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Effect of an AM fungal consortium and *Pseudomonas* on the growth and nutrient uptake of *Eucalyptus hybrid*

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Abstract *Eucalyptus* is an important tree species used for afforestation of large tracts of marginal and wastelands. *Eucalyptus*-arbuscular mycorrhizal fungal (AMF) interactions in seedling establishment and growth promotion have been inadequately dealt with. Efforts were made to assess the role of AMF-pseudomonad (PRS9, plant growth promotory fluorescent *Pseudomonas*) interactions in growth promotion and nursery establishment of *E. hybrid*. Seedlings were subjected to six different treatments: (i) uninoculated control, (ii) 400 AM spores, (iii) 800 AMF spores, (iv) PRS9 (v) 400 AMF spores + PRS9, (vi) 800 AMF spores + PRS9, with the different P regimes of 10, 20 and 30 ppm. Root length, shoot length, root and shoot fresh and dry weights were maximal at 400 AMF spores and 20 ppm soil P. Shoot P content was maximal at 800 AMF spores followed by 400 AMF spores and 400 AMF spores + PRS9. In general, plant growth was greater at 20 ppm P. Root P content increased significantly with 400 AMF spores followed by 800 at 20 ppm P. Independent of soil P levels, the quality index of mycorrhizal treatments without PRS9 was significantly higher than the treatments including PRS9 or PRS9 alone. Mycorrhizal inoculation efficiency was superior at 10 ppm P irrespective of the treatment. AM alone (400 spores) significantly improved the inoculation efficiency. PRS9 in association with AM fungi inhibited growth promotion and nutrient uptake

Key words *Eucalyptus* · PRS9 · Fluorescent · pseudomonad · AM fungi · Interaction

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Introduction

Although the rhizosphere is a rich reservoir of nutrients, especially sugars, amino acids and organic acids released from the root, competition among different microorganisms is high. Arbuscular mycorrhizal fungi (AMF) are a dominant component of the rhizosphere and transfer many assimilates to roots. This alters the root exudation pattern and hence changes the microbial population dynamics of the rhizosphere and rhizoplane regions (Bagyaraj and Menge 1978; Krishnaraj and Sreenivasan 1992). Available data indicate either an increase (Meyer and Linderman 1986), or a decrease (Ames et al. 1984) in the aerobic bacterial population in the rhizosphere of plants infected with mycorrhizal fungi or a neutral effect (Kothari et al. 1991). Mycorrhizal colonization also decreases the growth of bacteria in the rhizosphere as shown by tritium incorporation (Christensen and Jakobsen 1993), and might influence the species composition of the soil microbial community by stimulating select groups (Ames et al. 1984; Posta et al. 1994). The population density of fluorescent pseudomonads in the rhizosphere is usually reduced by AMF colonization (Ames et al. 1984; Waschkies et al. 1994). In a recent study, Ravnskov et al. (1999) examined the influence of *Glomus intraradices* Schenck & Schenck (BEG 87) on *Pseudomonas fluorescens* DF 57 in the hyphosphere and rhizosphere of *Cucumus sativus* L. (Aminex, F_1 hybrid) in a compartmentalized system. They concluded that the AMF significantly reduced the culture of the bacterium in both zones; however, the total number as measured by immunofluorescence microscopy only decreased in the hyphosphere soil.

Attempts have been made to improve the productivity of *Eucalyptus* through fertilization, breeding programmes or controlled mycorrhizal inoculation. Both ecto- and arbuscular mycorrhizal fungi have been reported in Eucalyptus (Boudarga et al 1990). However, most work has focussed on ectomycorrhizal fungi (Malajczuk et al. 1981; Adjoud et al. 1996). Furthermore,

AM reports are contradictory. For example, Schoeneberger (1984) reported a stimulatory effect of the AM fungus *Gigaspora margarita* on *E. regnans,* whereas Gomez et al. (1987) found no effect with 30 AM fungal isolates.

Eucalyptus is an important tree species in the afforestation programmes in India and large tracts of marginal and wastelands have been used for plantation. However, survival of *E. hybrid* (a hybrid between *E. camaldulansis* Dehnh. and *E. tereticornis* Sm.) in stressed soils is far from satisfactory. In order to improve nursery seedlings of *E. hybrid*, the influence of an AMF consortium alone or together with a growth-promotory fluorescent pseudomonad was studied in varying phosphorus regimes.

