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RESEARCH ARTICLE

Interactive effect of *Trichoderma* species with *Glomus intraradices* in growth promotion and wilt disease suppression of *Cajanus cajan*

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Abstract

The aim of present study was to investigate the effect of individual and co-inoculation of *Trichoderma* and *Glomus intraradices* on growth and wilt disease severity in pigeon pea (*Cajanus cajan* L Millsp). Five *Trichoderma* isolates i.e. *T. harzianum*, *T. viride*, *Th0126K*, *Th019K* and *Tv023K*, and *G. intraradices* individually and in different combinations were tested. *G. intraradices* alone was sufficient for growth promotion but it was not effective in terms of disease reduction. Among all the treatments tested, co-inoculation of *T. harzianum* and *G. intraradices* gave maximum growth and reduced the severity of disease (p<0.05). *Fusarium* considerably reduced the dry weight, nodulation and phosphorus (p) uptake. Results clearly showed that different species of *Trichoderma* produced varied results with *G. intraradices.*

Introduction

Soil is a complex habitat for a wide variety of organisms which includes bacteria, fungi, protozoans, nematodes, earthworms which function as populations or assemblage of similar organisms that interact with each other and contribute to plant nutrition, soil texture, soil fertility, decomposition of organic matter, cycling of nutrients and suppression of soil-borne pathogens. The plant roots with zone of intense microbial metabolic activity and high concentration of polysaccharides is called rhizosphere. Microbial communities in the rhizosphere influence ecological processes such as nutrient acquisition and fitness of plants through interaction with each other. *Trichoderma* species, arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR; *Pseudomonas florescence, P. putida, Paenibacillus, Burkholderia. Bacillus subtilis, B. circulense, Enterobacter* species, *Rhizobium* species) are beneficial organisms that are capable of influencing changes in rhizosphere functioning. However, *Fusarium* species are cosmopolitan population of soil, which causes wilt disease in major economic important crops.

Pigeon pea *(Cajanus cajan* L. Millsp.) is an important pulse crop of India. Its production has been decreasing mainly due to the complex disease-pest syndrome, which was estimated to be approximately 97,000 tons per year (Saxena et al., 2010). *F. udum* Butler causes wilt in *C. cajan* which survives through chlamydospores in seeds and dead plant debris. The fungus infects through roots and penetrates into the vascular system, and substantially reducing yields. Since *Fusarium* can survive in soil for several years, it is impracticable to control the disease by using fungicides. Several fungicides have been used to control plant diseases but abuse in their deployment has favored the development of resistant pathogens. In present context, disease management with biocontrol agents offers a great promise. A successful biological control agent should colonize the rhizosphere and should not leave toxic residues as opposed to chemicals (Dubey et al., 2007).

Trichoderma species are useful avirulent plant symbionts which act as biocontrol agents against phytopathogenic fungi by various mechanisms such as rhizosphere competition, mycoparasitism, by producing antibiotics and certain enzymes, induced resistance, and by promoting plant growth (Harman et al., 2004). AMF-mediated bioprotection has been exploited and accepted as a key practice for disease control (Garmendia et al. 2005; Garcia-Garrido 2009). Various workers have suggested that establishment of AMF in plant roots reduces the damage caused by pathogens and improves plant resistance to biotic stresses, but the underlying mechanism of bioprotection remain unknown (Pozo and Azcon-Aguilar 2007). According to Pozo et al. (2009), several mechanisms such as plant nutrition, damage compensation, and competition for sites/photosynthates, changes in root architecture and rhizosphere microbial populations, and activation of plant defense mechanisms, may play roles in plant protection by AMF.

The purpose of the present study was to isolate *Trichoderma* species and AMF from the rhizosphere of *C. cajan*, characterization of *Trichoderma* species on the basis of production of IAA, HCN, siderophore and cellulase production and to find out potential combination of *Trichoderma* with AMF for growth promotion and disease reduction of the same.

