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AM fungi shields *Coleus forskohlii* from root rot incidence

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Abstract

This study was taken up to determine the combined effect of arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR) in controlling root rot caused by *Macrophomina phaseolina* in *Coleus forskohlii*. AM root colonization was up to 70-73 per cent under combined inoculation of *Scutellospora* sp + *Pseudomonas fluorescens* + *Trichoderma viride* and 44-45 per cent under individual inoculation. A correlation analysis indicated that more the AM root colonization (73 per cent) less the root rot (28 per cent) incidence. The activity of the defense enzymes *viz.,* peroxidase, polyphenol oxidase and superoxide dismutase was found to be high at 30 days after inoculation of the pathogen in the co-inoculated treatments. Another correlation study between AM colonization and enzyme activity, showed low root rot index. There was a loss in the alkaloid content due to pathogen infection, yet, the combined treatments recorded a threefold increase in disease suppression.

Key words: AM fungi, plant growth promoting rhizobacteria, *Coleus forskohlii*, colonization, root rot index, peroxidase, polyphenol oxidase, superoxide dismutase, alkaloid.

Introduction

Plant response to colonization by mycorrhizal fungi can range from dramatic growth promotion to growth supression and the factors affecting this response include mycorrhizal dependency of the host crop, nutrient status of the soil, and the inoculum potential of the mycorrhizal fungi (Dalpe and Monreal, 2004). Besides, their ability to increase absorption surface of the roots and making the immobile ions available for growth, their antagonistic activity against the root rot pathogens is remarkable since it decreases disease susceptibility and increases tolerance against biotic and abiotic stress.

Coleus forskohlii is a medicinal herb, susceptible to root rot disease and as a result, the tubers get infected by pathogen like *Macrophomina phaseolina* that leads to reduction in its forskohlin content. Though many chemical formulations and many management practices are used to overcome this problem, a biocontrol practice is a must to support sustainable farming. With this necessity, studies were taken up using various inoculants like *Pseudomonas fluorescens, Trichoderma viride* and AM fungi to exploit the potential of AM fungus and *Pseudomonas* towards controlling the root pathogen *M. phaseolina*.

Materials and methods

An experiment was carried out at Department of Agricultural Microbiology, TNAU to study the effect of AM inoculation (*Scutellospora* sp.) with two PGPR isolates (*Pseudomonas* sp.) on *Coleus forskohlii* against *M. phaseolina*. Pots of 30 x 28 cm size with 10 kg soil having pH-8.18; EC-0.89 dSm^{-1}; available N - 219 kg/ha; P_2O_5 - 14.3 kg/ha and K_2O - 293.4 kg/ha was subjected to the following treatments. Treatments were replicated thrice. Design used was completely randomized block.

Treatments: *Scutellospora* sp. SSP 3 + *M. phaseolina; P. fluorescens* (CPF 1) + *M. phaseolina; P. fluorescens* (CPF 2) + *M. phaseolina; Trichoderma viride* (TV 1) + *M. phaseolina;* SSP 3 + *P. fluorescens* (CPF 1) + *T. viride* (TV 1) + *M. phaseolina;* SSP 3 + *P. fluorescens* (CPF 2) + *T. viride* (TV 1) + *M. phaseolina; M. phaseolina* alone.

Inoculants: The pathogen used for the study was *M. phaseolina* obtained from the Department of Plant pathology, Tamil Nadu Agricultural University, Coimbatore. The pathogen was multiplied in sand maize medium for 15 days (Riker and Riker, 1993) at the rate of 10 g per kg soil (*i.e.* 100 g/pot) before planting. The *Pseudomonas* isolates (CPF 1 and CPF 2) and the isolate SSP 3 (*Scutellospora* sp.) isolated from the rhizosphere of *C. forskohlii* were used as inocula for treatments. The AM fungus at the rate of 50 g pot^{-1} (containing 300-400 spores 100 g^{-1} inoculum) and talc based bio-control agent *T. viride* (TV 1) obtained from the Department of Plant pathology, Tamil Nadu Agricultural University (at the rate of 100 g pot^{-1}) were applied in soil before planting. *Pseudomonas* suspension (10^8 cells mL^{-1} broth) was inoculated (50 mL broth pot^{-1}) at the time of planting. Two to three leaved cuttings of *C. forskohlii* were used for planting. The control treatment was maintained with pathogen inoculation alone.