Materials and methods

Strain description

Fluorescent pseudomonad strain PRS9 (pea rhizosphere isolate) was obtained from the active gene pool of our department. This strain is positive for phosphate solubilization, HCN, indole acetic acid and siderophore (pyoverdine) production and has been evaluated extensively on crop plants for growth promotory and biocontrol activity (Rao and Johri 1999; Rao et al 1999).

Preparation and application of AMF inoculum

A native soil consortium of AMF spores dominated by *Acaulospora scrobiculata* Trappe*, Gigaspora albida* Schenck & Smith and *Glomus intraradices* Schenck & Smith (25:35:30) was used. Spores were recovered from the experimental soil by wet sieving and decanting (Gerdmann and Nicolson 1963). Spores were surface sterilized (0.1% streptomycin, 0.1% gentamycin, 0.2% chloramine-T and 0.1% Tween 80), washed several times with sterile distilled water and propagated on maize (*Zea mays* L.) for 6 months prior to use. The spores reisolated from the maize rhizosphere were again sterilized before application to *E. hybrid* seedlings.

Soil preparation and treatment

A native soil: sand: farmyard manure (FYM) (2:1:1 w/w) mixture (grey in colour, pH 7.4, 0.015% total N, 1.65% C, 10 ppm Olsen P, 2.89% organic matter) was used as potting mix. The soil was passed through 4-mm sieve, sterilized by drenching twice with 5% (v/v) formaldehyde, covered with a transparent polyethylene sheet for 10 days and then exposed to air for 15 days to remove the fumigant

Seed treatment and germination

Seeds collected from one plant were surface sterilized with 0.01% $HgCl_2$ for 30 s, rinsed several times with sterile distilled water and germinated on autoclaved soil: sand:FYM mix for 25 days. The seedlings were then transferred to polybags of 2 kg capacity containing the potting mix.

Application of AMF inoculum

AMF inoculum was applied as an overlay in a 1-inch hole in the mix. One seedling was planted per bag and the hole was covered with the potting mix.

Pseudomonas multiplication and seed bacterization

PRS9 was multiplied in succinate medium on a rotary shaker at 28 °C for 12 h (Rao et al. 1999). A charcoal carrier-based inoculum with population counts of 10^6–10^7 cfu g^{-1} was applied to the roots prior to transplantation.

Experimental details

The experiment was laid out in a completely randomized design pattern with six treatments: control, 400 AMF spores, 800 AMF spores, PRS9, 400 AMF spores + PRS9, 800 AMF spores + PRS9. Each treatment was replicated five times. Phosphorus in the form of single super phosphate (soluble phosphorus 16%) was used at the rate of 0 (existing P in the soil was 10 ppm), 20 ppm, and 30 ppm in the bags. The experiment was carried out in a polyhouse with day and night temperatures of 35 °C and 25 °C, respectively. Plants were irrigated as and when required and harvested after 6 months.

Sample analysis

The polybags were removed and plants were immersed in water along with the soil core. The adhering soil was removed by washing gently under running tap water and the roots were dried between layers of filter paper. The root and shoot portions were separated and fresh weight and length recorded. Roots were cut into 1-cm pieces and mixed thoroughly. A subsample of 100 segments was used for colonization rating and the remainder of the material was dried in a hot air oven and the dry weights recorded. Root segments were stained using the technique of Phillips and Hayman (1970) and colonization rating was performed according to Biermann and Lindermann (1981)

Plant samples were analyzed for Kjeldahl nitrogen on a semi-automatic nitrogen analyzer (M/S Gerhardt, Germany). The phosphorus content was estimated in acid-digested samples according to Jackson (1958)

Mycorrhizal inoculation efficiency

Mycorrhizal inoculation efficiency (MIE) (Bagyaraj 1992) was used as a measure of dependency on P acquisition for dry matter production calculated from the difference between inoculated and uninoculated plants relative to inoculated plants for each P level.

Quality index

Quality index (QI) (Prasad 1998) was determined as:

$$\frac{\text{Seedling Dry Biomass (g)}}{\frac{\text{Heigt (cm)}}{\text{Dia (mm)}} + \text{Top Dry Biomass (g)}}$$

Statistical analysis

Differences between treatments were determined using 2-factorial analysis of variance (ANOVA) for completely randomized design and significance among treatments was tested at the 5% probability level.