Materials and Methods

Plant material

Seeds of *C. cajan* (var. Bahar) procured from National Research Centre for Agroforestry, Jhansi were surface sterilized in 0.1% NaOCl for 2 minutes, washed several times in distilled water and germinated on sterilized sand.

Isolation of *F. udum*

Roots were washed with tap water to remove the soil particles and cut into small pieces (1 cm). Roots were surface sterilized in 0.2% mercuric chloride for 1 minute and washed several times with distilled water. Pieces of roots were placed on filter paper to remove excess water; and then, placed on potato dextrose agar (PDA). The plates were incubated at 28 $\pm$ 1 $^{\circ}$C for 7 days, and fungus was purified by hyphal tip culture technique.

Isolation of biocontrol agents

Soil samples were collected from rhizosphere of infected and healthy test plants, cultivated at selected sites. *Trichoderma* species were isolated on trichoderma selective media (TSM) containing 3 g glucose, 1 g NH_4NO_3, 0.9 g $Na_2H_2P0_4$, 0.2 g $MgS0_4.7H_20$, 0.15 g KC1, 20 mg $FeS0_4.7H_20$, 20 mg $ZnS0_4.7H_2O$, 20 mg $MnS0_4.H_20$, 30 µg rose bengal and 1000 ml distilled water, amended with 50 mg streptomycin sulphate, 50 mg chloramphenicol, 10 mg metalaxyl and 10 mg pentachloronitro benzene. The inoculated plates were incubated for 5 days at 28 $^{\circ}$C. Colonies of *Trichoderma* were isolated, purified and maintained on PDA.

G. intraradices Schenck and Smith, commonly occurring in pigeon pea fields of the region was used as AMF representative, in all experiments. The AMF inoculum used in this study consisted of sand with chopped root bits, spores and extramatrical mycelia. Taxonomic identification of AMF was matched with the description provided by the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (http://www.invam.caf.wvu.edu/). Selected AMF species was propagated as pure culture on *Zea mays* L. for 5 months in sterilized sand.

Pathogenecity of *F. udum*

The pathogenecity of isolates of *F. udum* was tested with susceptible cultivar of pigeon pea under net-house conditions. Dried soil was sterilized for 3 successive days. The pot soil was inoculated with *F. udum* 3 days before seed sowing. Seeds were sterilized with 0.2% mercuric chloride. Three seeds were sown in each pot. *F. udum* pathogenicity was confirmed after re-isolation of pathogen from the wilted seedlings.

In vitro plant growth promotion and antagonistic properties of *Trichoderma* species

IAA production was determined according to Gupta et al (2002), phosphate soubilization was done according to Vyas et al (2007), cellulases activity was determined according to Vyas (2005), HCN production was determined by Bakker and Schippers (1987). The biocontrol ability of selected isolates was studied against the *F. udum*, using dual culture technique (Dennis and Webster 1971). The mycelial discs (5 mm) of *Trichoderma* and *F. udum* from a 4 days old culture were placed at 5 cm away from each other on either side of PDA. Control plates contained only *F. udum* disc at the centre of plates. There were 5 replications for all the treatments and control. The plates were then incubated at 28 $\pm$ 1 $^{\circ}$C. The percent inhibition of *F. udum* was calculated according to the formula: $L = [(C - T)/C] \times 100$, where L is per cent inhibition of radial mycelial growth; C is radial growth measurement of the pathogen in control; T is radial growth of the pathogen in presence of *Trichoderma.*

***In vivo* growth promotion and disease suppression**

A completely randomized design was established in *Fusarium* infected soil with different treatments i.e. control (only *Fusarium* inoculated), *G. intraradices, T. harzianum, T. viride, Th0126K, Th019K, Tv023K, T. harzianum* +

