones of West Bengal.

Materials and Methods

Ten rhizospheric isolates of *Trichoderma* were obtained from different locations in West Bengal having diverse cropping history (Table 1) through dilution plate technique using modified *Trichoderma* specific medium (TSM; Saha and Pan, 1997). The isolates were characterised based on available literature and the monograph of Rifai (1969) and maintained on potato dextrose agar (PDA) slant at 4°C.

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Table 1. Trichoderma isolates collected from different agro-climatic locations of West Bengal and their *in vitro* antagonistic potential against *Rhizoctonia solani.*

Taxa	Isolates	Crops associated	Location#	Days required for point of contact	Bell's scale rating after (days)*			
					3	4	5	6
T. viride	T_1	potato	Falakata, Jalpaiguri (TZ)	2	S_2	S_1		
T. harzianum	T_2	chilli	Kakdwip, South 24 Pgs (CSZ)	2	S_2	$S_2 – S_1$	S_1	
T. viride	T_3	mustard	Bishnupur, Bankura (RLZ)	2	S_1			
T. harzianum	T_4	brinjal	Kalyani, Nadia (NAZ)	2	S_2	S_1		
T. harzianum	T_5	sunflower	Namkhana, South 24 Pgs. (CSZ)	2	S_2	S_1		
T. virens	T_6	gladiolus	Kalimpong, Darjeeling (HZ)	2	S_2	$S_2 – S_1$	S_1	
T. virens	T_7	jute	Arambagh, Hooghly (OAZ)	2	S_3	$S_3 – S_2$	S_2	S_1
T. virens	T_8	cabbage	Ranaghat, Nadia (NAZ)	2	S_2	S_1		
T. roseum	T_9	potato	Alipurduar, Jalpaiguri (TZ)	2	S_1			
T. roseum	T_{10}	rice	Sehara Bazar, Bardhaman (OAZ)	2	$S_2 – S_1$	S_1		

TZ= *Terai* zone, CSZ= Coastal saline zone, RLZ= Red lateritic zone, NAZ= New alluvial zone, HZ= Hill zone, OAZ= Old alluvial zone.

*S_1= the antagonist completely overgrew the pathogen (100% over growth), S_2= the antagonist overgrew at least 2/3 growth of the pathogen (75% overgrowth), S_3= the antagonist colonized on half of the growth of the pathogen (50% overgrowth), and S_4: the pathogen and the antagonist locked at the point of contact, $S_2 – S_1$= the stage between S_2 and S_1.

In vitro antagonistic potential of the biocontrol agent was evaluated against *Rhizoctonia solani* Kuhn through dual culture technique. For this, the pathogenic fungi *R. solani* was selectively isolated from sheath blight affected rice (*Oryza sativa* L.) plants. After purification, the culture was maintained on PDA. The isolates were further screened for their antagonistic potential against the pathogen on PDA by measuring the relative growth rates as a function of the incubation period. Five mm mycelial discs taken from the margin of young vigorously growing 4-day-old culture of the antagonists and the pathogen was inoculated at the margin of the petridish containing 20 ml sterilized PDA medium (opposite to each other). Observations were recorded up to 7 days of incubation (at 28±1°C). The treatments were replicated five times. Antagonistic activity was scored using a modified Bell's scale (Bell et al., 1982), after the pathogen and antagonist established contact. Hyphal interactions were also studied under microscope by growing them on a cellophane membrane placed over solidified PDA in petriplates.

The effect of non-volatile substances on pathogen was studied following the method of Dennis and Webster (1971a). Different antagonists were cultured in 100 ml sterile potato dextrose broth in 250 ml Erlenmeyer flask with intermittent shaking. After 10 days, the culture filtrate was passed through Whatman No. 42 filter paper and the filtrate was collected in sterile Erlenmeyer flasks. The culture filtrate was centrifuged at 3000 rpm for 10 min and sterilized by passing through millipore membrane filter paper (0.4 µm pore size). Different volumes of filtrates were added to the molten PDA medium to obtain final concentrations of 5 and 10% (v/v). The medium was poured into petriplate and inoculated with mycelial plug of pathogen from 4-day-old colonies. The petriplates were incubated at 28±1°C for 4 days. Control plates were maintained without culture filtrate. Radial mycelial growth of the pathogen (colony diameter) was measured at right angles to each other and the inhibition percentage calculated.

The effect of volatile metabolites from antagonistic fungi was studied following the method of Dennis and Webster (1971b). The antagonists were centrally inoculated in 9 cm petriplates containing PDA by placing 5 mm mycelial discs from the periphery of 4-day-old culture and incubated at 28±1°C for 4 days. The upper lid of each petriplate was removed and the

lower one sealed with the lower lid of PDA containing petriplate inoculated centrally with mycelial disc of the pathogen. Petriplates with PDA but without antagonist in the lower lid and inoculated with pathogen on the upper lid served as control. The pairs of each plate were sealed together with cellophane adhesive tape and incubated at 28±1°C. Radial mycelial growth of the pathogen was recorded and inhibition percentage of mycelial growth calculated.

