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Synergistic effect of a phosphate-solubilizing fungus and an arbuscular mycorrhizal fungus on leucaena seedlings in an Oxisol fertilized with rock phosphate

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Abstract: A greenhouse experiment was conducted to determine the effects of a phosphate-solubilizing fungus (*Mortierella* sp.) and an arbuscular mycorrhizal fungus (*Glomus fistulosum* (Skou and Jakobsen)) in enhancing plant Pi uptake and growth of *Leucaena leucocephala* (Lam.) grown in an Oxisol fertilized with graded amounts of rock phosphate (RP). For this purpose, a surface soil sample was fertilized with four levels of the Huila RP (P = 0, 150, 300, and 600 mg·kg^{-1}) and inoculated with none, one, or both fungi. In the unfertilized soil, leucaena plants grew poorly and there was no plant response to individual or dual inoculation. When RP was added *G. fistulosum* significantly increased plant Pi uptake and growth, the effect of inoculation was significantly higher at the P levels of 300–600 mg·kg^{-1}. *Mortierella* sp. was highly effective in increasing plant P uptake and growth of mycorrhizal leucaena, but it was ineffective in nonmycorrhizal leucaena across the RP gradient. The synergistic effects of dual inoculation were more evident on plant Pi uptake than on growth. The results indicate that the phosphate-solubilizing fungus effect was limited by soil Pi sorption. This limitation likely was overcome by the mycorrhizal association, which allowed a more efficient capture of the Pi released due to RP dissolution.

Key words: Mortierella, *Glomus fistulosum*, weathered soil, Oxisol.

Résumé : Les auteurs ont effectué une expérience en serre pour déterminer les effets d'un champignon solubilisateur de phosphate (*Mortierella* sp.) et un champignon mycorhizien arbusculaire (*Glomus fistulosum*) sur l'accélération de l'absorption du Pi et la croissance du *Leucena leucocephala* (Lam.) cultivé dans un oxisol fertilisé avec diverses quantités de phosphate de roche (PR). À cette fin, ils ont fertilisé un échantillon de sol de surface avec quatre concentrations de phosphate de roche de Huila (P; 0, 150, 300 et 600 mg·kg^{-1}), sans inoculation, avec l'un ou l'autre des deux champignons et avec les deux champignons. Dans le sol non fertilisé, les plants de leucaena montrent une faible croissance, sans qu'on puisse observer de réaction avec l'un des deux champignons ou avec les deux. Avec l'addition de PR, le *G. fistulosum* a stimulé significativement l'absorption du P aux concentrations de 300 et 600 mg·kg^{-1}. Le *Mortierella* sp est hautement efficace pour augmenter l'absorption du P et le croissance des leucaena mycorhizés mais reste sans effet chez les leucaena non mycorhizés, quelle que soit la concentration en PR. Les effets synergiques de la double inoculation sont plus évidents sur l'absorption du P que sur la croissance. Les résultats indiquent que l'effet du CSP est limité par l'absorption du Pi du sol. Il semble bien que cette limitation soit levée par l'association mycorhizienne permettant de capter plus efficacement le Pi relâché suite à la dissolution du PR. [Traduit par la Rédaction]

Mots-clés : Mortierella, *Glomus fistulosum*, sol altéré, oxisol.

Introduction

Phosphate (Pi) deficiency is a major constraint to plant productivity in many soils of the world, particularly in highly weathered soils of the humid tropics and acidic savannas (Oberson et al. 2006). In highly weathered soils, Pi can be strongly adsorbed by soil minerals and (or) precipitated with free ions in the soil solution (Al^{3+}, Fe^{2+}, Ca^{2+}) (Smith 2002). Because of this problem, high rates of Pi fertilizers are required to achieve normal plant growth (Hue and Fox 2010). Unfortunately, this is very expensive and not always affordable in many underdeveloped countries (Randhawa et al. 2006; Osorio 2008).

A viable alternative is the use of locally available rock phosphates (RP). However, the effectiveness of most of these materials is limited by their very low dissolution rate (Yusdar et al. 2007; Hamdali et al. 2010). There is an increasing interest in developing strategies to improve the effectiveness of RP as a Pi fertilizer in tropical soils (Msolla et al. 2005; Randhawa et al. 2006; Ojo et al. 2007; Shrivastava et al. 2007; Bhatti and Yawar 2010). One of these strategies is increasing Pi supply to plants in these soils by inoculating them with arbuscular mycorrhizal fungi (AMF) (Miyasaka and Habte 2001; Oberson et al. 2006). The much finer and far more extensive external hyphae of mycorrhizal fungi are more efficient than root hairs in taking up Pi because they can explore more of the available soil volume than would be possible with root hairs alone (Habte 2006). Mycorrhizal hyphae can take up Pi that is in solution (Bolan 1991) and, as recently shown by Cardoso et al. (2006), dissolve Pi from Fe-phosphate; however, a high amount of RP is required to obtain significant benefits from a mycorrhizal-dependent plant Pi uptake (Manjunath et al. 1989; Ba and Guissou 1996; Satter et al. 2006).