G. intraradices, T. viride + G. intraradices, Th0126K + G. intraradices, Th019 + G. intraradices, Th019 + G. intraradices, Tv023 + G. intraradices. Pots were inoculated with *G. intraradices* and *Trichoderma* species, individually and in combinations. All treatments were replicated 10 times. Thus, total 120 pots were maintained. All three inoculums i.e. *Trichoderma, G. intraradices* and *F. udum* (15 g each) were applied in plastic pots (size: 24 x 16 cm), filled with 3 kg autoclaved soil. Inoculum was applied in the cavity made in potting substrate, as per treatments. In co-inoculated pots, *Trichoderma, G. intraradices* and *F. udum* were mixed thoroughly and applied as mentioned above. Pre-germinated seedling of similar size was transplanted into the cavities. To reduce the risks of cross contamination, pots were kept on separate benches in a completely randomized design in net-house and watered as and when required. Half strength Hoagland's solution in de-ionized water was applied at weekly intervals. The composition of the solution was: 0.03 g/L KH_2PO_4, 0.51 g/L KNO_3, 0.246 g/L $Ca(NO_3)_2$, 0.245 g/L $MgSO_4.7H_2O$, 1.43 g/L H_3BO_3, 0.91 g/L $MnCl_2.7H_2O$, 0.11 g/L $ZnSO_4.5H_2O$, 0.04 g/L $CuSO_4.5H_2O$ and 0.04 g/L H_2MoO_4. Seedlings (5 per treatment) were harvested after 5 months growth (1 per replicate). Number of nodules per plant was counted and samples were dried in sun; and then, in hot air oven at 70 $^{\circ}$C for dry weight (g plant^{-1}) estimation. Phosphorus content of plant was determined using vanado-molybdo phosphoric yellow color method (Jackson 1973) with a UV-VIS spectrophotometer (Halo DB 20, Double Beam, made in Australia) at 420 nm, and expressed in mg plant^{-1} on the basis of dry weight.

Disease severity index

The disease severity was determined using 1 to 7 point scale where odd numbers 1= no wilting, 3= approximately 25% leaves showing yellowing, 5 = 50% leaves showing yellowing as well as shoot stunted or wilted, 7= severe wilting, resulting in death of plant. Even numbers i.e. 2, 4, 6 were given to plants whose symptoms were between two odd numbers.

Statistical analysis

Data was subjected to analysis of variance in which effect of various treatments was tested. For each factor analyzed, the means of the different treatments were compared and ranked using Fischer F-test (p<0.05).

Results

A sum of 129 *Trichoderma* isolates were obtained from soil samples collected from rhizosphere of *C. cajan*. 11 isolates were selected on the basis of their antagonistic activity against *F. udum* (Table 1). *T. harzianum*, *T. viride* and *Th0126K* showed significantly higher inhibition of *F. udum*, followed by *Th019K* and *Tv023K*. All selected isolates were found positive for phosphate solubilization, IAA and HCN production. These isolates form a clear zone around their growth after 36 hours of inoculation in phosphate solubilization culture media. IAA was confirmed by development of pink color in culture plates and HCN production was determined by the change of filter paper color (from yellow to reddish brown). Cellulases activity was significantly maximum in *T. harzianum* (1.629±0.09) followed by *T. viride* (1.483±0.05) and *Th0126K* (1.421±0.08), and minimum in *Th015K* and *Tv002K* (Fig 2). On the basis of the *in vitro* plant growth prompting and antagonistic activity, *T. harzianum*, *T. viride*, *Th0126K*, *Th019K* and *Tv023K* were selected for *in vivo* plant growth promotion and disease reduction experiments.

Results obtained from net-house experiment indicated that plants inoculated with *G. intraradices* showed significantly maximum growth promotion activities in terms of dry weight and P uptake either single or co inoculation conditions. Nodulation was maximum in plants co-inoculated with *T. harzianum* and *G. intraradices*. The values in terms of dry weight in different *Trichoderma* inoculated pots were comparable with each other. Significantly least dry weight, nodulation and P uptake were obtained in pots where *Fusarium* was inoculated alone (control). Results on remaining treatments showed that the values in terms of growth (dry weight and number of nodules) and P uptake was comparable with each other so that comparison could not be made. Further, results suggested that among all *Trichoderma* isolates, *T. harziamum* gave best response with *G. intraradices* which was followed by *T. viride* and *Th019K* (Table 2).

