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Research Article

In vivo interaction in antagonistic potential of *Trichoderma* spp. and *Pseudomonas fluorescens*

S. K. PAN* and AMRITA DAS

Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia 741 252, West Bengal, India.
**Corresponding author E-mail*: skpan_06@rediffmail.com

ABSTRACT: Studies were carried out to find out interaction between antagonism activity of *Pseudomonas fluorescens* on the different species of *Trichoderma*. The inhibition in growth of *Trichoderma* was 8.59% when *Trichoderma* was used first and 59.44% when *P. fluorescens* was used first in a dual culture test. Highest growth inhibition (59.44%) of *Trichoderma* (Th3) was recorded when *P. fluorescens* was first inoculated in King's medium B agar (KMB) 24h prior to inoculation of *T. harzianum*. The lowest inhibition (8.59%) was recorded in *T. viride* (Tv1) when the isolate was inoculated in KMB prior to inoculation of *P. fluorescens*. All other isolates of *Trichoderma*, irrespective of the species, exhibited intermediate growth inhibition ranging from 9.87% in potato dextrose agar (PDA) (Tv1) to 59.18% in KMB (Th3). When the cell-free culture filtrate (5%) of *P. fluorescens* was used against different species and isolates of *Trichoderma*, it also caused growth inhibition of fungal antagonist to different levels (5.55 for Tvs1 to 25.92% for Th3). In steamed soil, recoverable number of *Trichoderma* population gradually increased (90 x 10^5 CFU g^{-1} of soil for Th3 to 172 x 10^5 CFU g^{-1} of soil for Tvs1) irrespective of the species over a period of 14 days incubation.

KEY WORDS: Antagonistic potential, microbial interaction, *Trichoderma*, *Pseudomonas fluorescens*

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INTRODUCTION

The effect of interaction between *Trichoderma* and *Pseudomonas* can result in differential suppression of plant pathogens. Very limited literature is available on interaction of antagonistic bacterial and fungal agents. One of the important ways of improving biocontrol in the rhizosphere may be to add mixtures or combination of biocontrol agents, particularly if they exhibit different or complementary modes of action or abilities to colonize root microsites (Whipps, 1997; Burges, 1998; Mathare *et al*., 1999). Significant biotic factors may include soil bacteria or fungi that are antagonistic to biocontrol fungi. Some strains of fluorescent pseudomonads inhibit many soil fungi, including *Trichoderma* spp., due to the production of siderophores or antibiotics (Loper, 1988). Production of antifungal metabolites like phenazine-1 carboxamide (PCN) by fluorescent pseudomonads inhibits the growth of many biocontrol fungi including *Trichoderma* (Chin *et al*., 1998). However, Dandurand and Knudsen (1993) indicated that 2-79RNo strain of *Pseudomonas fluorescens* did not have a significant detrimental effect on the biocontrol activity of *T. harzianum* in spermosphere and rhizosphere of pea. So, the present investigation was conducted to test the type of interaction(s) that occurred between antagonistic *Trichoderma* spp. and fluorescent pseudomonads.

MATERIALS AND METHODS

Interaction between P. fluorescens *and* Trichoderma

Ten isolates of *Trichoderma* including three of *T. harzianum*, two of *T. roseum*, three of *T. virens* and two of *T. viride* were used in this study (Table 1). *Pseudomonas fluorescens* was isolated from the rhizosphere soil of potato on King's B medium enriched with Cetrimide and its antagonistic potential against *Rhizoctonia solani* was evaluated under laboratory conditions as described earlier (Pan, 2009; Pan and Jash, 2009). It was maintained at 15°C in 15% glycerol and grown on King's medium B agar (KMB) (King *et al.,* 1954). A variation of the methods described by Weller *et al*. (1988) was used to test the ability of *P. fluorescens* to inhibit growth of *Trichoderma* isolates on agar. In the first experiment, an agar disc of 5 mm dia. was aseptically cut from a 5-day-old culture of *Trichoderma* and seeded at one side of the Petri plate

containing KMB without selective antibiotics or potato dextrose agar (PDA) separately and incubated for 24h at 28 ± 1°C for its establishment. After 24h, a sterilized filter paper disc (5 mm dia.) impregnated in bacterial suspension containing 10^8 CFU ml^{-1} was placed at the opposite side in the same plate. In the second experiment this process was reversed and the bacterial strain was seeded first and after their establishment (after 24h) *Trichoderma* was inoculated. Each treatment was replicated thrice. After incubation for 5 days at 28 ± 1°C, radial growth of *Trichoderma* isolates and the inhibition zones were measured. The zone of inhibition was defined as the distance between the edge of the bacterial colony and the nearest edge of the fungal colony. Uninoculated control without bacterial strain served as check. Per cent inhibition of growth of *T. harzianum* caused by *Pseudomonas* strain was computed.

