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Phosphorus solubilization by ectomycorrhizal *Pisolithus tinctorius* in pure culture and in association with *Acacia mangium*

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Abstract
Ability to utilize insoluble form of P by different isolates of ectomycorrhizal *Pisolithus tinctorius* (E3418, M270, Pt441 and P53) was observed from their growth on agar plates containing insoluble P (tricalcium phosphate). Inorganic P solubilizing efficiency and surface-bound and extra-cellular phosphatase activities were also measured in pure cultures. All the 4 isolates of *P. tinctorius* had significantly lower P concentration in their mycelia when grown on agar plates containing insoluble P. However, only the isolate E3418 showed poor mycelial production. The colony morphology (area and tissue density) of isolates M270 and Pt441 was significantly altered in the presence of insoluble P, while colonies of the isolate P53 did not show any change. Inorganic P solubilizing efficiency and phosphatase activities were highest in isolates P53 and Pt441, respectively. Ability to solubilize rock phosphate by different isolates of *P. tinctorius* when grown in association with *Acacia mangium* was also studied. Dry matter production, and N and P contents were significantly higher in *P. tinctorius*-inoculated *A. mangium* seedlings when compared to uninoculated seedlings. Seedlings inoculated with the isolate E3418 had significantly lower N and P contents when compared to seedlings inoculated with other isolates of *P. tinctorius*. Seedlings inoculated with isolates M270, Pt441 and P53 had similar N and P contents. However, seedlings inoculated with the isolate M270 produced significantly lower dry matter when compared to seedlings inoculated with isolates Pt441 and P53. The results are discussed in terms of variations in P solubilizing efficiencies, carbon consumption by ectomycorrhizal fungi, and carbon loss via the ectomycorrhizal hyphae.

Keywords: *Pisolithus tinctorius*, *Acacia mangium*, P solubilization

1. Introduction

Available P constitutes less than 1% of the total soil P and this can support plant growth for only a short time (Bolan, 1991). A major fraction of the inorganic form of P is tightly adsorbed to soil particles and is not available for plant uptake (Blake et al., 2000; Sanyal and De Datta, 1991; Beckett and White, 1964). The organic form of P must first undergo an enzymatic hydrolysis to inorganic P to become available to plants (Pant and Warman, 2000). Plants have developed numerous morphological, physiological, biochemical and molecular adaptations to acquire P from the soil (Raghothama, 1999); however, at least a part of the P needed for plant growth should be compensated from other sources. Ability to transform poorly soluble P into soluble forms is reported in many soil microorganisms, which include P solubilizing bacteria and fungi (Toro et al., 1997; Mikanova and Kubat, 1994) and P solubilizing and mobilizing mycorrhizal fungi (Wallander and Hagerberg, 2004).

Pisolithus tinctorius (Pers) Coker and Couch, is an ectomycorrhizal gasteromycete with a widespread global distribution (Marx, 1977). Seedlings inoculated with *P. tinctorius* significantly out perform uninoculated ones in nurseries (Marx and Bryan, 1975), in severely disturbed plantation sites (Valdés, 1986), and in reforestation sites (Marx et al., 1977). Variation in growth stimulation by isolates of *Pisolithus* sp. inoculated on to *Eucalyptus grandis* (Burgess et al., 1994) and Douglas-fir and lodgepole pine seedlings (Molina, 1979) have been reported. Also, variations in phosphate solubilizing and phosphatase activities by isolates of *P. tinctorius* in pure cultures have

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been reported (Lapeyrie et al., 1991; Ho, 1987). Trappe (1977) stressed the need to test many ecotypes of a particular mycorrhizal fungus both in pure culture and in association with the host plant before wide scale nursery inoculations.

Acacia mangium Willd. (Pedley, 1987) is a fast growing leguminous tree that can form symbiotic associations with both nitrogen-fixing rhizobia and mycorrhizal fungi. *Acacia mangium* has gained popularity in the last two decades for reforestation of degraded lands (National Academy of Sciences, 1983; Turnbull, 1986), because of its ability to grow well on degraded soils. Also, *A. mangium* is the most widely planted *Acacia* species in Asia (Lee and Nguyen, 1991) and Africa (Kessy, 1991). In the present study, laboratory experiments were designed to measure the P solubilizing efficiency of different isolates of *P. tinctorius* in pure cultures. Also, a seedling inoculation experiment was conducted to find out the ability of *P. tinctorius*-inoculated *A. mangium* seedlings to utilize rock phosphate as the sole P source. A comparison was made between the performances of different isolates of *P. tinctorius* in pure cultures and in association with *A. mangium* seedlings to find out whether the isolate that had higher P solubilizing efficiency in pure culture was able to perform well in association with the host plant.

