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Effect of Arbuscular Mycorrhizal Fungi and *Trichoderma* on Plant Health and Disease Suppression in Cotton

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ABSTRACT

The experiments were carried out to study the effect of AMF and biocontrol agents on improving plant health and suppressing plant diseases in cotton cultivar, MCU-5-VT. Inoculation of both AMF and *Trichoderma* reduced disease severity. Biochemical concentrations in plant tissues were significantly influenced by AMF and biocontrol agent inoculation. Higher biochemical concentrations with maximum accumulation of proteins, amino acids and phenols were observed in plants inoculated with the tested microorganisms. Defense related enzyme activity, peroxidase and phenyl alanine ammonia-lyase, increased with inoculation of both AMF and the pathogen. Generally enzyme activities reached their maximum at early stages of plant growth and were reduced subsequently. Soil microbial activity, measured in terms of soil respiration rate and dehydrogenase activity, increased significantly in inoculated soils.

Key words : Arbuscular mycorrhizal fungi (AMF), *Trichoderma*, Biocontrol, *Fusarium*, Cotton

Introduction

The control of soil-borne plant diseases with fungal biocontrol agents has elicited considerable recent research interest in sustainable agriculture as it is based on the management of a natural resource, i.e. certain rhizosphere organisms, which are the common components of ecosystem known to develop antagonistic activities against harmful organisms. Arbuscular mycorrhizal fungal (AMF) associations have been shown to reduce damage caused by soil borne plant pathogens. The degree of protection varies with the pathogen involved and can be modified by soil and other environmental conditions [1].

Species of *Trichoderma* are common inhabitants of the rhizosphere and are well recognized as biocontrol agents against soil borne plant pathogens [2,3]. Defense responses were demonstrated during the early stages of root colonization by *Trichoderma* [4,5]. The positive characteristics of *Trichoderma* include mycoparasitism, tolerance to dry conditions, effective root colonization and resistance to fungicides [6]. The importance of *Trichoderma* like saprophytic fungi lies in the large microbial biomass they supply to soil.

Considering these facts, the present work was proposed to study the effect of specific soil microorganisms on disease suppression and biochemical changes in cotton plants.

Materials and Methods

1. Experimental setup

The experiment was conducted at the nursery of the Department of Botany, Bharathiar University (latitude 11˚02′N and longitude 76˚58′E 409 m. a.s.l.), located at the foothills of Maruthamalai, in Coimbatore, Tamil Nadu, India. The cotton cultivar MCU 5 VT was selected for the study based on its wide cultivation. The native microbial population was: *Trichoderma harzianum* - 2.16×10^3 CFU, *T. viride* - 1.93×10^3 CFU, *T. virens* - 1.87×10^3 CFU, *Fusarium oxysporum* f. sp. *vasin-*

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fectum - 0.22×10^3 CFU, *Glomus geosporum* - 10.10 spores per gram, *G. fasciculatum* 11.2 spores per gram.

2. Source of inoculum

Two AMF, *Glomus geosporum* Nicol. & Gerd. and *G. fasciculatum* (Thaxter) Gerd. & Trappe emend. Walker & Koske isolated from cotton growing-soil, multiplied and maintained using *Allium cepa* L. in sterile sand: soil (1 : 1) and served as inocula of AMF. For each AMF species, ten thousand propagules were applied to respective treatment. Treatments devoid of AMF received soil filtrate of an equal amount of soil suspended in 1 L water and passed through 38 μm sieve.

Trichoderma harzianum Rifai, *T. viride* Persifr and *T. virens* Miller Giddens & Foster were isolated from cotton growing-soil using selective medium. Isolated and identified cultures were grown on Potato Dextrose broth, transferred to sterile talc base powder and the inoculum was made containing *ca.* 5×10^5 propagule per gram.

3. Source of pathogen

F. oxysporum was isolated from infected cotton plant roots in Czapek-Dox medium and identified using standard manuals. The pathogen multiplied in Czapek-Dox broth served as source of pathogen for inoculation. One mL of the broth containing *ca.* 1×10^3 propagules per mL of the pathogen was inoculated directly to the soil using a syringe.