Effect of culture filtrates

Effect of culture filtrate of *P. fluorescens* on the growth of *Trichoderma* was studied as per the technique of Dennis and Webster (1971a). For inoculum production, the bacteria were first grown on KMB agar at 28°C for 48h. The cells were scraped from plates and suspended (10^6–10^7 cells ml^{-1}) in sterile phosphate buffer, pH 7.0. One ml of bacterial suspension was inoculated in an Erlenmeyer flask (500 ml) containing 100 ml succinate broth (SB) medium and subjected to mechanical agitation. After 48 h of incubation at 28 ± 1°C, the culture was centrifuged at 3000 rpm for 5 min and the supernatant was collected. The culture filtrate was heat killed at 50°C for 15 min and sterilized by passing it through Millipore membrane filter using a vacuum pump. The cell-free culture filtrate so obtained was used in the experiment. The fungus growing on PDA medium amended with 5% SB without bacterial culture filtrate served as the check. Each treatment was replicated thrice.

In vitro *interaction*

Soil was steamed in an autoclave by adding steam until a temperature of 100-110°C was reached and then holding the temperature of 90-110°C for 1h following the method of Knudsen and Bin (1990). This method nearly eliminated the resident fungi (for example, *Rhizopus* and *Penicillium*) and was necessary to help distinguish the hyphae of *Trichoderma* in soil. Similarly, soil bacterial populations were reduced but not totally eliminated. The soil was air dried under a transfer hood. The bacterial suspension was hand mixed with 120 g of steamed soil to obtain a moisture content of 60% and bacterial population level of 3 x 10^7 CFU g^{-1} of soil. A glass Petri plate (15 cm diam) was half filled with approximately 60 g of the soil preparation. A single mycelial disc of 5 mm diameter was placed on the soil surface at the centre of the plate. The mycelial disc of *Trichoderma* isolates was overlaid with two layers of nylon mesh (1mm mesh) and then covered by the remainder of the soil preparation. The petriplates were placed in a plastic bag with a wet paper towel to maintain high humidity and incubated at 28°C in the dark for 14 days. The experiment was performed initially with 3 replicates per treatment. The soil from both upper and lower layers in each sampled Petri plate was thoroughly mixed in a plastic bag and then a sample (1g) was randomly taken for dilution plate technique (Harris and Sommers, 1968). The population of *Trichoderma* was determined after 7 and 14 days of incubation at 28°C by counting colonies on the Petri plate containing TSM for *Trichoderma* isolates.

RESULTS AND DISCUSSION

All the isolates of *Trichoderma* were inhibited by *P. fluorescens* (Table 2). In the first experiment when *Trichoderma* was inoculated 24h before the *Pseudomonas* strain on KMB agar, growth inhibition of *Trichoderma* varied with isolates used. *T. viride* (Tv1) and *T. virens* (Tvs1) showed high levels of tolerance against *Pseudomonas*

Table 1. Isolates of *Trichoderma* investigated and their sources

Isolate (no)	Taxa	Crop associated	Location	Bell's scale rating against *R. solani*
Th1	*T. harzianum*	Black pepper	Andaman and Nicobar islands	S_3
Th2	*T. harzianum*	Lentil	Mizoram	S_2
Th3	*T. harzianum*	Red gram	Mizoram	S_3
Tr1	*T. roseum*	French Bean	Meghalaya	S_2
Tr2	*T. roseum*	Rice bean	West Bengal	S_1
Tvs1	*T. virens*	Bengal gram	Mizoram	S_2
Tvs2	*T. virens*	Cowpea	Meghalaya	S_2
Tvs3	*T. virens*	Soybean	Meghalaya	S_2
Tv1	*T. viride*	Green gram	Andaman and Nicobar islands	S_1
Tv2	*T. viride*	Pea	Andaman and Nicobar islands	S_3