Bengal gram (*Cicer arietinum* Linn.) cv. 'Mahamaya-1' was used for rhizosphere and non-rhizosphere colonization experiments. The effect of root exudates and root extracts on growth of different isolates of *Trichoderma* was studied according to the method of Sullia (1973). For this, the root exudates were collected by growing Bengal gram plants in sand cultures. The seeds were surface sterilized with 0.1% $HgCl_2$ solution, and aseptically transferred to 250 ml Erlenmeyer flasks containing acid washed sand wetted with sterile distilled water. Fifteen seeds were transferred to each flask and three replications were maintained. After two weeks of growth under diffused light, root washings were collected after removing the roots carefully from the sand. The washings from the sand were also collected, but volatiles were not encountered. All washings were bulked and concentrated in a water bath at 60°C to a final volume of 10 ml.

To obtain root extracts, roots of the above plants grown in sand were homogenized in 200 ml sterile distilled water. The extract was centrifuged at 3000 rpm to remove debris and concentrated as above to a final volume of 10 ml. Both the root exudates and extract were sterilized through millipore filter. The effect on fungal growth was assessed by linear growth rate method using Czapek dox agar medium at pH 6.0. The inoculated plates were incubated at 28±1°C and radial mycelial growth was measured in two directions at right angles to each other. For each treatment, three replications were maintained and the Student *t* test was used to compare the treatments.

Population dynamics of *Trichoderma* both in the rhizosphere and non-rhizosphere soil were studied during the *rabi* season with Bengal gram. The spore suspension of different strains of antagonist (10^7 conidia ml^{-1}) was introduced into each pot containing 2 kg sandy loam soil (pH 6.3) so that the final preparation contained 10^5 colony forming unit per gram (cfu g^{-1}) soil. Ten Bengal gram seeds (cv 'Mahamaya-1') were sown in each moistened pot with three replications per isolate and maintained at 20% moisture content. Rhizosphere soil was collected at 10-day interval by gently uprooting the plants and the soil adhering to the roots was collected by brushing. Serial dilution technique was adopted for quantification of *Trichoderma* colony on the TSM plate. The fungal population count was expressed as cfu g^{-1} of soil and the data were analyzed using ANOVA for randomized block design. In case of non-rhizosphere soil, samples were collected from 5 cm lateral distance within the root zones.

Results and Discussion

All isolates evaluated were antagonistic to *R. solani* and overgrew the pathogen colony within 3 to 6 days (Table 1). However, *T. viride* collected from Bishnupur (red and lateritic zone) and *T. roseum* of Alipurduar (*terai* zone) were the most antagonistic and fast growing as they overgrew the pathogen in 3 days after incubation. *T. viride* from Falakata, *T. harzianum* from Kalyani and Namkhana, and *T. virens* from Ranaghat were also effective against the pathogen and reached the S_1 stage (antagonist completely overgrowing the pathogen) on the 4th day of incubation. Very low antagonistic activity was noted for *T. virens* from Arambagh, and it took 6 days to reach the S_1 stage. In general, the pathogen was not highly sensitive to the volatile exudates from *Trichoderma* isolates compared to the non-volatile substances (Table 2). The highest mycelial growth inhibition of the pathogen through production of volatile and non-volatile substances was noted for *T. viride* of Falakata origin.

Microscopic examination of the hyphal interactions revealed that the hyphae of *Trichoderma* coiled around that of the pathogen. At the point of contact, sometimes, knob-like protuberances were formed, which seemed to penetrate the pathogen hyphae. The ultimate result

of mycoparasitism was partial to complete digestion of the protoplasmic contents and lysis of the pathogen hyphae. Lu et al. (2004) reported that *in situ* branching of *T. atroviride* hyphae is an active, probably chemotactic response to the presence of host, whereas papilla-like structures at the hyphal tips occurred both in the presence and absence of direct contact with *Pythium ultimum*. Previously, Bartnicki-Garcia et al. (1995) stated that papilla formation might be caused by exudates from the host mycelium capable of displacing the *spitzenkorper* (a phase-dark body found at the tip of elongating hyphae of higher fungi), and results in the apex becoming rounded and broad.