The quantity of RP required can be substantially reduced by increasing its dissolution rate (Osorio 2008). One of the biological approaches of achieving this objective is through the use of P-solubilizing microorganisms (PSM) (Barea et al. 2002; Delvasto et al. 2006; Duponnois et al. 2006; Vyas et al. 2007; Zaidi et al. 2009; Vassileva et al. 2010). However, most of the research on PSM has

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been conducted in lowly weathered soils that exhibit low P sorption capacity (Mollisols, sandy soils) (Kucey 1988; Asea et al. 1988; Kucey and Leggett 1989; Gleddie 1993; Peix et al. 2001; Pramanik et al. 2009), and little has been studied in highly weathered soils (Osorio and Habte 2001; Stamford et al. 2007; Oliveira et al. 2009) where the need to alleviate Pi deficiency is greatest. Moreover, most researchers working in this area have focused their attention on plant responses to inoculation with PSM and very few studies have been undertaken on the interaction of these microorganisms and soil factors that control P availability. Consequently, the effectiveness of PSM in increasing plant P uptake in highly weathered soils and the variables governing it are not clearly understood.

The hypothesis tested in the current study was that in highly weathered soils, the effectiveness of PSM to increase plant Pi uptake and growth may be enhanced by the presence of mycorrhizal association, the extensive hyphal network of AMF would avoid the Pi resorption by soil minerals. The objective of the investigation was to determine the synergistic interaction of a phosphate-solubilizing fungus (PSF, *Mortierella* sp.) and a mycorrhizal fungus (*Glomus fistulosum* (Skou and Jakobsen)) in enhancing plant growth and Pi uptake by *Leucaena leucocephala* (Lam.) in an Oxisol fertilized with graded amounts of RP.

Materials and methods

Soil preparation

A surface soil sample (A horizon, 0–15 cm) was collected from the Agricultural Experiment Station of the International Center for Tropical Agriculture (Carimagua; 4°34′N, 71°20′W) in the eastern plains of Colombia (supplied by Carmen-Rosa Salamanca from the Colombian Institute of Agriculture, Villavicencio). The soil was classified as a clay loam, kaolinitic, isohypertermic Typic Haplustox by the soil survey staff of the Colombian Geographic Institute Agustin Codazzi (IGAC 1991). Soil chemical and physical properties were determined in the Soil Laboratory of the Universidad Nacional de Colombia at Medellin with the following results: 20% sand, 50% silt, 30% clay; texture clay loam (Bouyoucos); soil pH 4.9 (w, 1:1); soil organic matter content, 3.5% (Walkley and Black); aluminum, 3.1 $cmol_c \cdot kg^{-1}$ (1 mol/L KCl); calcium, magnesium, and potassium were 0.1, 0.1, 0.05 $cmol_c \cdot kg^{-1}$ (1 mol/L ammonium acetate), respectively; cation exchange capacity at pH 7.0, 13.9 $cmol_c \cdot kg^{-1}$; phosphorus, 2 $mg \cdot kg^{-1}$ (Bray II); soluble phosphorus, 0.003 $mg \cdot L^{-1}$ (0.01 mol/L $CaCl_2$); sulfate, 5 (0.008 mol/L CaH_2PO_4); iron, manganese, copper, zinc were 188, 2, 1, 1 $mg \cdot kg^{-1}$ (Olsen-EDTA), respectively; boron, 0.1 $mg \cdot kg^{-1}$ (hot water); nitrate, 1 $mg \cdot kg^{-1}$ (0.025 mol/L $Al_2(SO_4)_3$); ammonium, 54 $mg \cdot kg^{-1}$ (1 mol/L KCl). A soil mineral-P fractionation protocol (Kuo 1996) indicated that labile-Pi, aluminum-Pi, iron-Pi, and calcium-Pi contents were 0.2, 2.0, 22.4, and 1.3 $mg \cdot kg^{-1}$, respectively (which represented 1.0%, 7.8%, 86.3%, and 5.0%, respectively, of the total mineral P).

The soil was passed through a 4 mm aperture sieve, its soil pH was adjusted to 6.0 with $Ca(OH)_2$ following the procedure of Uchida and Hue (2000), and then mixed with quartzitic sand (3:1 *w*/*w*). This was autoclaved twice (120 °C, 0.1 mol/L Pa for 1 h) and transferred into plastic pots measuring (11.5 cm × 15.5 cm), which were filled with 900 g (dry mass) of the mixture.

A soil P isotherm using the method of Fox and Kamprath (1970) indicate that the $P_{0.2}$ value (amount of P in $mg \cdot kg^{-1}$ required to obtain a soil solution P concentration of 0.2 $mg \cdot L^{-1}$) was 417 $mg \cdot kg^{-1}$. According to Juo and Fox (1977), the soil had a medium P sorption capacity.