(inhibition of 12–14%) and were followed by *T. virens* (Tvs2). The remaining isolates of *Trichoderma* also had reduced the growth (28-59%). Inhibitory effects of *P. fluorescens* were comparatively more when the bacterial strain was applied 24h before inoculating with *Trichoderma*. Growth of all the isolates of *Trichoderma* spp. was significantly reduced by the bacterial antagonist irrespective of the medium used. *T. viride* (Tv1) was highly tolerant to *Pseudomonas* in dual culture technique followed by *T. virens* (Tvs1) and *T. viride* (Tv1).

The culture filtrate (5%) of *P. fluorescens* caused significant reduction in the growth of *Trichoderma* isolates. *Trichoderma virens* (Tvs1) and *T. viride* (Tv1) isolates were highly tolerant to *P. fluorescens* showing less growth inhibition (6–7%). The growth of *T. harzianum* (Th3), however, was affected by 26%. The rest of the isolates showed intermediate reaction against the bacterial antagonist.

Effect of *P. fluorescens* on the population density of *Trichoderma* isolates was assessed in steamed soil (Fig. 1). Recoverable numbers of *Trichoderma* population increased over a period of 14 days in all treatments. After 14 days, the highest population density was recorded in *T. virens* (Tvs1) isolate.

More rapid growth and sporulation of biocontrol fungi from biocontrol formulations may significantly enhance efficacy in the field. Some strains of fluorescent pseudomonads inhibit many soil fungi, including *Trichoderma* spp., due to the production of siderophores or antibiotic compounds (Loper, 1988). The fungistatic property of fluorescent pigment was due to its ability to chelate iron from the environment, creating an iron deficiency (Misaghi *et al*., 1982). Fravel (1988) discussed the possibility of deleterious effects of antibiotics and antibiotic-like compounds, produced by biocontrol agents, on beneficial micro-organisms. In the present investigation, *P. fluorescens* strain inhibited radial growth of *Trichoderma* on both PDA and KMB agar *in vitro* through dual culture technique and also production of culture filtrate. Inhibition on PDA, a relatively high iron medium, suggests that production of phenazine 1-carboxylic acid may have been

Table 3. Effect of culture filtrate of *Pseudomonas fluorescens* on the growth of different isolates of *Trichoderma* spp.

Isolates	Growth inhibition over control (%)
Tv1	7.30
Th1	20.73
Tv2	10.36
Th2	18.51
Th3	25.92
Tvs1	5.55
Tvs2	17.77
Tvs3	18.14
Tr1	25.55
Tr2	17.77
SEd	2.33
CD (P = 0.01)	6.63

Table 2. Effect of *P. fluorescens* on growth of *Trichoderma* spp. in dual culture

Isolates	*Trichoderma* inoculated first				Fluorescent pseudomonad inoculated first			
	Radial diam (mm)		Inhibition (%)		Radial diam (mm)		Inhibition (%)	
	KMB	PDA	KMB	PDA	KMB	PDA	KMB	PDA
Tv1	74.00	78.00	11.96	8.59	70.00	76.00	15.66	9.87
Th1	61.00	64.33	26.79	24.61	58.00	62.33	30.12	26.08
Tv2	64.66	67.33	22.40	21.09	58.33	66.00	29.72	21.73
Th2	43.33	48.66	48.00	42.97	39.66	46.66	52.21	44.66
Th3	34.00	38.33	59.19	55.08	33.66	35.66	59.44	57.71
Tvs1	72.00	76.33	13.59	10.54	67.00	74.66	19.27	11.46
Tvs2	58.00	58.66	30.39	31.25	57.00	58.33	31.32	30.83
Tvs3	49.00	52.00	41.19	39.06	47.33	49.66	42.97	41.11
Tr1	36.00	39.66	56.79	53.52	35.33	37.33	57.43	55.73
Tr2	59.66	66.66	28.40	18.67	54.66	62.66	34.14	25.69
SEd	2.75	2.41			1.69	1.33		
CD (P = 0.01)	7.84	6.87			4.81	3.79		