4. Treatments

Individual inoculum of each microorganism was added to the soil and twenty days later the pathogen and seeds were introduced. The combinations used were:

T_1-Uninoculated soil; T_2-*Trichoderma harzianum* (T.h.) + *Fusarium oxysporum* f. sp. *vasinfectum* (F.o.); T_3-*Trichoderma* viride (T.v.) + F.o.; T_4-*Trichoderma virens* (T.vi.) + F.o.; T_5-*Glomus geosporum* (G.g.) + F.o.; T_6-*Glomus fasciculatum* (G.f.) + F.o.; T_7-F.o.

5. Development of cotton seedlings

The experiments were carried out in a complete randomized block design, consisting of 7 treatments. Six cotton seeds were sown in each polybag (30 × 11 cm) containing ca. 1.5 kg black soil. The soil had an initial pH of 7.8 and an electric conductivity of 47.35 mS cm^{-1}. The soil contained 0.135 mg kg^{-1} of total Nitrogen, 0.017 mg kg^{-1} of available Phosphorus and 0.10 mg kg^{-1} of exchangeable Potassium and 4.01% organic carbon. Immediately after emergence the seedlings were thinned to one seedling per polybag. The polybags were arranged in 2×2 m^2 sandpits and the sandpits were drenched with water as and when necessary in order to maintain humidity. Each treatment was replicated three times.

6. Disease severity assessment

Disease severity was assessed by grading the plant and leaves according to Dimond et al. [7].

Grade 0 : No disease symptom
Grade 1 : Epinasty and/or slight yellowing of leaf
Grade 2 : 20 to 50 % yellowing of leaf area or stunted growth with small leaves
Grade 3 : Complete yellowing or partial wilting
Grade 4 : Leaf fallen or dried

The average grade was computed for the plant as a whole and divided by 0.04 to give a maximum value of 100.

7. Sampling and analysis of soil microbial activities

Rhizosphere soil and root samples were collected every 30 days upto 120 days after emergence (DAE) of seedlings. Rhizosphere soil was collected by removing the loose soil attached to the roots. Root samples were fixed in FAA and analyzed later.

Indirect analysis of the microbial activities in the soil was done by assessing the dehydrogenase activity and respiration in the soil. Dehydrogenase activity was assessed according to Öhlinger [8]. Soil samples were suspended in triphenyltetrazolium chloride solution and incubated for 16 h at 25˚C. The triphenyl formazan (TPF) produced was extracted with acetone and measured photometrically at 546 nm. Soil respiration was assessed according to Öhlinger [8]. The CO_2 produced from the soil sample incubated in a closed vessel was absorbed in NaOH and quantified by titration against diluted HCl.

8. Preparation of roots for AM assessment

Roots fixed in FAA were washed thoroughly to remove FAA and observed under dissection microscope (×20) to examine AM fungal spores attached to them. After examination, the roots were cut into 1 cm bits, cleared in 2.5% KOH [9], acidified with 5 N HCl and stained with trypan blue (0.05 % in lactoglycerol). The roots were kept overnight immersed in stain for staining. One hundred root bits were used for examination. The stained root bits were examined with a compound

microscope (×200-400) for AM fungal structures and the percentage of total root length colonization was estimated according to magnified intersection method [10].

9. Enumeration and isolation of AMF spores

One hundred gram soil was dispersed in 1 L water and the suspension was decanted through a series of 710- to 38-µm sieves, filtered and stained [11]. Spore morphology was also compared with the INVAM culture database (http://invam.caf.wvu.edu).

10. Biochemical studies

Proteins were extracted in 0.02 M phosphate buffer (pH 7.0) and quantified using Folin Phenol reagent [12]. Amino acid was estimated by using ninhydrin reagent. One mL of the alcohol extract was added with 1 mL ninhydrin, heated and the intensity of colour developed was read at 625 nm in a UV-VIS spectrophotometer (Spectronic D20) using glycine standard graph [13]. Total phenol was estimated according to Bray and Thorpe [14]. One mL of the tissue alcohol extract was mixed with 1 mL Folin-ciocalteu reagent followed by 2 mL sodium carbonate, heated and colour intensity was read at 650 nm using UV-VIS spectrophotometer (Spectronic D20). Phenols were estimated from a standard graph using catechol as standard.

11. Enzyme studies

Peroxidase activity was assessed using Guaiacol as substrate for assay according to Sadasivam and Manickam [15]. Phenylalanine ammonia-lyase activity was measured as the amount of trans-cinnamic acid produced [16].