Observations: Root samples were taken after 45 days of planting and AM colonization was assessed in roots (Phillips and Hayman, 1970). Based on the disease incidence, root rot index was calculated (Kesavan and Choudhary, 1977) at 45th DAP.

Root rot index: The root rot index was calculated by employing the formula given below:

$$\text{Root rot index} = \frac{\text{Summation of individual scores}}{\text{Maximum grade x Total number of plants}} \times 100$$

Assay of defense related proteins and chemicals induced by bio-inoculants

Peroxidase and polyphenol oxidase: The peroxidase enzyme activity was assayed using pyrogallol and the intensity of

formation of yellow colour due to the enzyme activity was measured at 430 nm spectrophotometrically (Hammerschmidt *et al.,* 1982). For estimation of polyphenol oxidase enzyme activity, catechol was used as the substrate and the activity was measured as change in absorbance at 495 nm spectrophotometrically.

Superoxide dismutase (SOD): SOD activity was determined by measuring inhibition of the photochemical reduction of NBT (Nitro Blue Tetrazolium) and the absorbance was read at 560 nm (Beauchamp and Fridovich, 1971).

Forskohlin estimation: Forskohlin was estimated at harvest using HPLC. Fresh root tissue (200 mg) was powdered, soaked in 2 mL of absolute methanol (HPLC grade) for 24 h and centrifuged at 10,000g for 10 min at 4 °C. The supernatant obtained was concentrated by vacuum centrifugation for 5 h. The concentrate was dissolved in HPLC grade methanol and then filter sterilized using membrane filter (0.2 µm). Forskohlin estimation was done using High Performance Liquid Chromatographic system (HPLC) employing a mixture of acetonitrile and water (50 : 50 v/v) as the mobile phase and 250 x 4.6 mm C 18 column (Octadecyl silane - 5µ size) as the stationary phase. The flow rate was 1.5 mL/min and the wavelength was 220 nm. Forskohlin - 100 ppm concentration was used as the standard. From the area of the peak obtained in the graph, the content of forskohlin present in the samples was calculated. The biometric observations were recorded along with enzyme related studies in the roots of *C. forskohlii* as well as in the rhizosphere soil. The data were subjected to statistical analysis by variance (P= 0.05) with mean separation by Least Significant Difference (LSD), as per the methods detailed by the Panse and Sukhatme (1978). The analysis for microbial population was based on the *log* and *arc sine* transformed values.

Results

AM colonization: Treatments significantly influenced the root colonization percentage than when compared to the control. Inspite of the presence of *M. phaseolina* the inoculants were not only able to reduce the root rot but also influence the colonization rate positively. AM fungi SSP3 under combination with *P. fluorescens* and *T. viride* (73.5 and 70 % in T5 and T6 respectively) still showed remarkable impact than when inoculated in single (66.8%) (Fig. 1)**.**

Root rot index: A significant reduction in root rot index was observed in all the treatments. *T. viride* performed better by reducing the root rot index up to 34.2 %, followed by AM inoculation (38.2%) and *Pseudomonas* CPF 1 (40.6%). *T. viride* and AM inoculation expressed significant reduction of root rot index over the control. In combination, these inoculants' performance was superior, which recorded the lowest root rot index of 28-29% (Table 1). A simple correlation between AM

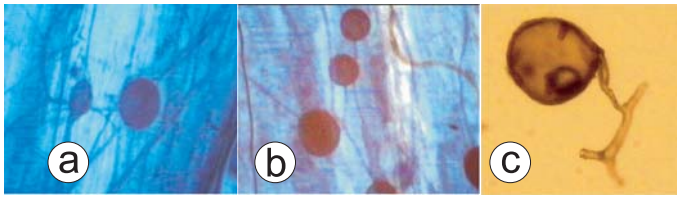

Fig. 1. Colonization of *Scutellospora* spp. SSP 3 in infected roots of *C. forskohlii*; a & b: Hyphae with vesicles; a spore of *Scutellospora*

Table 1. Effect of combined inoculation of AM fungus and PGPR organisms on root rot index and AM root colonization in *C. forskohlii* inoculated with *M. phaseolina* at 45 days after inoculation