Results of the present study are generally consistent with that of Bose et al. (2005), who reported strong antagonism by *Trichoderma* spp. against a range of soil-borne plant pathogens. Higher growth rate and greater competitive ability of the selected strains are clear indications of the better antagonistic potential. Although the results of *in vitro* studies reflecting the antagonistic potential of microorganisms are not always correlated with the degree of antagonism observed in the field, such studies are important for screening the antagonists' effectiveness. In the present experiment, strong selectivity of *Trichoderma* isolates in their antagonistic efficiency was noted. Strain specificity against a particular pathogen, however, is one of the major deterrents to the commercial use of antagonists.

Although the type and quantity of volatile substances were not measured, our results suggest that there might be production of different volatile substances. Selective activity of both volatile and non-volatile substances released by the *Trichoderma* isolates also was noticed. Yet it may be possible to detoxify or degrade the compounds released by the antagonistic fungi by the metabolites of pathogen origin. Selectivity in volatile and non-volatile substances released by *Trichoderma* against many pathogens has been previously reported (e.g., Jash and Pan, 2004a, b).

Root exudates and extracts showed stimulatory effects on the linear growth of *Trichoderma* (Table 3). The degree of stimulation was far greater in the case of root extracts (p= 0.01 in most cases) than the exudates. And the highest growth stimulation was found in *T. harzianum* collected from Kalyani by both root exudates and extract. However, highest radial growth was found in *T. virens* from Kalimpong (84 mm and 88 mm by root exudates

Table 2. In vitro antagonistic potential of *Trichoderma* isolates against *R. solani* through production of volatile and non-volatile substances.

Isolates	Volatile substances		Non-volatile substances			
	Radial mycelial growth (mm)	Inhibition (%)	Radial mycelial growth (mm) at variable concentrations		Inhibition (%) at different concentrations	
			5%	10%	5%	10%
T_1	57.3	36.3	24.3	15.6	72.9	82.6
T_2	61.3	31.8	35.6	24.0	60.3	73.3
T_3	72.6	19.2	38.0	28.6	57.7	68.1
T_4	68.0	24.4	44.0	31.0	51.1	65.5
T_5	81.3	9.6	49.0	36.3	45.5	59.6
T_6	69.6	22.6	37.0	27.0	58.8	70.0
T_7	69.3	22.9	39.3	28.3	56.3	68.5
T_8	81.0	9.8	31.6	27.3	64.8	69.6
T_9	60.6	32.6	42.6	21.6	52.6	75.9
T_{10}	66.6	25.9	29.0	17.0	67.7	81.1
Control	90.0	-	90.0	90.0	-	-
CD (p=0.05)	5.7		5.3	4.3		
CD (p=0.01)	7.8		7.2	5.9		

For a description of the isolates (T_1 to T_{10}) see Table 1.

Table 3. Effect of root exudates and extract of Bengal gram on the linear growth of rhizosphere fungi *Trichoderma* spp.

Isolates	Radial mycelial growth (mm)			Increased over control (%)	
	Control	Exudates	Extract	Exudates	Extract
T_1	71.0	74.6	74.6	5.1	6.5
T_2	70.6	80.0*	85.6**	13.2	21.2
T_3	74.0	80.3*	81.6**	8.5	10.3
T_4	62.0	71.3*	76.6*	15.0	23.6
T_5	68.0	73.6	75.6	8.3	11.2
T_6	74.0	84.0	88.0**	13.5	18.9
T_7	75.6	82.3	84.3*	8.8	11.4
T_8	73.0	77.0	80.0**	5.4	9.5
T_9	62.6	68.6	72.3*	9.5	15.4
T_{10}	63.6	70.6*	73.6*	9.9	15.7

For a description of the isolates (T_1 to T_{10}) see Table 1; *Significant at 5% level, **Significant at 1% level.

and extract, respectively), followed by *T. virens* from the old alluvial zone of Arambagh. The observation that root extract has a greater stimulatory effect on antagonistic fungi than exudates is important in understanding the rhizospheric effects of biocontrol fungi.

In rhizosphere soil, all the isolates grew profusely up to 20 days after sowing and then declined gradually (Table 4). However, the slow growing *T. harzianum* (Kalyani and Namkhana origin) and *T. virens* from Kalimpong grew rapidly up to 30 days after sowing and then declined. *T. harzianum* of Kalyani origin greatly colonized the rhizosphere soil and maintained their maximum population (224 x 10^6 cfu g^{-1} of soil) at 30 days after sowing while very low rhizosphere colonizing ability was found in *T. roseum* of Bardhaman.

On the contrary, colonization of non-rhizospheric soil by *Trichoderma* isolate was very low compared to the rhizospheric soil (Table 4). For all or most isolates, the population increased slowly and reached a peak at 20 days after sowing, which was followed by a very gradual decline. *T. viride* of Falakata had only 44.6 x 10^6 cfu g^{-1} of soil at 20 DAS and *T. roseum* from Bardhaman just had 28.3 x 10^6 cfu g^{-1} of soil at 40 days after sowing. An apparent fluctuation in the population dynamics was subsequently recorded especially towards the later stages.