12. Statistical analysis

Activity data were analyzed using a one-way analysis of variance (ANOVA) (Microsoft Excel for Windows), and presented as means ± standard error. All measurements were replicated three times. P-values less than 0.05 were considered to be statistically significant.

Results

1. Disease severity

Disease severity of the plants under various treatments showed the influence of AMF and *Trichoderma* in controlling the disease. The greatest incidence of disease was recorded in treatment with artificial infestation of the pathogen alone, and it was clear that the incidence was reduced to the greatest extent in cotton plants inoculated by AMF and *Trichoderma* (Table 1).

2. AMF colonization

There were significant differences in total AMF colonization (% RLC), AMF inoculated plants showed significantly higher AMF colonization among the treatments (Table 2).

3. Tissue biochemical contents

Leaf and root proteins, amino acids and leaf phenol concentrations were significantly differed between treatments, but root phenol concentration was not affected by inoculation regimes (Figs. 1-3).

Table 1. Disease severity index of MCU 5 VT at 30-120 days after emergence (DAE).

	30 DAE	60 DAE	90 DAE	120 DAE
T_1	2.5	2.5	3.5	8
T_2	14	22.5	18.5	18.8
T_3	16.5	19	20	21.5
T_4	**9**	**9.5**	**9**	**9.5**
T_5	9	9.5	8.5	10
T_6	10	10.5	9.5	7
T_7	31.5	44.5	48.5	50.5
SE	0.200627	0.306826	0.325824	0.339325

Treatments: T_1-Uninoculated soil; T_2-*Trichoderma harzianum* (T.h.) + *Fusarium oxysporum* f. sp. *vasinfectum* (F.o.); T_3-*Trichoderma viride* (T.v.) + F.o.; T_4-*Trichoderma virens* (T.vi.) + F.o.; T_5-*Glomus geosporum* (G.g.) + F.o.; T_6-*Glomus fasciculatum* (G.f.) + F.o; T_7-F.o. *SE*: *Standard error*

Table 2. Percent root AMF colonization levels in MCU 5 VT at 30-120 days after emergence (DAE).

	% RLC			
	30 DAE	60 DAE	90 DAE	120 DAE
T_1	60.20	61.80	62.90	63.70
T_2	61.40	65.80	66.10	65.80
T_3	62.10	64.90	65.20	65.60
T_4	63.60	66.60	67.20	66.80
T_5	**72.60**	**79.60**	**80.40**	**81.20**
T_6	**71.60**	**78.80**	**79.80**	**80.45**
T_7	59.80	62.10	62.75	63.10
SE	0.109514	0.153403	0.155581	0.159736

Treatments: T_1-*Uninoculated soil*; T_2-*Trichoderma harzianum* (T.h.) + *Fusarium oxysporum* f. sp. *vasinfectum* (F.o.); T_3-*Trichoderma viride* (T.v.) + F.o.; T_4-*Trichoderma virens* (T.vi.) + F.o.; T_5-*Glomus geosporum* (G.g.) + F.o.; T_6-*Glomus fasciculatum* (G.f.) + F.o; T_7-F.o. *SE*: *Standard error*

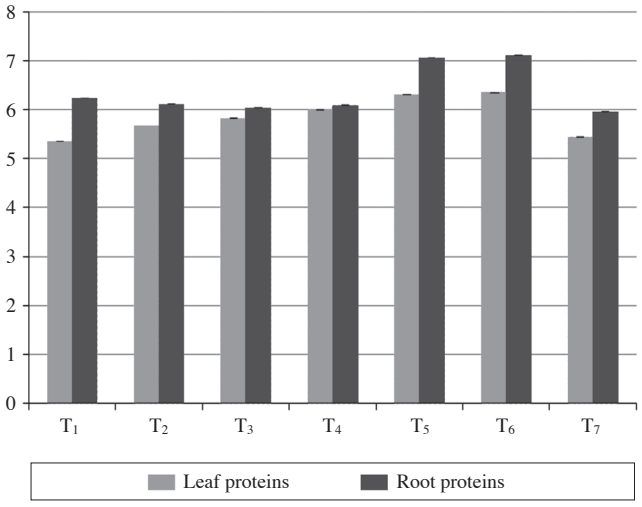

Fig. 1. Total Proteins (mg per one gram of sample) [Error bars stand for the standard error]