2. Materials and Methods

Pisolithus tinctorius *culture maintenance*

Four isolates of *P. tinctorius* namely E3418, M270, Pt441 and P53 were used in the present study. All the *P. tinctorius* isolates were cultured on modified Melin-Nokrans (MMN) (Marx, 1969) agar plates. Twenty-day-old colonies on MMN agar plates incubated at 27°C were used as the mother culture. Stock cultures were stored at 4°C, and sub-cultured every 2 months.

Colony morphology on insoluble P

Ten replicate plates of 25 ml MMN agar each containing either 25 mg KH_2PO_4 (soluble P) or 125 mg $Ca_3(PO_4)_2$ (tricalcium phosphate or insoluble P) per plate were prepared for each isolate of *P. tinctorius*. Mycelial plugs were cut with a sterile cork borer (8 mm diameter) from one or more mother culture plates, and one mycelial plug was placed on each MMN agar plate prepared as described above. All plates were sealed with parafilm and incubated in the dark at 27°C. After 20 d, the diameters of the colonies were measured. The intact colonies were removed from agar plates, washed and transferred to 0.1 M citrate buffer (pH 5.1), and left for 1 h at room temperature. The mycelia were then removed from solution, washed with distilled water and blotted dry. The mycelia were then oven dried at 45°C for 24 h and weighed. The colony area and tissue density were calculated. The oven-dried mycelia were digested (Novozamsky et al., 1983), and P concentrations were estimated by stannous chloride reaction method (Allen, 1989).

Inorganic P solubilizing efficiency

Five mycelial plugs were cut with a sterile cork borer (8 mm diameter) from one or more mother culture plates, and transferred to a 250 ml conical flask containing 100 ml of basal nutrient solution (Cairney and Smith, 1992), 60 mg NH_4NO_3 and 200 mg $Ca_3(PO_4)_2$. Uninoculated flasks served as the control. Seven replicates were maintained for each isolate of *P. tinctorius* and control. The flasks were incubated in a shaker incubator at 27°C for 72 h. After 72 h, the mycelia were separated from culture filtrate by using dried and desiccated membrane filters. A gentle vacuum was applied to aid filtration. The culture filtrate was then centrifuged at 3,000 rpm for 2 min at 27°C and the clear supernatant was removed. Soluble P was estimated by the stannous chloride reaction method (Allen, 1989). The procedure was repeated for the control flasks. The control value was deducted from the value obtained for each isolate. The separated mycelia were gently washed in running tap water, blotted-dry and oven-dried at 45°C for 24 h. The dry weights were recorded. The carbon content in the oven-dried fungal mycelia was estimated by rapid titration method (Allen, 1989). Similarly, the initial dry weight and carbon content were estimated from samples of mycelial fungal plugs used for inoculation. Carbon utilized by the fungus was calculated. The results were expressed as mg tricalcium phosphate solubilized per mg of carbon utilized.

Surface-bound and extra-cellular phosphatase activities

Five mycelial plugs were cut with a sterile cork borer (8 mm diameter) from one or more mother culture plates, and transferred to a 250 ml conical flask containing 100 ml of basal nutrient solution (Cairney and Smith, 1992), 60 mg NH_4NO_3 and 0.013 mg KH_2PO_4. Seven replicate flasks were prepared for each isolate of *P. tinctorius*, and were incubated in a shaker incubator at 27°C for 24 h. After 24 h, the mycelia were separated from culture filtrate by using dried and desiccated membrane filters. A gentle vacuum was applied to aid filtration. Surface-bound and extra-cellular phosphatase activities were estimated, based on the hydrolysis of *p*-nitrophenol (Tibbett et al., 1998). Mycelial dry weight and total organic carbon in the mycelium were measured. The results were expressed as mmoles *p*-nitrophenol liberated per h per mg of mycelial carbon.