The observation on disease severity index showed that it was highest (6.63±0.15) in *Fusarium* inoculated pots (control). Among all treatments except control, maximum disease severity was recorded in *G. intraradices* (4.46±0.12) and *T. viride* (4.60±0.26) inoculated pots. Inoculation of *Trichoderma* with *G. intraradices* was efficient in disease reduction. Disease severity was least (1.30±0.10) in pots co-inoculated with *T. harziamum* and *G. intraradices*, followed by *Th019K + G. intraradices* (2.06±0.53) and *Th0126K + G. intraradices* (2.8±0.05) inoculated pots. However, remaining treatments showed that disease severity index were comparable with each other (Figure 2).

Table 1 Assay of selected isolates of *Trichoderma* for percent inhibition of mycelia growth of *Fusarium udum*.

Isolates	Percent inhibition of radial growth of *Fusarium udum*	
	IV day	VII day
Control	0.00	0.00
T. harzianum	29.00±2.01	46.23±2.49
T. viride	23.66±2.51	39.32±2.08
Th0126K	27.33±1.52	35.66±3.54
Th007 K	9.66±2.08	20.63±1.31
Th016 K	7.33±1.43	16.33±1.42
Th019 K	14.00±1.00	2963±2.07
Th015 K	11.00±1.09	23.00±1.03
Tv004 K	8.66±0.57	20.00±1.00
Tv023 K	11.33±2.08	23.46±2.51
Tv002 K	4.33±1.56	14.41±3.51
Tv011 K	6.34±1.09	10.54±1.30
$LSD_{0.05}$	13.11	17.8

Values are means of three replicates±SD

Fig 1Cellulase activity of *Trichoderma* isolates, values are means of three replicates which represent amount of glucose removed in µm/min/ml. Bars represent standard error of means(SE) at *p<0.05*

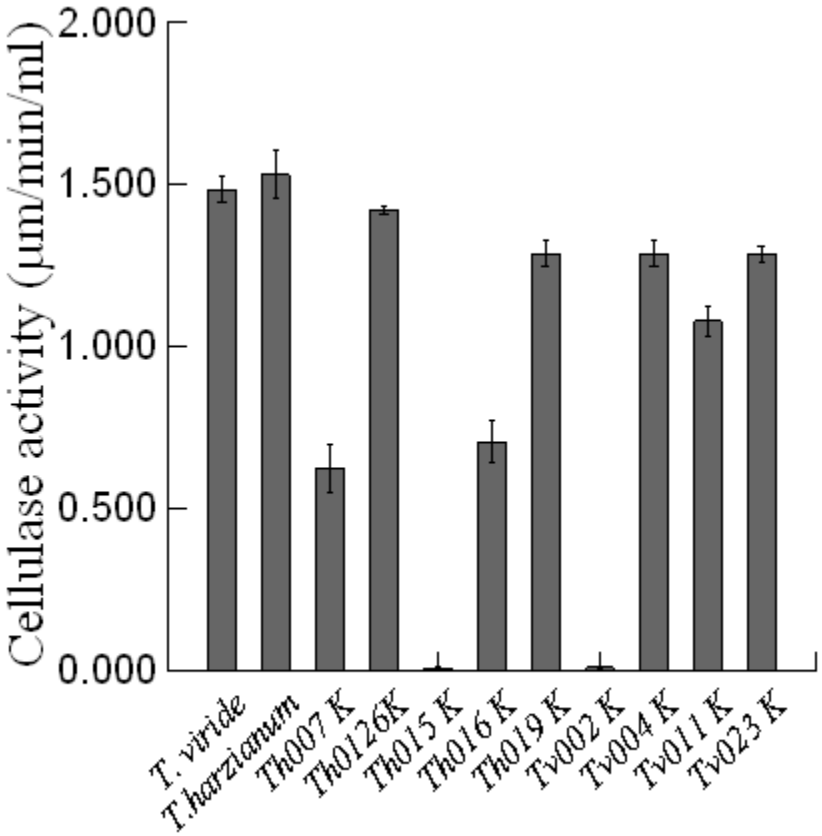

Fig 2 Effect of *Trichoderma* spp and *G. intraradices* on disease severity index of wilt disease. Bars represent standard error of means (SE) at *p<0.05*