Results

Mycorrhizal colonization

Irrespective of the treatment, mycorrhizal colonization of roots was significantly higher in plants grown at 20 ppm soil P (64.67%)than other P levels. At 20 ppm P, the highest degrees of colonization were recorded with 400 and 800 AMF spore treatments but the values were not significantly different from those for 400 AMF spores at 10 ppm P (58.76%) and 400 AMF spores + PRS9 at 20 ppm P level (60.73%) (Table 1).

Table 1 Percent root colonization of *Eucalyptus hybrid* at different soil phosphorus levels (*AM1* 400 spores, *AM2* 800 spores)

Microbial treatment	Soil phosphorus level (ppm)		
	10	20	30
Control	–	–	–
AM1	58.76	64.67	45.09
AM2	57.18	63.88	43.75
PRS9	–	–	–
AM1 + PRS9	51.05	60.73	50.47
AM2 + PRS9	50.56	57.37	43.25
CD 5%	7.23		

Root, shoot length and collar diameter

The root and shoot lengths were maximal at an inoculum dose of 400 AMF spores per plant at 20 ppm P. However, there was no significant difference in these parameters between plants treated with either 800 AMF spores at 20 ppm P or 400 spores at 10 ppm P (Table 2). A significant decrease in root and shoot length was recorded at 30 ppm P level. The results of PRS9 inoculation alone or dual inoculation with 400 or 800 spores were not significantly different to the control. However, a significant improvement in shoot length was observed in treatments with single inoculation of 400 or 800 AMF spores. PRS9 inoculation exerted an additive effect on shoot length when used in combination with 400 or 800 AM fungal spores. Treatment with PRS9 alone resulted in a considerable improvement in shoot length at 10 ppm P compared with the control; however no significant difference was observed at 20 or 30 ppm P.

Irrespective of the treatment, collar diameter was significantly higher at 20 ppm P, whereas no significant difference was observed with the control either at 10 or 30 ppm soil P. Maximal collar diameter was recorded with the 400 AMF spore treatment, but this was not significantly different from that with 800 AMF spores or with 400 AMF spores + PRS9. PRS9 alone did not improve collar diameter at any P level; however, in combination with AM fungi it imparted an additive effect (Table 2).

Fresh and dry weight of root and shoot

Maximal root and shoot fresh and dry weights were recorded in plants treated with 400 AMF spores. A soil P level of 20 ppm was optimal in terms of fresh and dry weights. (Table 3). Mycorrhizal performance was adversely affected at 30 ppm P. Inoculation of PRS9 did not significantly improve growth over the uninoculated control. An additive effect, however, was observed in plants treated with PRS9 + AM fungi.

Phosphorus contents of roots and shoots

Irrespective of the initial soil P level, phosphorus content was maximal in plants inoculated with 800 AMF spores, followed by 400 AMF spores + PRS9. 20 ppm P was better than 10 or 30 ppm P. Treatment with 800 AMF spores at 20 ppm P gave the maximal shoot P content, followed by 400 AMF spores and 800 AMF spores + PRS9. In contrast, 400 and 800 AMF spores at 20 ppm P resulted in maximal root P content, which was comparable with the P content of plants receiving the PRS9 treatment at 30 ppm P. The uninoculated

Table 2 Effect of different treatments on root and shoot length and collar diameter of *E. hybrid* at different soil P levels [*AM1* 400 spores, *AM2* 800 spores, *Collar* collar diameter (cm), *RL* root length (cm), *SL* shoot length (cm)]

Microbial treatment	Soil phosphorus level (ppm)								
	10			20			30		
	RL	SL	Collar	RL	SL	Collar	RL	SL	Collar
Control	20.00	89.52	0.36	23.48	125.20	0.63	24.40	129.64	0.74
AM1	30.64	145.40	0.66	33.48	155.44	0.82	24.20	138.16	0.54
AM2	26.00	136.42	0.60	31.44	145.20	1.00	24.88	128.00	0.42
PRS9	21.28	127.58	0.36	20.32	130.00	0.50	24.98	131.58	0.50
AM_1 + PRS9	24.28	130.00	0.62	26.06	148.64	0.75	24.98	136.32	0.49
AM2 + PRS9	23.00	114.32	0.52	26.00	138.32	0.71	23.72	109.28	0.38
CD 5%	4.44	6.43	0.10	4.44	6.43	0.10	4.44	6.43	0.10