Pisolithus tinctorius *inoculum preparation*

Vegetative mycelial inoculum for *P. tinctorius* isolates were prepared according to the procedures described by Marx and Bryan (1975). Two-litre-flasks were filled with 1,450

ml of vermiculite and 50 ml of peat moss. The content was moistened with 800 ml of MMN solution and autoclaved for 1 h at 120°C. The flasks of vermiculite and peat moss were then aseptically inoculated with 50 ml of mycelial slurry. Control flasks were left uninoculated. After 11 weeks of growth at 27°C, the content (vermiculite-peat moss mixture and mycelial growth) was removed from the flask and leached with 15 times its volume of sterile tap water to remove unused nutrients. The rinsed content (inoculum) was placed in sterile plastic bags and stored over night at 5°C.

Seedling inoculation experiment

Acacia mangium seeds were surface sterilized in 95% sulphuric acid for 30 min, rinsed with sterile distilled water, and germinated on 1% water agar at 25°C in the dark. One 14-d-old healthy seedling free of contamination was aseptically transferred into a plastic cup containing the rooting mixture, which includes 200 g of sterilized, water-washed river sand, 56 mg of mussori rock phosphate (insoluble P) and 40 g of vegetative mycelial inoculum. The uninoculated controls received 40 g of moistened vermiculite-peat moss mixture. A total of 15 replicate plants were maintained for each isolate of *P. tinctorius* and for uninoculated control. The plants were grown in a net house and supplied with 30 ml of sterile N free and P free nutrient solution (modified from Broughton and Dilworth, 1971) once in 15 d. Ammonium nitrate (NH_4NO_3) was applied in solution to all the seedlings at the rate of 20.4 mg per seedling at 15 d interval. The seedlings were watered with sterile distilled water to maintain moisture conducive to seedling growth. After 6 months, the plants were harvested and dried in an oven at 60°C for 72 h, and dry weights were recorded. After digestion (Novozamsky et al., 1983), the N content in the oven-dried plant samples was analyzed by indophenol blue method (Novozamsky et al., 1974), and the P content was analyzed by stannous chloride reaction method (Allen, 1989).

Statistical analysis

The data obtained were analyzed using the SPSS program. A single factor analysis of variance (one way ANOVA) was used to test for significant differences in treatments. A multiple range analysis was used to test for significant differences between treatments using Duncan's procedure at $P \leq 0.05$. The effect of P availability on the colony morphology of each fungal isolate was assessed by comparing their growth on soluble and insoluble P and using a student t-test at $P \leq 0.05$.

3. Results and Discussion

All the isolates of *P. tinctorius* had significantly lower P concentration in their mycelia when grown on agar plates containing insoluble tricalcium phosphate (Fig. 1a). However, only the isolate E3418 produced significantly lower dry matter (Fig. 1b) indicating that P deficiency is a limiting factor for its growth. *Pisolithus tinctorius* isolates Pt441 and M270 significantly increased their respective colony area (Fig. 1c) followed by a significant decrease in the tissue density (Fig. 1d) when grown on insoluble P, while the isolate P53 did not show any change in its colony morphology. Variations in the colony morphology of *P. tinctorius* isolates as a result of P stress are reported for the first time. However, similar changes in *P. tinctorius* isolates as a result of N stress have been reported earlier by Dickie et al. (1998). According to them, mycorrhizal fungi with lower hyphal density and higher colony area can explore a large volume with minimal investment in tissue biomass, while mycorrhizal fungi with higher hyphal density can utilize the resources efficiently. The same interpretation is applicable to the four *P. tinctorius* isolates studied here.

The inorganic P solubilizing efficiency and specific phosphatase activity of a mycorrhizal fungus are important when P is deficient in the soil. The isolate Pt441 showed higher surface-bound and extra-cellular phosphatase activities when compared to other isolates of *P. tinctorius* (Table 1). The enzyme phosphatase is involved in the conversion of organic P to an available form (Dighton, 1991) indicating that the isolate Pt441 is suitable for inoculation in soils with high organic P content. On the other hand, organic acids present in the fungal metabolites play a major role in acidification of the surrounding medium and solubilization of recalcitrant mineral P (Wallander, 2000; Nahas, 1996; Paris et al., 1995). *Pisolithus tinctorius* isolate P53 showed a significantly higher inorganic P solubilizing efficiency (Table 1) indicating that this isolate is suitable for soils rich in recalcitrant mineral P.

