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In vivo evaluation *of Trichoderma* spp. for Survival and their Population in Rhizosphere Soil of French Bean

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The initial populations of *Trichoderma* spp. in black, red and laterite soils were 5.2, 3.6 and 4.8 X10$_3$ cfu / g of soil. The soil from rhizosphere of French bean was collected and the population of *Trichoderma* was assessed by dilution plating technique using TSM. The population was estimated at 15, 30 and 45 days after sowing. At 15$_{th}$ day the population of *Trichoderma* isolates were maximum in P+ Z5TH8(*Trichoderma harzianum*)+Z7TV5(*Trichoderma viridae*) + Z10TVS10(*Trichoderma virens*) treatment (26 x10$_3$ cfu/g of soil) which was followed by P+ Z5TH8 treatment (23.33 x10$_3$ cfu / g of soil) and Z5TH8 (23.0 x10$_3$ cfu/g of soil) treatment. Lowest population was observed in natural soil (9.67 x10$_3$ cfu / g of soil). The populations of *Trichoderma* isolates differed significantly from the control.

Key words: *Trichoderma*, rhizosphere, population, french bean.

Trichoderma species are imperfect filamentous fungi with teleomorphs belonging to the Hypocreales order of the Ascomycota division. The ecological role of this genus is that *Trichoderma* strains take part in the decomposition of plant residues in the soil (Herper and Lynch, 1985) and also a good biocontrol agent (Chet, 1987; Haran *et al*., 1996; Schirmbock *et al*., 1994). *Trichoderma* and other potential biocontrol fungi proliferate abundantly in various natural soils when added as young mycelia in intimate contact with food base (Lewis and Papavizas, 1984).

Trichoderma spp. have received considerable attention as potential biological control agents against a wide range of soil-borne plant-pathogenic fungi (Chet, 1987) in both green house (Lewis and Papavizas, 1987) and field (Elad *et al* ., 1983). However, the efficacy of *Trichoderma* spp. as biocontrol agents in natural soils may be limited by soil fungistastis (Lai, *et al.,* 1968), competition by other soil microorganisms (Harman, *et al*., 1993), poor plant root colonization (Ahmad and Baker, 1987), or unfavorable environmental conditions. Identification and quantification of ecological factors affecting the establishment and the population dynamics of introduced *Trichoderma* strains in natural habitats may provide more predictable and effective biocontrol of plant diseases. For effective biological control of soil borne plant pathogens, a major consideration is antagonist proliferation after introduction into the soil or rhizosphere. Successful antagonist should produce inoculums in excess to survive and proliferate in soil and rhizosphere (Baker and Cook, 1974). However, the phenomenon of antagonist establishment and proliferation in soil in relation to biological control is important and deserves consideration. The survival ability of biocontrol agents, including population size, survival period and distribution in or on crops, need to be surveyed and associated with biocontrol effects.

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Materials and Methods

There are conflicting reports regarding the ability of *Trichoderma* spp. to survive in different soils under different soil conditions. Hence an experiment was conducted to study the population dynamics and survival of *Trichoderma* isolates in prominent soil types of Karnataka *viz.,* black, red, and laterite soils.

Three *Trichoderma* spp. were evaluated against soil borne fungal pathogens and for plant growth promotion under glasshouse conditions.

Collection and processing of soil sample

Four soil samples of 500 grams each were collected randomly from top six-inch layer of soil samples *viz.,* black, red and laterite soils from Bangalore regions were collected in polyethylene bag and transferred to lab for further studies. The soil samples were dried in the laboratory at 28$_o$C. Four soil samples collected from each soil type were mixed well to get a pooled soil sample. Soil sample was sieved through a 1000µ mesh and subjected for analysis of physico-chemical properties of soils like soil texture, pH, electrical conductivity, organic matter content, Major nutrients like N, P, K, Ca, Mg and micronutrients like Fe, Mn, Cu and Zn using different methods.

Isolation of Trichoderma spp.

The laboratory experiments were conducted

under aseptic condition in the laminar flow cabinet. *Trichoderma* spp. was isolated by using *Trichoderma* selective medium (TSM) developed by Elad and Chet (1983). Ten grams of soil sample was taken separately and suspended in 100ml of sterile water and stirred well to get 1:10 dilution (10^{-1}). One ml from this was transferred to test tube containing 9ml of sterile distilled water to get 1:100 (10^{-2}) dilution. Likewise the dilution of the sample was prepared up to 1:10000 (10^{-4}). One ml of the final dilution of each soil sample was aseptically pipetted out into each sterile Petri plate separately to which a quantity of 20 ml of sterilized and cooled *Trichoderma* selective medium was poured and gently rotated for uniform mixing and plates were incubated daily for appearance of *Trichoderma* colonies. *Trichoderma* colonies were initially white in color and later turned green. Later the colonies were purified by hyphal tip isolation method.