Table 3 Effect of different treatments on root and shoot dry weight of *E. hybrid* at different soil P levels [*AM1* 400 spores, *AM2* 800 spores, *RDW* root dry weight (g), *SDW* shoot dry weight (g)]

Microbial treatment	Soil phosphorus level (ppm)					
	10		20		30	
	RDW	SDW	RDW	SDW	RDW	SDW
Control	1.91	7.50	3.15	13.43	5.23	19.70
AM1	7.46	26.62	7.96	32.04	4.75	18.45
AM2	5.32	22.34	7.05	24.33	4.26	16.19
PRS9	1.90	8.54	4.53	15.09	6.20	21.72
AM1 + PRS9	5.74	25.50	6.47	28.72	4.60	16.05
AM_2 + PRS9	5.32	22.43	6.04	23.71	3.75	15.10
CD 5%	0.57	1.40	0.57	1.40	0.57	1.40

Table 4 Effect of different treatments on root and shoot P content of *E. hybrid* at different soil P levels [*AM1* 400 spores, *AM2* 800 spores, *RPC* root P content (mg per root), *SPC* shoot P content (mg per shoot)]

Microbial treatment	Soil phosphorus level (ppm)					
	10		20		30	
	RPC	SPC	RPC	SPC	RPC	SPC
Control	0.33	1.87	0.51	3.12	1.10	5.83
AM1	1.23	4.37	1.95	7.27	0.84	3.75
AM2	1.12	4.10	1.82	9.48	1.41	3.68
PRS9	0.33	1.70	1.13	3.82	1.71	5.92
AM_1 + PRS9	1.01	3.48	1.54	6.46	0.93	3.69
AM2 + PRS9	1.18	3.74	1.67	7.42	1.11	3.52
CD 5%	0.13	0.34	0.13	0.34	0.13	0.34

control and PRS9 treatment showed an increases in root and shoot P content at increased soil P levels. In contrast, the increase resulting from a rise from 10 to 20 ppm P was not sustained at 30 ppm P in treatments associated with AM fungi (Table 4).

Nitrogen contents of roots and shoots

Root and shoot nitrogen content was highest at 20 ppm P (Fig. 2). Independent of the initial soil P level, treatment with 400 AMF spores resulted in maximal N content (Table 5). The PRS9 treatment was superior to the uninoculated control in terms of root and shoot nitrogen content. Treatment of *E. hybrid* seedlings with 400 AMF spores alone resulted in maximal % root nitrogen, followed by 800 AMF spores at 20 ppm P. Root and shoot N contents were highest at 20 ppm P in plants treated with 400 AMF spores. Inoculation with AM fungi alone resulted in better performance than dual inoculation of AM fungi with PRS9. Thus PRS9 did not improve N content, and 30 ppm P was inhibitory in mycorrhizal plants.

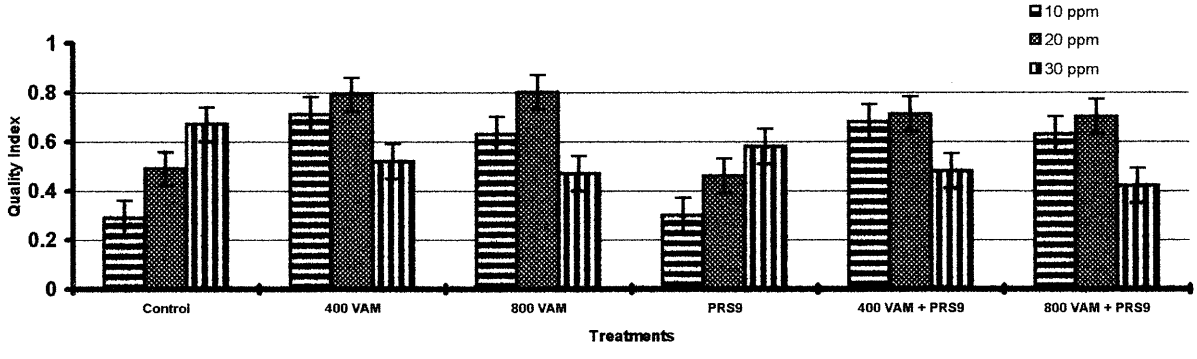
Table 5 Effect of different treatments on root and shoot N content of *E. hybrid* at different soil P levels [*AM1* 400 spores, *AM2* 800 spores, *RNC* root N content (g per root), *SNC* shoot N content (g per shoot)]