Treatments	Root rot index (%)	AM root colonization (%)
T1: *Scutellospora* sp. (SSP 3)	38.2	66.8
T2: *Pseudomonas fluorescens* (CPF 1)	40.6	48.2
T3: *Pseudomonas fluorescens* (CPF 2)	53.3	44.6
T4: *Trichoderma viride* TV 1	34.2	45.5
T5: SSP 3 + CPF 1+ TV 1	28.5	73.5
T6: SSP 3 + CPF 2 + TV 1	29.5	70.0
Pathogen inoculated Control	79.3	40.0
LSD (P=0.05)	2.0	2.3

Table 2. Correlation analysis between AM colonization in roots of *C. forskohlii*, root rot index and defense enzymes activity under pot culture condition inoculated with *M. phaseolina*

Enzyme activities	AM root colonization
Root rot index	-0.700
Peroxidase activity	0.803
Poly phenol oxidase activity	0.819
Super oxide dismutase activity	0.944
Catalase activity	0.884

colonization and root rot index was found to be negative (r = -0.719) (Table 2).

Enzyme activities: Significant peroxidase activity was found at 20 days after inoculation (DAI) in all the treatments, where the combined inoculation of SSP 3 + CPF 1 + TV 1 registered 2-3 fold increase in peroxidase activity, which was 48-87 per cent higher than the individual inoculations. The induction of polyphenol oxidase was higher (85.5-112.2 per cent increase over control) in combined inoculations, while among the individual inoculations, SSP 3 recorded a significant increase over pathogen inoculated control. The defense related enzyme activity in the infected roots was maximum at 20 DAI. The 'Native page' analysis revealed that the induction of peroxidase as well as polyphenol oxidase iosenzyme was prominent under combined inoculations (Fig. 2). The SOD activity showed 2-3 fold increase over pathogen inoculated control (T_8) because of the combined inoculation of SSP 3 +CPF 1 +TV 1. Correlation analysis between AM colonization and the enzyme activities showed positive 'r value' (Table 2).

Forskohlin content: Root rot infection led to reduction of the forskohlin, but the loss of the alkaloid due to the pathogen severity was mitigated by the combined inoculation of AM fungus with *T. viride* and *Pseudomonas* (Table 3, Fig. 5).

Table 3. Effect of combined inoculation of AM fungus and PGPR organisms on forskohlin content in roots of *C. forskohlii* inoculated with *M. phaseolina*

S No.	Treatments	Forskohlin content (%)
1.	Pathogen inoculated control	0.001
2.	SSP 3 + CPF 1 + TV 1 + Pathogen	0.004
3.	SSP 3 + CPF 2 + TV 1 + Pathogen	0.002

Discussion

Generally AM plants have high concentrations of 'P' than non AM plants. This leads to the reduction in root exudates by altering the membrane permeability and hence the pathogen population may get reduced. This favours the negative correlation of AM colonization and root rot index (Table 2). The reduction in disease