Identification and maintenance of Trichoderma isolates

The fungal isolates were subjected to morphological tests as listed in 'The Manual of Soil Fungi (Gilman, 1961) and 'A revision of the genus *Trichoderma* III', Bisset, 1991). Formation of characteristic colonies by fungal isolates and other characters were taken as a tool for preliminary identification by comparing with the standard (Table 1). Cultures grown on potato dextrose agar were stained with cotton blue stain. The structure of the fruiting bodies and spore arrangement of the isolates were first observed under 100X compound microscope and then they were photographed using phase contrast microscope.

Cultures of soil borne fungal pathogens, antagonistic *Trichoderma* spp. and bacterial isolates were grown for seven days at room temperature on potato dextrose agar (PDA) slants respectively and subsequently stored at 5°C in a refrigerator and maintained by sub culturing once in a month. One set of all cultures were preserved in mineral oil to serve as stock culture.

In vitro evaluation of Trichoderma isolates against soil borne fungal pathogens

The *Trichoderma* isolates were evaluated for their antagonistic effect *in vitro* against *Rhizoctonia solani, Sclerotium rolfsii, Fusarium oxysporum*, *Pythium* sp. and *Phytopthora* sp. by dual culture method (Dennis and Webster, 1971b) on potato dextrose agar (PDA) medium. Fifteen ml of PDA medium was poured into sterile petri plates and allowed for solidification. Five mm discs from actively growing colony of pathogen was cut with a sterile cork borer and placed near the periphery of PDA plate. Similarly antagonistic fungi was placed on the other side i.e., at an angle of 180°C. Plates with no antagonists placed were served as control for the pathogen. The plates were incubated at 27 ±1°C for seven days. Each treatment was replicated thrice.

The extent of antagonistic activity by *Trichoderma* isolates i.e., growth after contact with fungal plant pathogens was recorded after incubation period by measuring growth of fungal plant pathogens in dual culture plate and in control plate (Table 2). The per cent inhibition of fungal plant pathogens was calculated as suggested by Vincent (1927).

$$I = \frac{100\ (C-T)}{C}$$

Where,

I = Per cent inhibition

C = Growth of fungal plant pathogens in control (mm)

T = Growth of fungal plant pathogens in dual culture plate (mm)

Greenhouse evaluation

In greenhouse a pot culture experiment was conducted in the Department of Agricultural Microbiology, UAS, GKVK, Bangalore, to study the population dynamics and to evaluate antagonistic effect of *Trichoderma* spp. against soil borne fungal pathogens and plant growth parameters in French bean.

Preparation of Pathogen Inoculum

Fungal pathogens viz., *Rhizoctonia solani* and *Sclerotium rolfsii* were first grown on potato dextrose agar in sterile plates. A mixture of 940 gram sand and 60 gram crushed sorghum (i.e., sand:sorghum; 94:6% w/w) were mixed by adding tap water till the sand could form ball. Then the mixture was filled to autoclavable polybags and the opening of the bags was sealed using rubber band with cotton plug and autoclaved. After autoclaving 5 mycelial discs of 5 mm diameter were cut from the margin of actively growing pathogens and transferred aseptically to the polybags and incubated at 27±1°C for 15 days. The bags were carefully shaken periodically in order to permit uniform growth.

Preparation of biocontrol agents formulation

Fungal Biocontrol agents viz., *Trichoderma harzianum (*Z5TH8*), Trichoderma viridae(*Z7TV5*)* and *Trichodema virens* (Z10TVS10) were grown on potato dextrose agar sterile plates and then 5 mm mycelial disc of biocontrol agent was transferred to sterile PDB aseptically and incubated at 28±2°C on a rotary shaker at 150 rpm for 5 days; After 7 days the biomass was thoroughly mixed in a mixer grinder to break the mycelial bits and formulated using talc as a carrier material (Talc: liquid culture of biocontrol agent @ 2:1 w/v) with 10 g carboxy methyl cellulose (CMC) per kg carrier material as adhesive.