Microbial treatment	Soil phosphorus level (ppm)					
	10		20		30	
	RNC	SNC	RNC	SNC	RNC	SNC
Control	0.01	0.04	0.02	0.08	0.05	0.15
AM1	0.08	0.42	0.12	0.62	0.04	0.31
AM2	0.04	0.28	0.08	0.42	0.03	0.18
PRS9	0.01	0.05	0.04	0.12	0.06	0.29
AM_1 + PRS9	0.06	0.39	0.07	0.46	0.04	0.22
AM_2 + PRS9	0.04	0.26	0.06	0.37	0.03	0.20
CD 5%	0.006	0.07	0.006	0.07	0.006	0.07

Quality index

Seedling quality index (QI) was highest in mycorrhizal treatments without PRS9, independent of the soil P level (Fig. 1). Within the treatments, 20 ppm P was most suitable for improvement of seedling QI, and 400 AMF and 800 AMF spores at 20 ppm P were optimal. The bacterial treatment interacting with AM fungi reduced QI.

Fig. 1 Quality index of *E. hybrid* seedlings treated with AM fungi and PRS9 at different soil P levels



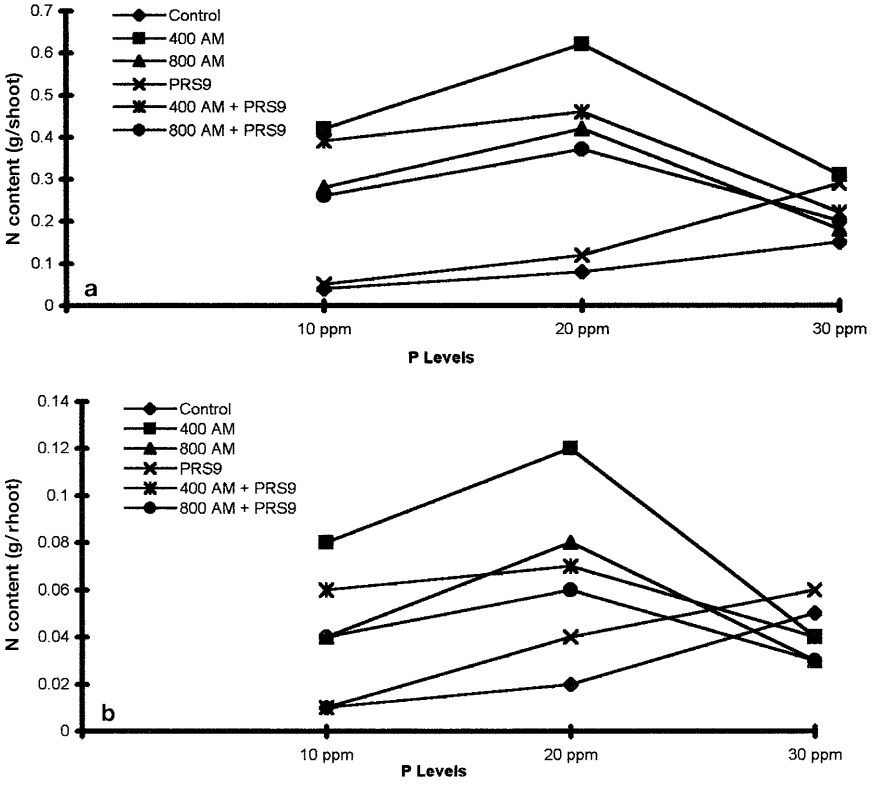


Fig. 2 N content of (**a**) shoots and (**b**) roots of *Eucalyptus hybrid* plants treated with AM fungi and fluorescent pseudomonad PRS9 at different soil P levels