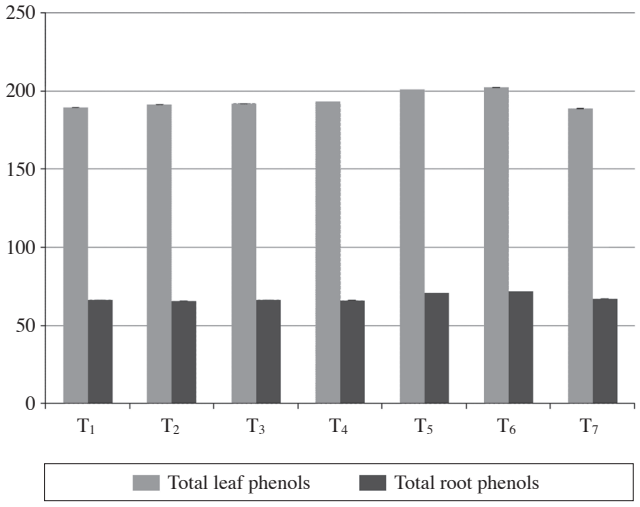

Fig. 3. Total Phenols (mg per g sample) [Error bars stand for the standard error]

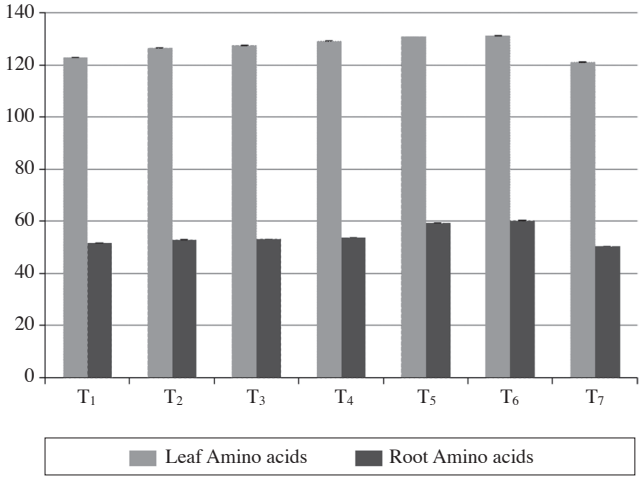

Fig. 2. Total Amino acids (mg per gram sample) [Error bars stand for the standard error]

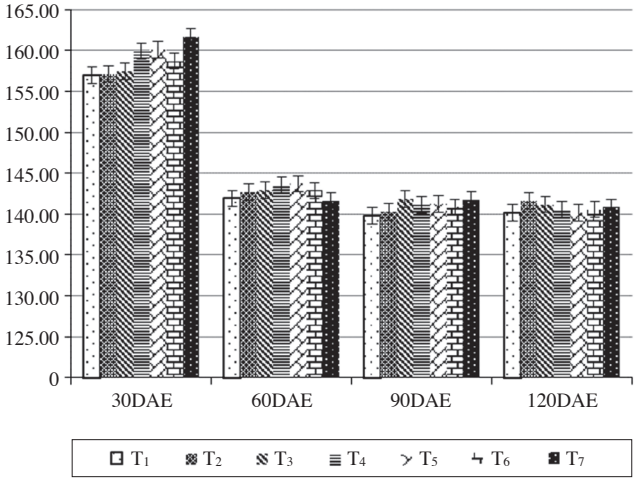

Fig. 4. Peroxidase activity (enzyme activity per unit liter) [Error bars stand for the standard error]

4. Plant enzyme activities

PAL and peroxidase activities were highest during early stages of mycorrhizal development or pathogen infection. At 30 and 60 DAE, PAL activity differed significantly between cultivars, but at 90-120 DAE, it differed significantly with treatments also. There were significant differences in peroxidase activity between treatments (Figs. 4-5). Enzyme activities generally improved with pathogen inoculation at the early stages.

5. Soil microbial activities

Soil microbial activity were measured in terms of soil dehydrogenase activity and soil respiration rate varied significantly between treatments (Figs. 6-7). Inoculation of *Trichoderma* improved soil microbial activities compared to inoculation of AMF species.

Discussion

Inoculations of AMF and *Trichoderma* spp. in this study were promising in disease suppression as well as nutrient acquisition and plant growth. This is in accordance with the well-known bioprotective effect of AMF and *Trichoderma* [1,17].