Greenhouse experimental details

Green house experiment in French bean crop (Arka Komal) with 10 treatments and 3 replications was conducted at Department of Agricultural Microbiology, GKVK, Bangalore

Details of treatments imposed in soils (Red, black and laterite)

T_1- Uninoculated control (natural soil)

T_2- Pathogen control (*Rhizoctonia solani* and *Sclerotium rolfsii)* (p)

T_3- Z5TH8 (*Trichoderma harzianum*)

T_4- Z7TV5 (*Trichoderma viridae*)

T_5- Z10TVS10 (*Trichoderma virens*)

T_6- P + Z5TH8

T_7- P + Z7TV5

T_8- P + Z10TVS10

T_9- P + Z5TH8+ Z7TV5+ Z10TVS10

T_{10}- P+ Bavistin

Preparation of pots and sowing

Twenty gram of the inoculum from each of the pathogen (*Rhizoctonia solani* and *Sclerotium rolfsii*) was mixed in 4 kilograms of sieved non-sterile field soil and later filled in poly bags. The seeds were first surface sterilized with mercuric chloride (0.1%) for one minute followed by five washings with sterile water. The surface sterilized seeds were then treated with talc formulations @ 10 g per kilogram seeds, shade dried for 30 minutes and sown immediately. Ten treated seeds were sown in each plastic pot.

Estimation of Trichoderma spp. Population in Soil under Green House Condition

The population density of *Trichoderma* spp. in the rhizosphere was calculated by serial dilution plate count method. Ten gram of soil from each replication was drawn from the rhizosphere of French bean and transferred to 100ml water blank, shaken well for 2-3 minutes to get 10^{-1} dilution. One ml from this dilution was transferred to 9ml water blank, shaken well to get 10^{-2} dilution. Further the same process was continued to get required dilutions. One ml from the appropriate dilution was pipetted out and transferred to sterile Petri plates and 20ml of appropriate cooled molten sterile *Trichoderma* specific medium (TSM) was poured and gently rotated in clockwise and anticlockwise direction to let the suspension distribute uniformly in the medium.Three replications were maintained for each dilution.

The plates were incubated at 27°±1°C for 5 days and colonies were counted on a colony counter and population was estimated and expressed as colony forming units (cfu) per gram of soil.

Results and Discussion

The initial populations of *Trichoderma* spp. in black, red and laterite soils were 5.2, 3.6 and 4.8 $X10_3$ cfu / g of soil. The soil from rhizosphere of French bean was collected and the population of *Trichoderma* was assessed by dilution plating technique using TSM. The population was estimated at 15, 30 and 45 days after DAS.

Table 1. Key characteristic used for the identification of *Trichoderma* isolates

Characteristics	*Trichoderma* spp.	*T. harzianum*	*T. viride*	*T. virens*
Colonies	Loose tufts in shades of green or yellow orless frequently white, yellow pigment may be secreted into the agar(PDA) characteristic sweet or coconut odour	Growing rapidly, white green, bright green to dull green	Fast growing, dark green, smooth, hairy, typical coconut odour	Coinidia effuse covering entire plate,conidia typically formed moderately well, no odour detected
Mycelium	Long, Septate, thin walled, colourless, smooth and hyaline, branched	Septate, colorless, smooth	Hyaline, smooth, septate much branched	smooth, septate thin walled
Chlamydospores	Abundant, Globose to subglobose, ellipsoidal intercalary, terminal,	Mostly globose, smooth	Intercalary, globose	Abundant, terminal and intrcalary, subglobose to ellipsoidal
Conidiophores	Highly branched, loosely or compactly tufted, often formed concentric rings , produces lateral branches paired or not, paired branches appeared pyramidal aspect , terminatesin one or few phialides.	Loose stuff, main branch produces numerous side branches	Arise in compact or loose tuft, main branch produced side branches , all branch stand at wide angles	Arise in clusters from aerial mycelium, branching towards to the tip, each branch terminating in a pencillius
Phialides	Typically enlarged in the middle but may be cylindrical nearly subglobose. Held in whorls, variously penicillate, clustered on wide main axis or solitary	Short, narrow at the base, pointed neck	Curved, pin shaped, narrow at the base	Arise in closely appressed whorls, less frequently in pairs or singly, base constricted, swollen in the middle, attenuate at the tip
Phialophores	Globose to subglobose, ovoid, some shades of greenish yellow or colourless	Acuminated at the tip of phialides, subglobose, short and smooth, pale green	Globlose, pale green, smooth	Produced singly or in pairs and accumulated at the tip

Population dynamics of Trichoderma isolates in the rhizosphere of black soil

The population of *Trichoderma* isolates were maximum in P+Z5TH8+ Z7TV5+Z10TVS10 treatment (26 $x10_3$ cfu/g of soil) after 15 days of sowing. It was followed by P+ Z5TH8 treatment (23.33 $x10_3$ cfu / g of soil) and Z5TH8 (23.0 $x10_3$ cfu/g of soil) treatment. Lowest population was observed in natural soil (9.67 $x10_3$ cfu / g of soil) (Fig 1). The same trend was observed at 30 and 45_{th} days after sowing.