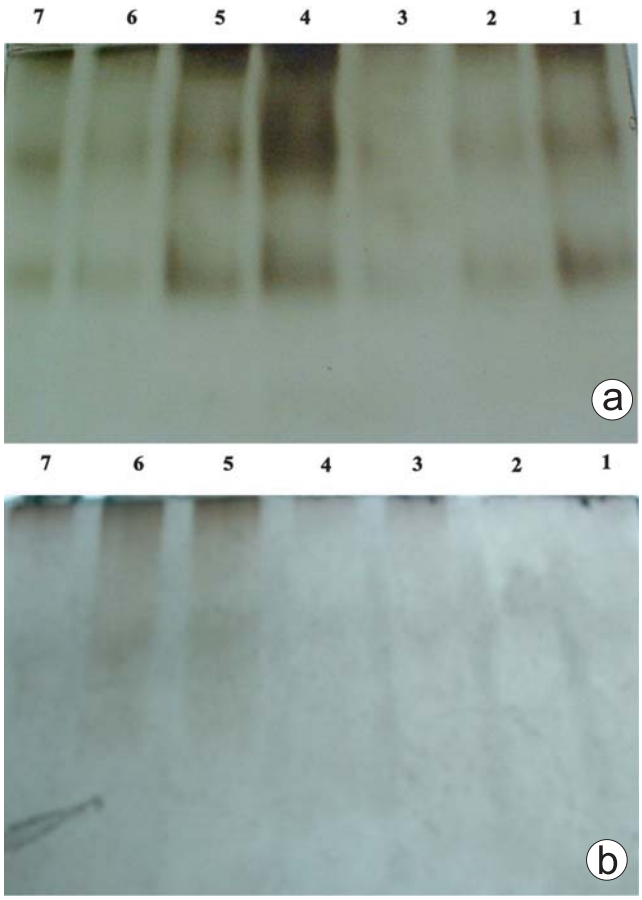

Fig. 2. Native PAGE analysis profile of peroxidase (a) and polyphenol oxidase (b) isoforms induced due to combined inoculation of AM fungus and PGPR organisms on 20 DAI in *C. forkohlii*; Lane (In order from the right end): T1 - *Scutellospora* spp. SSP 3; T2 - *P. fluorescens* CPF 1; T3- *P. fluorescens* CPF 2; T4 - *T. viride* TV 1; T5 - SSP 3 + CPF 1 + TV 1; T6 - SSP 3 + CPF 2 + TV 1

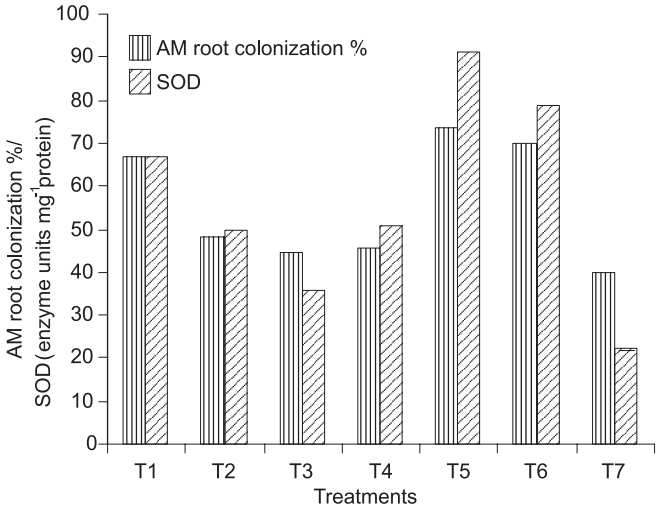

Fig. 3. Effect of combined inoculation of AM fungus and PGPR organisms on AM root colonization and SOD acivity in *C. forskohlii* inoculated with *M. phaseolina*

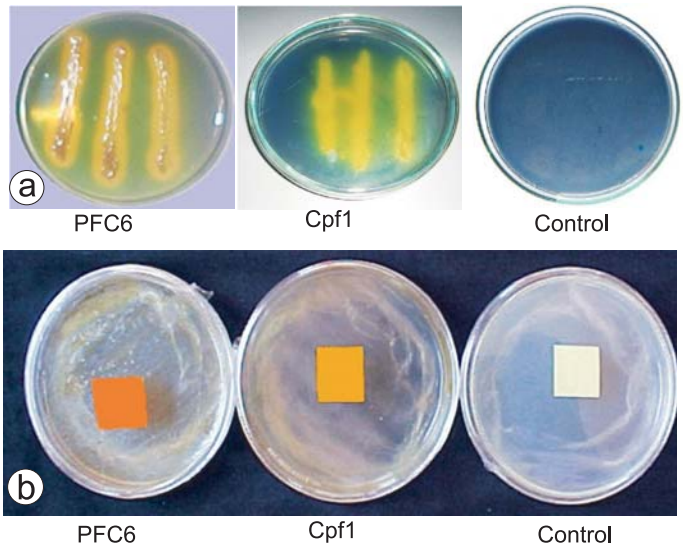

Fig. 4. Fig. 4. Siderophore production by *Pseudomonas* isolates; a. From the left, PFC6, CPF1 and control; b. From the left, PFC6, CPF1 and control