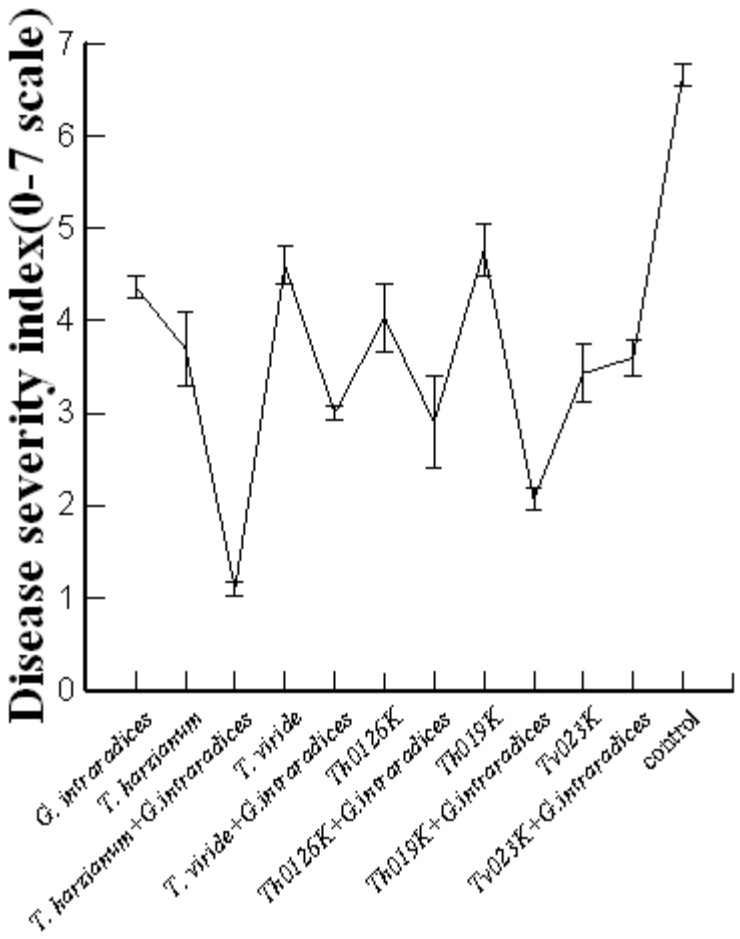

Table 2 Effect of treatments on growth and phosphorus uptake of *Cajanus cajan*

Treatments	Dry weight(mg/plant)	Number of root nodules/plant	Phosphorus uptake(mg)
Control	4.1±1.52	2.03±0.98	1.007±0.25
G. intraradices	23.66±1.46	11.33±3.55	4.50±0.40
T. harzianum	17.61±3.50	8.66±0.54	3.63±0.37
T. viride	13.66±1.15	7.50±2.00	2.73±0.20
Th0126K	14.00±1.50	15.00±3.00	3.93±0.058
Th019K	16.00±1.01	11.00±1.00	3.86±0.45
Tv023K	10.66±1.51	13.00±1.00	4.20±0.88
T.harzianum+G.intraradices	22.00±1.00	22.00±1.00	6.80±0.24
T. viride+G.intraradices	28.66±1.52	19.33±1.52	4.26±0.45
Th0126K+G.intraradices	19.00±1.06	16.00±1.00	4.13±0.20
Th019K+G.intraradices	25.00±1.20	14.00±1.00	3.40±0.55
Tv023K+G.intraradices	21.33±1.22	8.33±1.52	5.30±0.26
$LSD_{0.05}$	12.75	13.67	3.41