Population of Trichoderma spp. in the rhizosphere soil of French bean in red soil

The data presented revealed that the populations of *Trichoderma* isolates were significantly differed from the control treatment. At

15th day significantly higher population was observed in treatment P+ Z5TH8+ Z7TV5 + Z10TVS10 (23 x10$_3$ cfu / g of soil) and less population was observed in untreated and pathogen control (10 and 12 x10$_3$ cfu /g of soil) respectively (Fig 2). Whereas, all other treatments were on par with each other except in chemical control. In chemical control the population of *Trichoderma* decreased at all the intervals of incubation.

Table 2. Antagonistic properties of *Trichoderma* isolates against common soil borne fungal pathogens

Trichoderma isolate	Soil borne inhibited fungal pathogens				
	Rhizoctonia solani	*Sclerotium rolfsii*	*Fusarium oxysporum*	*Pythium* sp.	*Phytopthora* sp.
Z1TH1	++	++	+	+	++
Z2TH2	++	+	++	+++	++
Z3TH5	+++	++	++	++	+
Z3TH4	+++	++	++	++	+++
Z4TH5	++++	+++	++	+++	++
Z4TV1	+++	+++	++	++	+
Z4TVS1	+++	++	++	+++	+
Z5TH6	+++	++	++	++	+
Z5TV2	+++	+++	+++	+	++
Z5TVS3	+++	+++	++	++	++
Z5TV3	+++	+++	++	++	+
Z5TH8	++++	++++	++++	++++	++++
Z5TVS4	+++	+	++	++	++
Z6TH9	++	+++	+++	++	++
Z6TV4	+++	+	+++	++	++
Z6TVS5	++	++	+++	+++	++
Z6TVS6	+++	++	++	++	++
Z7TH11	++	++	++	++	++++
Z7TH12	++	+++	++	++	+++
Z7TV5	+++	++++	++++	++++	+++
Z7TV6	++	++	+++	++	+
Z7TVS7	++	++	+++	+++	+++
Z8TH13	+++	+	+++	++++	+
Z8TV7	+++	++	+++	+	+++
Z8TVS8	+++	+++	++	++	++
Z8T9	++	++	++++	+++	++
Z8T10	++	+++	+	+	+++
Z9TH14	++++	+	+++	+++	+
Z9TV8	++	+++	+++	++	++
Z9TVS9	++	+++	+++	++++	++
Z9TV9	+++	+++	+++	+++	+++
Z10TV10	++++	++	+++	+	++
Z10TVS10	++++	+++	++++	++++	++++

Note: + = < 25 % inhibition over control; ++ = 25 – 50 % inhibition over control; +++ = 50 - 75 % inhibition over control; ++++ = >75 % inhibition over control

Population dynamics of Trichoderma sp in the rhizosphere of laterite soil

In treatment P+ Z5TH8+ Z7TV5+Z10TVS10, maximum population (20.67 x10$_3$ cfu/g of soil) was observed at 15th day followed by Z10TVS10 treatment (20.33 x10$_3$ cfu/g of soil) (fig 3). All the treatments significantly differ when compared to pathogen control and but the results in other treatments were on par with each other. At 45th day maximum population was observed in P+ Z5TH8+ Z7TV5 + Z10TVS10 (45.67 x10$_3$ cfu/g of soil) which was followed by Z5TH8 treatment (40.67 x10$_3$ cfu/g of soil) and least population was observed in pathogen control (26.00 x10$_3$ cfu/g of soil).