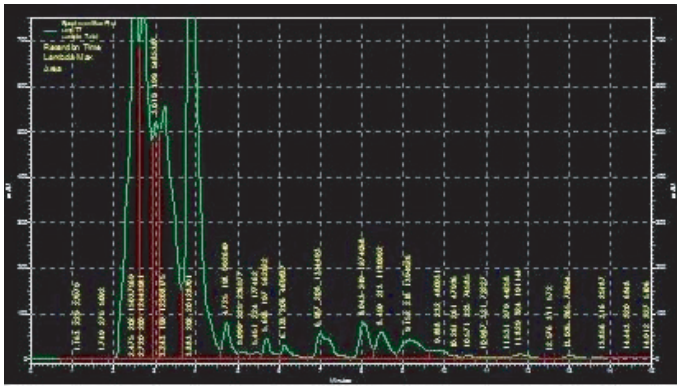

Fig 5. Effect of combined inoculation of AM fungus and PGPR organisms on forskohlin content *C. forskohlii* inoculated with *M. phaseolina*

severity could be explained by morphological alterations in host plants or by physiological changes due to concentration of phenols, aminoacids etc. (Bagyaraj, 1989) or by the elicitation of certain defense related molecules during AM colonization (Gianinazzi *et al.*, 1996). Increased peroxidase activity was noticed in a number of interactions involving plant pathogenic fungi, bacteria and viruses (Chen *et al.*, 2000). Accumulation of peroxidase has been correlated with induced systemic resistance in several plants (Hammerschmidt *et al.*, 1982) and with deposition of phenolic materials into plant cell wall during resistance interactions (Graham and Graham, 1991), which serve as a barrier that prevents the pathogenic fungal spread. Loganathan (2002) reported that application of bioformulation mixture induced several isoforms of polyphenol oxidase (PPO) in cabbage and cauliflower against *Plasmodiophora brassicae*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. Enhanced PPO activity in a mixture of biocontrol agents have been reported by several workers. Accumulation of PPO was higher in the combination of *Pseudomonas* strains (EPB22 + Pf-1) treated banana plants and PPO activity was significantly enhanced in rice plants pretreated with PGPR isolates (Radjacommare, 2005). This oxidative enzymes alter substances related to pathogen infection and thereby reduce the infection. Palma *et al.* (1993) studied the presence of two isoenzymes (Cu-Zn SOD and Mn-SOD) in the red clover roots due to the inoculation of *G. mosseae* in soil, as a result of which, there was an increase in O_2(-) radicals in root. Arines *et al.* (1993) showed the presence of an additional protein in the mycorrhizal extracts of red clover which was a plant induced SOD. These oxidative enzymes are grouped as active oxygen species (AOS) detoxifying enzymes; their production is associated with stress for water as well as for pathogen infections. Pathogenesis affects the biological oxidations in plant tissues

which alter the concentrations of various substrates and products of these reactions which in turn, are closely associated with plants' defense mechanisms.

In this study, there was a positive correlation between AM root colonization and isoenzyme activity in the infected roots, which proved the enzyme induction induced systemic resistance in plants (Fig. 3). The correlation values between the defense enzymes (peroxidase, polypheol oxidase and SOD) and the AM colonization percentage were positive (r = 0.8 to 0.9), which was due to the influence of proteins produced during biotic stress in the roots of the plants (Table 2). Several other reports showed the role of AM fungus in accumulating the secondary metabolites in crops like cucumber (Akiyama and Hayashi, 2002). *C. forskohlii* being the nursery raised crop, AM inoculation offers better scope to produce the disease free healthy seedlings and to reduce the use of chemicals. *C. forskohlii* has gained popularity by virtue of its exclusive constituent 'forskohlin' which has shown positive effect in treating glaucoma, congestive heart failure, intestinal spasms, insomnia, convulsions, hypertension, asthma and certain type of cancers, by its ability to increase the synthesis of 3'5' cyclic ester of AMP (cAMP), by the action of adenylate cyclase enzyme (Vishwakarma *et al*., 1988; De Souza, 1991).

Volatile and non volatile antibiotics and chitinase produced by *T. viride* may inhibit the root pathogens (Ezziyanni *et al.*, 2007). It is now, the fluorescent *Pseudomonas* sp. that are considered as an alternative to agrochemicals for controlling plant diseases and increasing plant development (Gloria and Leda, 2006). Their antagonistic property is attributed to the production of compounds like, siderophore and hydrogen cyanide. Further these results were supported by the field study, where in combined inoculation of *T. viride with G. mosseae* gave the best results with less root rot index of 32.28% in *C. forskohlii* (Boby and Bagyaraj, 2003).

The study revealed that there was no reduction in AM colonization in plants receiving *Pseudomonas*/*Trichoderma* inoculants along with the root-rot pathogen. The study also showed the compatability of AM fungi with *Trichoderma* as well as *Pseudomonas*. Forskohlin content was higher in the combined inoculation of AM with PGPR organisims.

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