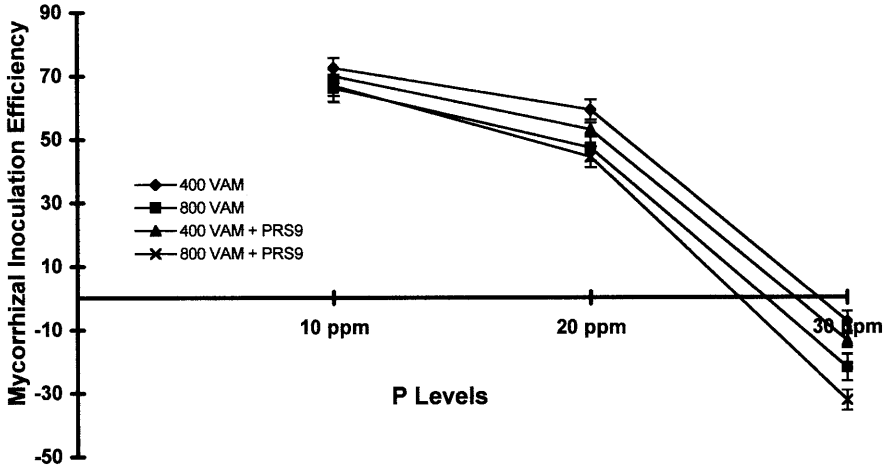


Fig. 3 Mycorrhizal inoculation efficiency of AM fungi and its interaction with PRS9 in *E. hybrid* at different soil P levels

Mycorrhizal inoculation efficiency

In contrast to other parameters, e.g., root and shoot length, weight, QI, P and N uptake, MIE was highest at 10 ppm soil P irrespective of the treatment. Thus, 400 AMF spores + 10 ppm P resulted in higher MIE than 400 AMF spores + PRS9 (Fig. 3). MIE values at 30 ppm P were below the threshold of the uninoculated control. MIE declined with increasing soil P level.

Discussion

AM are of high ecological significance in the eucalypt forest ecosystem (Malajczuk et al. 1981). Promotion of the growth of *E. hybrid* by mycorrhizal fungi has been reported by several workers (Chilvers et al. 1987; Boudarga et al. 1990; Adjoud et al. 1996). In the present study, increased uptake of N and P was only observed with mycorrhizal-colonized plants. Ibijbijen et al. (1996a,b) reported that biological N fixation in legume hosts may be enhanced indirectly by AMF through improved P supply. Here, N and P uptake was highest at 20 ppm P with a sharp decline at higher P; mycorrhizal P uptake efficiency was reduced drastically at 30 ppm P. Bagyaraj and Machado (1996) found that an increased soil P concentration had an adverse effect on mycorrhizal colonization. This was confirmed in the present study by the lower colonization at 30 ppm than at 10 and 20 ppm P. Reduced mycorrhizal micronu-

trient uptake and suppression of AM activity at high P levels has been reported also by Habte and Manjunath (1991). A low soil P level enables the endophyte to take up sufficient phosphorus for plant growth (Dhinakaran and Savithri 1997). Increase in the AMF inoculum dose produced no significant effect or retarded the plant growth. Growth retardation may be due to excess carbon consumption by the endophyte.

At any given P level, root colonization was reduced in plants treated with AMF + PRS9. The colonization was higher in plants treated with AMF alone. This may be due to bacterial metabolites or is a consequence of an alteration of the root exudation pattern by AM fungi which affected the growth of *Pseudomonas* (PRS9) or vice-versa (Stanley et al. 1992). The resultant growth responses were also reduced by significant amounts in PRS9 interactive mycorrhizal plants. The possible role of PRS9 in AMF-pseudomonad interactions as a true helper bacterium or a growth promotory rhizobacteria needs to be investigated further under these conditions. While PRS9 is well established in crop plants as a plant growth promoting rhizobacterium (PGPR), a change in behaviour under tree rhizosphere conditions can not be ruled out. In accordance with our results, high colonization of the endophyte may suppress the deleterious effect of moderate P levels to a large extent. High levels of HCN and antibiotic production under these circumstances may be antagonistic in an interactive environment. The result of inoculation of PRS9 alone was more or less comparable with the uninoculated control. However, at higher P levels (30 ppm) this produced a significantly higher biomass than in the uninoculated control or AMF-inoculated plants. Considerable differences in biomass production were observed within the treatments at various P levels as well as between treatments at any one P level. Such large differences have been reported by Kormanik et al. (1982) in hardwood species within mycorrhizal and nonmycorrhizal treatments.

The present study indicates a potentially significant role for AMF in growth promotion and quality improvement of *E. hybrid* seedlings. In contrast, it is necessary to reconsider the role of PGPRs in seedling improvement programmes based on AMF. Although two different growth-promotory components (pseudomonads and AMF) may be positive when used independently, they are not necessarily synergistic when used in association. The same dual inoculation has also been tested for improvement of other tree species like *Dalbergia sissoo* and *Acacia catechu*. Such studies may provide information about the behaviour of these microorganisms under different rhizosphere environments. Study of other growth-promotory pseudomonad strains would also be useful.

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