Table 3. Population dynamics of *Trichoderma* spp. in rhizosphere of French bean in black soil under green house condition

Treatment	Population density (10$_3$ cfu /g of soil)		
	15th day	30th day	45th day
Natural Soil	9.67	19.33	38.00
Pathogen control (R. s+S. r)	10.67	25.00	33.00
Z5TH8	23.00	31.66	42.67
Z7TV5	22.00	32.00	40.33
Z10TVS10	20.00	27.00	37.00
P+Z5TH8	23.33	31.00	44.33
P+Z7TV5	21.00	31.66	36.00
P+Z10TVS10	20.00	29.33	40.67
P+Z5TH8+Z7TV5+Z10TVS10	26.00	37.66	48.00
P+Bavistin	23.66	21.66	20.00
LSD(P<0.05)	4.874	6.463	4.963

Note: DAS: Days after sowing; P: Pathogens (*Rhizctonia solani* + *Sclerotium rolfsii*) Bavistin: Seed treatment @2g/kg seeds

Trichoderma isolates were studied in different soil types at different intervals. The maximum population of *Trichoderma isolates* was found in Z5TH8 +Z7TV5 +Z10TVS10 followed by Z7TH8 treated soil compared to pathogen treated soils. The earlier methods using Martins medium (Elad *et al,* 1981)

Table 4. Population dynamics of *Trichoderma* spp. in rhizosphere of French bean in red soil under green house condition

Treatment	Population density (10$_3$ cfu /g of soil)		
	15th day	30th day	45th day
Natural Soil	10.00	23.00	35.33
Pathogen control (*R. s+S. r*)	12.00	22.33	26.33
Z5TH8	21.00	29.33	42.33
Z7TV5	22.67	32.00	39.66
Z10TVS10	18.00	28.33	39.33
P+Z5TH8	20.00	28.00	37.66
P+Z7TV5	19.00	29.66	40.33
P+Z10TVS10	20.33	29.00	40.66
P+Z5TH8+Z7TV5+Z10TVS10	23.00	28.66	39.66
P+Bavistin	18.00	25.00	21.66
LSD(P<0.05)	7.141	7.949	6.067

Note: DAS: Days after sowing; P: Pathogens (*Rhizctonia solani* + *Sclerotium rolfsii*); Bavistin: Seed treatment @2g/kg seeds

and soil extract agar supplemented with rose Bengal (Mughogho, 1968) failed to eliminate undesirable contaminants. In these media both *Trichoderma* and

Table 5. Population dynamics of *Trichoderma* spp. in rhizosphere of French bean in laterite soil under green house condition

Treatment	Population dynamics (10$_3$ cfu /g of soil)		
	15th day	30th day	45th day
Natural Soil	11.00	27.00	29.33
Pathogen control (*R. s+S. r*)	8.00	21.33	26.00
Z5TH8	17.67	25.67	40.67
Z7TV5	18.67	25.33	37.00
Z10TVS10	20.33	24.33	39.33
P+Z5TH8	17.00	21.67	36.67
P+Z7TV5	13.33	25.67	35.00
P+Z10TVS10	20.00	24.33	36.33
P+Z5TH8+Z7TV5+Z10TVS10	20.67	29.00	45.67
P+Bavistin	18.00	22.00	23.00
LSD(P<0.05)	5.075	6.139	5.024

Note: DAS: Days after sowing; P: Pathogens (*Rhizctonia solani* + *Sclerotium rolfsii*); Bavistin: Seed treatment @2g/kg seeds

Gliocladium was also very fast growing posing unsuitability for selective and qualitative enumeration of these organisms.

Among these media TSM was finally selected for further work. TSM appeared better in qualitative and quantitative enumeration of *Trichoderma* and *Gliocladium* was compact and restricted in growth (3-4mm diameter), dirty green and dark green in colour respectively compared to other media (Saha and Pan, 1997). The *Trichoderma* introduced treatments showed maximum population of *Trichoderma* spp in their respective soils with regardless of soil types. In all the three types of soil viz., red, black and laterite soils, the population of Z5TH8, Z7TV5 and Z10TVS10 goes on increasing with the crop growth period. But the population was decreased in the treatments of pathogen control, absolute control and chemical control.

Lewis and Papavizas (1984) also demonstrated the potentiality of various *Trichoderma* spp aggregates to form chlamydospores readily and in great number in natural soil and in fragments of organic matter. After the introduction of the fungus to the soil as conidia, they suggested that introduced isolates have the potentiality and aggressiveness to colonize and establish themselves in organic matter in natural environment. *Trichoderma* and *Gliocladium* added to soil as dry formulation appeared to proliferate greatly (Papavizas *et al*., 1982), increasing from an initial amount of $5x10_3$ to a maximum of $6\text{-}7x10_6$ conidia/g of soil of various organic matter content.

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