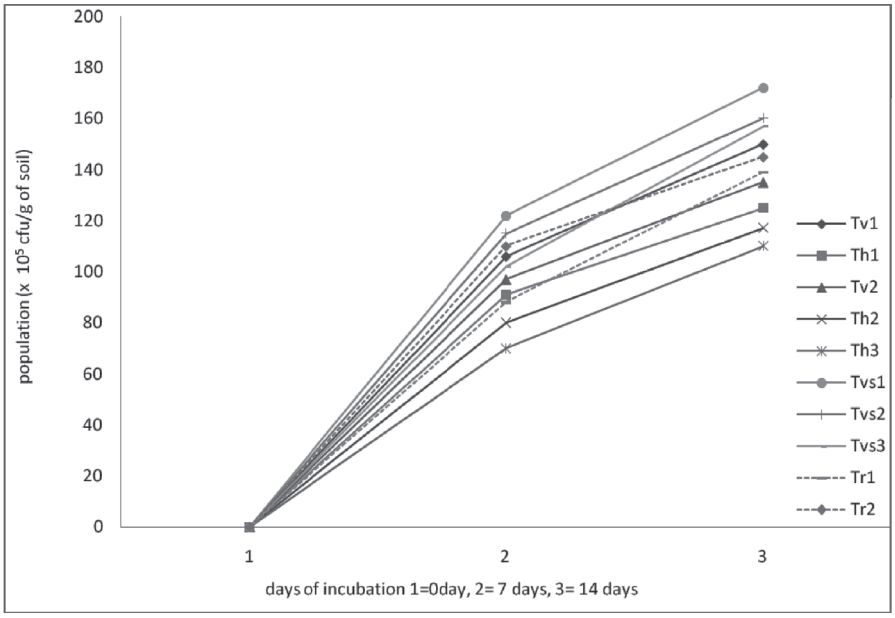

Fig. 1. Population of *Trichoderma* in steamed soil inoculated with *P. fluorescens*

a mechanism of inhibition (Weller *et al*., 1988), although zones of inhibition were narrower than those observed on KMB. Synergistic and antagonistic interactions between an introduced biocontrol agent and the indigenous microflora can influence their performance in the rhizosphere. For example, two groups of micro-organisms that occupy the same ecological niche and have the same nutritional requirements are bound to compete for nutrients (Janisiewicz and Bors, 1995). Hubbard *et al*. (1983) describe the negative effects of endemic *Pseudomonas* spp. strain on the biocontrol agent *Trichoderma hamatum*. They suggested that these negative effects were caused by effective competition for iron by the *Pseudomonas* spp. strains, because addition of iron suppressed growth inhibition of *T. hamatum* by *Pseudomonas* strains *in vivo*.

Table 4. Population of *Trichoderma* (x 10^5 cfu g^{-1} of soil) in steamed soil inoculated with *P. fluorescens*

Isolates of *Trichoderma*	7 days of incubation	14 days of incubation
Tv1	106	150
Th1	91	125
Tv2	97	135
Th2	80	117
Th3	70	110
Tvs1	122	172
Tvs2	115	160
Tvs3	102	157
Tr1	88	139
Tr2	110	145

By using steamed soil in the laboratory experiment, it was attempted to eliminate the possibility of significant direct effect of microbes other than introduced *P. fluorescens*. This bacterial strain reduced the population of *Trichoderma* to some extent in the soil. Although the reduction in growth and proliferation of different strains of *Trichoderma* in the presence of relatively high population of the bacterial antagonist was found, it is questionable whether this reduction would significantly reduce the potential biocontrol efficacy of the fungus. Lumsden *et al*. (1999) pointed out that the importance of biomass in ecological interactions is difficult to assess and proposed that the biocontrol activity of *T. harzianum* is linked primarily to a transient increase in biomass, so that high propagule numbers may not be needed to achieve control. Results of this experiment clearly revealed that fungal and bacterial biocontrol agents could not be always compatible and it is quite possible that the situation would be different if both organisms were present in the rhizosphere, where conditions may be favourable for growth and antibiotic production by *P. fluorescens*. This is a logical area for future investigation.

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