Table 1. P solubilizing efficiency of different isolates of *Pisolithus tinctorius* in pure cultures. Values are means ± SE (n=5). Values followed by same letter do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test.

P. tinctorius isolates	Inorganic P solubilizing efficiency[a]	Phosphatase activity	
		Surface-bound[b]	Extra-cellular[b]
E3418	0.0026±0.0003a	0.98±0.04a	0.08±0.01a
M270	0.0048±0.0004b	1.30±0.02b	0.10±0.02a
Pt441	0.0067±0.0005c	1.50±0.05c	0.26±0.01b
P53	0.0119±0.0005d	1.30±0.02b	0.23±0.01b

[a]mg tricalcium phosphate solubilized per mg of carbon utilized; [b]mmoles *p*-nitrophenol liberated per h per mg of mycelial carbon.

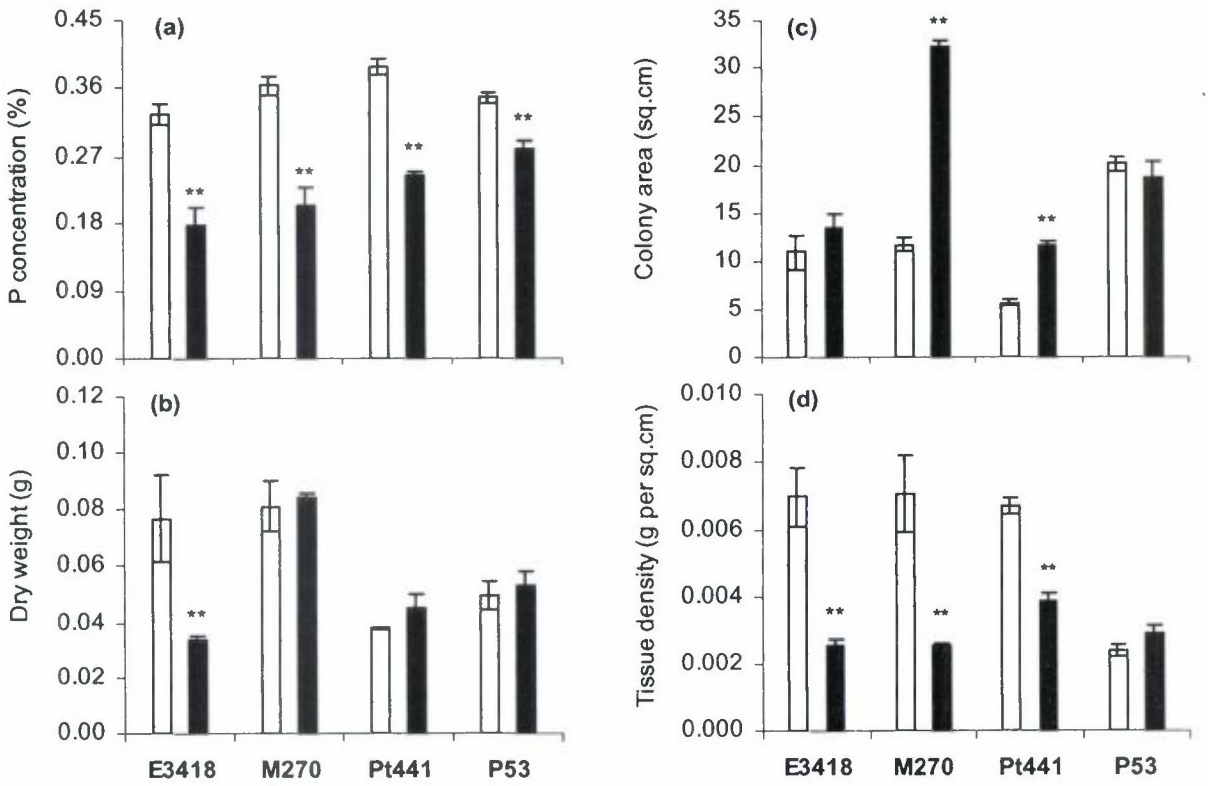

Figure 1. Effects of P availability on (a) mycelial P concentration (b) mycelial dry weight (c) colony area and (d) mycelial tissue density of different isolates of *P. tinctorius* grown on agar plates. ❑ Soluble P, ■ Insoluble P or tricalcium phosphate. Data are means and bars indicate SE (n=10). **Significantly different from soluble P at $P \leq 0.05$.