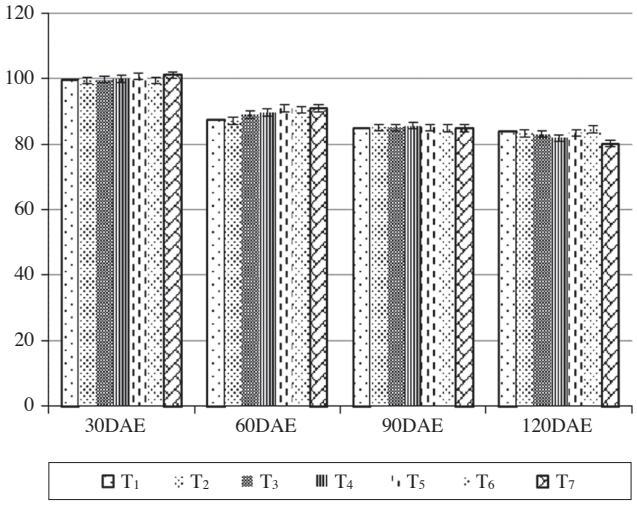

Fig. 5. PAL activity (µ mols *T*-cinnamic acid per hour per mg protein) [Error bars stand for the standard error]

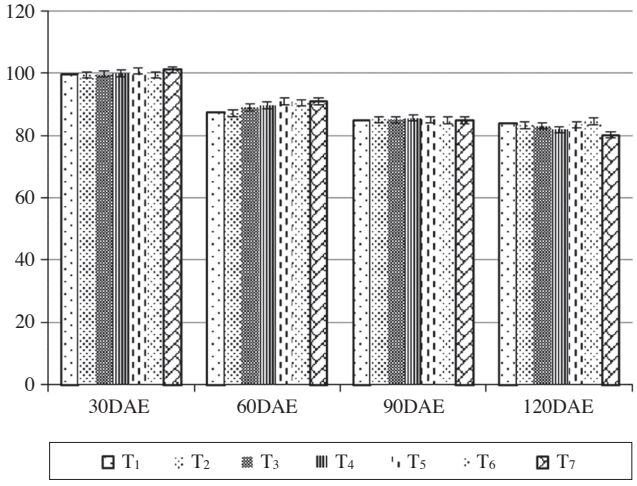

Fig. 6. Soil dehydrogenase activity (mg CO_2 per gram dry matter at 24 hours) [Error bars stand for the standard error]

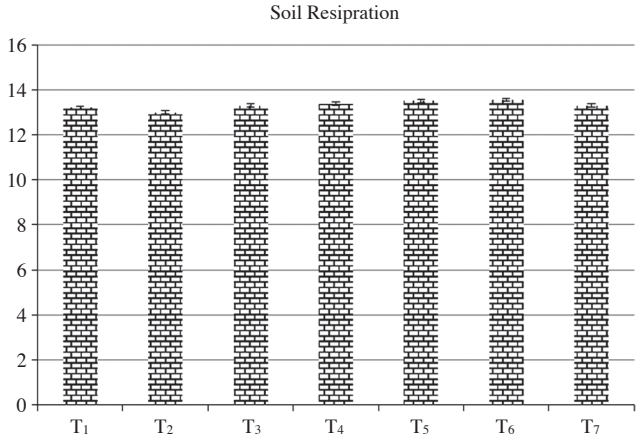

Fig. 7. Soil respiration rate (µg TPF per gram drymatter soil at 16 hours) [Error bars stand for the standard error]

All the tested bioinoculants proved to be effective in disease reduction compared to the controls. Similar effects of AMF on reduction in disease incidence were reported by many workers in different plant species [18]. Often inoculation with AMF prior to challenging with a pathogen is necessary to achieve disease reduction. This is because many root pathogens germinate and grow more rapidly than AMF [19]. Similar results were already reported by many workers [20,18]. Cordier et al. [20] demonstrated that induced resistance against *Phytophthora parasitica* in mycorrhizal tomato roots resulted from both localized defense enzymes in arbuscule-containing cells and systemic defense responses in non-mycorrhizal parts of mycorrhizal roots. The reduced propagule number of pathogen in AMF treated soils is also in parallel with the findings of St-Arnaud et al. [21] where *Pythium ultimum* propagule density was reduced in soil inoculated with *Glomus intraradices*.