Discussion

Present study demonstrated the effect of 5 isolates of *Trichoderma* and *G. intraradices* individually and in combination on growth response and wilt disease incidence. Results suggested that interaction between *Trichoderma* and *G. intraradices*, and their effect on growth or disease reduction varied with different species of *Trichoderma. T. harzianum* exhibited better results among *Trichoderma* species with reference to growth and disease reduction. Evidence of IAA or HCN production and phosphate solubilization indicated that these isolates could be potential for growth promotion. Cellulase activity of biocontrol agents directly correlated with the antagonism against pathogens, isolates taken in the proposed study were potential cellulose producers. *T. harzianum* inoculated seedlings showed maximum dry weight and P uptake per plant over control. Harman (2000) reported that *Trichoderma* increases root development, crop yield, proliferation of secondary roots and seedling fresh weight. Growth promotion activities of *Trichoderma* might be a direct consequence of colonization, enhanced positive interaction between beneficial fungi and plant, increased nutrient uptake by plant or by reducing pathogen activity (Shoresh et al., 2010). Combined inoculation of *T. harzianum* and *G. intraradices* had additive effects on plant growth and nodulation as compared to their single inoculations. Calvet et al. (1993) has also reported a synergistic effect on the growth of marigold and percentage of AMF internal colonization as a result of combine-inoculation of *T. aureoviride* and *G. mosseae*. This suggests that possible mechanism for growth stimulation by *Trichoderma* and AMF could be the solubilisation of plant nutrients and colonization of roots, which increase growth and P uptake (Yedidia et al., 2001). Synergistic effect of *Trichoderma* and *G. intraradices* could also be due to the release of volatile compounds by *T. harzianum* (Calvet et al., 1992) which stimulate the formation of auxiliary cells (Fracchia et al., 1998) and leads to enhanced growth of plants. However, *G. intraradices* alone was also significantly enhanced growth and P uptake. Different species of AMF produce different response with same species of *Trichoderma*. In present study, AMF consortium was used which might be responsible for such results. According to Linderman (1992), the establishment of AMF symbiosis in plants is known to change physiological and biochemical properties of the host plant and these changes may alter the composition of root exudates which play a key role in the modification of the microbial population qualitatively and quantitatively in the mycorrhizosphere.

Results obtained from disease severity index revealed that single inoculation of all *T. harzianum* was effective in disease suppression as compared to single inoculation of *G. intraradices* compared to the control. Similar results were obtained by Chandanie et al. (2009). According to them, *Trichoderma* species antagonizes the rhizosphere pathogenic population strongly and it is indicated that the antagonistic process relies mostly on the production of antibiotics and/or hydrolytic enzymes (Harman *et al.,* 2004). *Trichoderma* function as antagonists of many phytopathogenic fungi, thus protecting plants from diseases by means of induced systemic resistance or localized resistance (Harman et al. 2004). *Trichoderma* induced production of phytoharmones such as jasmonic acid (JA), ethylene and salicylic acid (SA) which play a major role in the resistance (Martinez-Medina et al. 2010).

Further, our results suggested that single inoculation of *G. intraradices* was not effective for disease reduction. Such results suggested that only a well-established AMF symbiosis could reduce damage caused by root pathogens. Co-inoculation of *T. harzianum* and *G. intraradices* significantly reduced disease severity either than all treatments. *Trichoderma* facilitated positive interactions in the rhizosphere which reduces biotic and abiotic damage (Shoresh et al., 2010). Earllier reports suggested that *T. harzianum* and AMF establishment in plant roots induces hormonal changes, which activate systemic defence responses.

On the basis of results obtained, it could be stated that combination of different group of microorganisms activate several mechanism such as competition, altered root exudations, anatomical and morphological changes in the root system, antibiosis and induced plant defense systems in the presence of pathogen.

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