Table 2. Dry matter production, and P and N contents in 6-month-old A. mangium supplied with mussori rock phosphate as the sole P source and inoculated with different isolates of *P. tinctorius*. Values are means ± SE (n=15). Values followed by same letter do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test.

Treatments	Seedling dry weight (g)	Seedling P content (mg)	Seedling N content (mg)	Shoot P concentration (%)	Shoot N concentration (%)
Uninoculated control	2.50±0.24a	0.50±0.07a	34.1±3.7a	0.034±0.001a	1.9±0.08a
E3418	4.93±0.30b	1.33±0.13b	90.4±3.8b	0.043±0.003ab	2.5±0.12c
M270	5.67±0.37b	1.83±0.17c	118.2±7.7c	0.051±0.003b	2.8±0.09d
Pt441	6.61±0.31c	1.89±0.21c	117.7±7.3c	0.046±0.004b	2.3±0.08bc
P53	6.85±0.31c	1.79±0.21c	118.2±7.3c	0.042±0.003ab	2.2±0.10b

In addition to their role in phosphorus solubilization, organic acids secreted into the soil serve as a carbon source for native microorganisms present in the soil (Garbaye, 1991). Ignatov et al. (2000) reported that treatment of soybean plants with organic acids during inoculation with *Bradyrhizobium japonicum* enhanced the adhesion of bacteria and nodule formation. Co-inoculation of N-fixing rhizobia with the *P. tinctorius* isolate P53 may enhance nodule formation without affecting the carbon economy of the host plant when compared to the other isolates of *P. tinctorius*. However, the effect of organic acids secreted by the mycorrhizal fungi on the rhizosphere pH has to be taken into account before such a practice is recommended because nodulation is very sensitive to low pH (Whelan and Alexander, 1986; Wolff et al., 1993).

Inoculation experiment showed that *P. tinctorius*-inoculated *A. mangium* seedlings had significantly higher growth rate, and N and P contents when compared to

uninoculated seedlings (Table 2). Functional compatibility between *Pisolithus* sp. and *A. mangium* has been reported by Duponnois and Bâ (1999). According to them, inoculation with *Pisolithus* sp. significantly enhanced *A. mangium* growth in soils with low available P. This study shows that inoculated seedlings were able to utilize rock phosphate as the sole P source. The inoculated seedlings had 2.6 to 3.7 times higher seedling P content when compared to uninoculated seedlings, which supports the above conclusion. Similarly, utilization of unavailable form of P by plants inoculated with AM fungi (Guissou et al., 1998; Rahman and Parsons, 1997) and other ectomycorrhizal fungi (Wallander, 2000; Wallander et al., 1997) has been reported earlier.

Inoculation experiment also revealed that *P. tinctorius* isolates Pt441 and P53, which showed higher P solubilizing efficiencies in pure cultures, significantly increased the dry matter production in *A. mangium* seedlings when compared to the other two isolates (E3418 and M270) (Table 2). The poor growth of isolate E3418-inoculated seedlings can be attributed to the poor P solubilizing efficiency of this isolate and its inability to utilize insoluble form of P for its own growth. Seedlings inoculated with the isolate M270, in spite of recording N and P contents similar to those seedlings inoculated with Pt441 or P53, produced significantly lower shoot dry weight. This can be due to relatively higher carbon allocated to the fungal partner by these seedlings. Although higher allocation of carbon to mycorrhizal root systems and their associated mycelia increases nutrient acquisition (Rouhier and Read, 1998), it also increases respiration rate resulting in carbon loss (Ek, 1997). The amount of nutrient taken up per unit of carbon allocated to the mycorrhizal mycelium is an important measure of the efficiency of the symbiosis and of the benefit to the plant (Fahey, 1992; Jones et al., 1991). Hence, the cost-benefit relationship in mycorrhizal symbiosis (Fitter, 1991) should be considered while selecting potential isolates for inoculation with host plants.

Finally, from the present study it is evident that isolates of *P. tinctorius* varied significantly in their ability to solubilize P, and the variations in pure cultures were representative of the variations when grown in association with *A. mangium*. Therefore, in future, the cultural characteristics can be used for screening isolates of *P. tinctorius* to assess their performance in low P soils.

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