In this study, among the two AMF tested, *G. fasciculatum* was superior to *G. geosporum* in disease suppression. These differences in interaction between different AMF and pathogen show that each pathogen-AMF-plant combination is unique.

Trichoderma species are proved to be efficient biocontrol agents against various soil borne plant pathogens [22]. In the present study, *T. virens* and *T. harzianum* were found to be more effective than *T. viride*. Further, Larkin and Fravel [23] demonstrated that *T. harzianum* and *T. virens* significantly reduced *Fusarium* wilt of tomato by 37-75% under in greenhouse conditions. Howell et al. [24] reported an induction of terpenoid synthesis and improved peroxidase activity in cotton roots by seed treatment with *T. virens* against *Rhizoctonia solani*. Similarly Katragadda and Murugesan [25] demonstrated the hyperparasitic potential of *T. harzianum* over *Fusarium oxysporum* f. sp. *vasinfectum*. Hanson [26] showed a reduction in *Verticillium* wilt symptoms in cotton following seed treatment with *T. virens*. As rapid colonization contributes to the effectiveness of an AM association, high inoculum densities may be required for maximum plant growth response [27]. The spatial relationships of the propagules and roots will affect the amount of root available for infection spread [28]. AMF inoculated plants showed improved nutrient acquisition and growth compared to the uninoculated plants in unsterilized soil. This could be due to the increased size of population of the AMF species available to them.

Tissue nutrient acquisition was highest when AMF and biocontrol agents applied. In these treatments, AMF inoculated plants in general showed higher amount of tissue N which is in agreement with earlier studies [29]. The increased N may be taken up directly from different sources such as the indigenous soil N pool, N-fertilizers or plant-unavailable N pool [30]. Similarly P acquisition was also positively correlated mycorrhizal levels.

An improved tissue nutrient content in *Trichoderma* inoculated plants could be due to the transfer of nutrients from the soil to the roots in a way analogous to mycorrhizal effect. Similar results were already reported [31].

Proteins, aminoacids and phenols are key molecules found associated with mycorrhizal colonization patterns. Plant phenolics are the most widespread classes of secondary metabolites known to be involved in plant-microbe interactions. One of the best ways to assess the implication of phenolics in mycorrhizal enhanced resistance or tolerance of plants to soilborne pathogens is to compare the accumulation of phenolic compounds in mycorrhizal and non-mycorrhizal roots when challenged by a pathogen. The analysis of total root phenol may be an indication of plant reaction to a given mycorrhizal fungus [32]. Dehne and Schonbeck [33] shown that the simultaneous inoculation of *G. mosseae* and *F. oxysporum* increased the total phenol contents of the roots than the mycorrhizal fungus alone or the pathogen alone. Similarly in the present study, AMF colonization and inoculation of both AMF species correlated positively with total phenol contents in leaf and root.

Differences in qualitative and quantitative expression of proteins were observed in AMF inoculated plants. AMF inoculation increases the expression of low molecular weight proteins as suggested by Dumas et al. [34] and Pacovsky [35]. Furthermore, the ratios of the protein concentration in mycorrhizal roots were increased as the percentage of root colonization increased [36]. In the present study both leaf and root proteins were improved quantitatively in comparison with the control which could account for the earlier reports.

The response of plants to AMF infections involves a temporal and spatial activation of different defense mechanisms. The activation and regulation of these defenses have been proposed to play a role in the mechanism of mutualistic status of the association. Generally defense-related enzymes such as peroxidases, polyphenol oxidases and phenyl amine ammonia-lyase are elicited as a result of AMF colonization. Peroxidases are oxido-reductive enzymes that participate in the wall-building process such as oxidation of phenols, suberization and lignification of host plant cells during defense reaction against pathogens. In the present study the activity of the defense related enzymes, PAL and peroxidases were improved at early stages of colonization by both AMF and the pathogen and later their activity was reduced to a minimum level. Similarly an induction of defense responses was detected at an early stage of root colonization followed by suppression at a later stage of the symbiosis [37-39].

Conclusion

In conclusion, cotton plants inoculated with AMF and *Trichoderma* individually showed substantial increase in plant growth, nutrient acquisition and reduction in disease incidence. These responses were either marginal or reached upto many folds when inoculated plants were compared with the uninoculated plants. Inoculation of cotton plants with a combination of AMF and *Trichoderma* confirmed improved growth of the plants, which in turn raises the possibility of improved productivity.

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