Root exudates and extract of Bengal gram stimulated growth of *Trichoderma* significantly. However, there were distinct variations among the isolates in terms of the stimulatory effect caused by the exudates and extract, which can be explained by the variations in their nutritive constituents. Sullia (1973) stated that root extracts induced a high degree of stimulation in growth rate of *Trichoderma*. In the natural environment, actually the root exudates provide a major energy source to the microbes. Thus, colonization of this region in the rhizosphere might reduce infection by *R. solani* that penetrates the vascular system of the host through undifferentiated xylem at the root tip.

In the present investigation, a rapid increase in the antagonist population of the rhizosphere soil was observed, i.e., up to 20 days and in a few cases up to 30 days, followed by little or no fluctuations thereafter. A similar trend in population proliferation was discernible for the non-rhizosphere soil also; despite the rate of population increase was of lower magnitude. The initial increase in population might be due to germination of different spore forms and their subsequent proliferation with or without food base. Odunfa and Oso (1979) also found that initial increase of antagonist population in rhizosphere could be due to the abundance of sugar and amino acids exuded from roots while the subsequent decrease could be ascribed to a reduction or change in composition of root exudates as the plants age. From the population dynamics of *Trichoderma* spp. in the rhizosphere soils that included both roots and soil, it is,

Table 4. Root colonization of different isolates of *Trichoderma* spp. in natural soil.

Isolates	Population (x 10^6 cfu g^{-1}) at different time period							
	10 DAS		20 DAS		30 DAS		40 DAS	
	RC	NRC	RC	NRC	RC	NRC	RC	NRC
T_1	50.0 (1.69)*	26.6 (1.42)	143.0 (2.15)	44.6 (1.64)	123.3 (1.08)	42.0 (1.61)	115.6 (2.06)	31.0 (1.48)
T_2	62.0 (1.79)	51.0 (1.70)	187.6 (2.27)	75.0 (1.87)	168.6 (1.22)	70.6 (1.84)	148.6 (2.17)	41.3 (1.61)
T_3	43.0 (1.62)	40.6 (1.60)	107.0 (2.02)	79.6 (1.90)	100.0 (1.99)	58.3 (1.76)	71.6 (1.85)	28.6 (1.45)
T_4	90.3 (1.95)	61.6 (1.78)	198.0 (2.29)	71.6 (1.85)	224.0 (2.34)	49.3 (1.69)	172.0 (2.23)	35.0 (1.54)
T_5	67.0 (1.82)	45.6 (1.65)	122.3 (2.08)	64.6 (1.81)	127.6 (2.10)	66.0 (1.81)	123.3 (2.08)	48.0 (1.67)
T_6	84.3 (1.92)	40.0 (1.59)	176.0 (2.24)	81.3 (1.90)	181.3 (2.25)	73.3 (1.86)	143.0 (2.15)	29.3 (1.45)
T_7	77.6 (1.89)	24.6 (1.38)	165.0 (2.21)	51.0 (1.70)	159.0 (2.20)	45.6 (1.65)	164.6 (2.21)	30.6 (1.48)
T_8	59.0 (1.76)	27.0 (1.43)	133.3 (2.12)	59.0 (1.76)	161.0 (2.20)	41.3 (1.61)	122.3 (2.08)	34.3 (1.53)
T_9	72.0 (1.85)	42.0 (1.62)	139.6 (2.14)	75.0 (1.87)	177.3 (2.24)	60.3 (1.77)	136.0 (2.13)	37.3 (1.56)
T_{10}	53.0 (1.71)	32.6 (1.51)	118.0 (2.07)	50.6 (1.70)	130.3 (2.11)	30.6 (1.48)	96.3 (1.98)	28.3 (1.45)

CD (p=0.01) Rhizosphere: Isolate = 0.043, days = 0.027, Isolate x days = 0.086Non-rhizosphere: Isolate = 0.057, days = 0.036, Isolate x days = 0.114.

For a description of the isolates (T_1 to T_{10}) see Table 1; * Figure in parenthesis is log-transformed value, RC= rhizosphere colonization, NRC= non-rhizosphere colonization; DAS= days after sowing.

however, not clear whether the antagonist colonized the rhizoplane, the rhizosphere soil, or both. Yet another limitation of the present investigation is that it was carried out by using sun-dried non-sterile soils and the results on rhizosphere colonization in sterile soil are not always well correlated with that of field experiments. Nevertheless, application of suitable biocontrol agents capable of competing with other microbes in the soil vis-à-vis their ecological fitness can result in great success of biological control of crop diseases.

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