Inoculum preparation

Glomus fistulosum was obtained from the Biogeochemistry Laboratory at the Universidad Nacional de Colombia. It was multiplied using a mixture of sorghum (*Sorghum bicolor* (L.) Moench) and kudzu (*Pueraria phaseoloides* (Roxb.) Benth.) grown as nurse plants, which were grown in a substrate composed of soil and quartzitic sand (2:1, by mass) for 4 months as described by Habte and Osorio (2001). The inoculum consisted of spores, hyphal fragments, and pieces of mycorrhizal roots in the soil–quartz matrix. The crude inoculum contained 40 infective propagules per gram, which was determined by the most probable number technique (Porter 1979).

Mortierella sp. was originally isolated from the roots of *L. leucocephala* growing in an Andisol of Hawai'i (Osorio and Habte 2001) and has been maintained on YMA slants at 4 °C. For this study, the fungus was multiplied in Petri dishes on YMA medium for 3 days at 28 °C. Then, its mycelium was removed with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed. Plate counting on YMA medium indicated that there was 5.8×10^6 colony-forming units$\cdot mL^{-1}$.

Host plant

Seeds of *L. leucocephala* cv. K-11 were scarified in concentrated H_2SO_4 for 30 min to facilitate their germination and then rinsed several times with deionized water. They were placed onto sterile moist paper towels to germinate for 2 days. Two germinated seeds were planted into each pot.

Treatments

At planting, the potted soil–sand mixture was either unamended or amended with four P levels (0, 150, 300, and 600 $mg \cdot kg^{-1}$) using Huila rock phosphate (HRP) as P source, which was passed through a 500 μm aperture sieve. The P content of HRP was 120 $g \cdot kg^{-1}$, and its empirical formula, $Ca_{9.69}Na_{0.22}Mg_{0.09}(PO_4)_{5.14}(CO_3)_{0.86}F_{2.34}$, was obtained from Chien and Hammond (1978). Concurrently, the soil–sand mixture was either uninoculated or inoculated with 34 g per pot of the crude inoculum of *G. fistulosum*. The mycorrhizal inoculum was uniformly mixed with the upper half of the potted soil. Uninoculated pots received 34 g of sterilized soil–sand mixture (1:1) along with 10 mL of washings from the crude inoculum after removal of mycorrhizal propagules with Whatman No. 1 filter paper.

Soil was also either uninoculated or inoculated with 10 mL of a suspension (in 5% glucose) containing 5.8×10^6 colony-forming units$\cdot mL^{-1}$ of *Mortierella* sp., which was directly pipetted into the planting holes (2 cm diameter, 3 cm depth) prior to transplanting. Control plants received 10 mL of a 5% glucose solution.

At planting, the following nutrient sources and rates were applied (rates in $mg \cdot kg^{-1}$ of soil) $(NH_4)_2SO_4$ (475), K_2SO_4 (240), MgO (414), Zn–EDTA (111), Cu–EDTA (55), $Na_2B_8O_{13} \cdot 4H_2O$ (4), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.9). The nutrient sources were mixed thoroughly with substrate.

The plants were grown under natural light in the greenhouse of the Universidad Nacional de Colombia, Medellin, Colombia (6°15′N, 75°35′W, and 1495 m altitude). Air temperature ranged from 16 to 37 °C (mean = 22 °C) and relative humidity from 30% to 94% (mean = 74%). The substrate was watered to maintain it at 50%–60% of maximum water holding capacity. Twenty-five mL per pot of P-free Hoagland's solution were applied to each pot once a week.

Measured variables

Phosphorus status of *L. leucocephala* leaves was monitored as a function of time by determining P content of the 4th pinnule (counting from the base of the pinna) of the youngest fully expanded leaf (Habte et al. 1987) at 14, 24, 32, 37, and 47 days after transplanting. Plants were harvested at the end of 47 days of growth; shoots were dried at 70 °C for 48 h before shoot dry mass was measured. Shoot P content was determined colorimetrically by the molybdate-blue method (Murphy and Riley 1962) after dry-ashing samples at 500 °C for 3 h in a muffle furnace and dissolving the ash in 2.5 mL of solution containing 0.3 mmol/L antimony potassium tartrate, 3.89 mmol/L ammonium molybdate, 24.3 mmol/L

Table 1. Significant *P* values of ANOVA tests.

	Pinnule P content (days after transplanting)								
Treatment	14	24	32	37	47	SDM	SPC	MC	PSFR
RP (A)	<0.001	0.008	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS
AMF (B)	NS	NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PSF (C)	NS	NS	NS	NS	0.023	NS	NS	<0.001	<0.001
A × B	NS	NS	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	NS
A × C	NS	NS	NS	NS	NS	0.037	0.026	<0.001	NS
B × C	NS	NS	NS	0.033	<0.001	NS	NS	<0.001	<0.001
A × B × C	NS	NS	NS	NS	0.006	0.039	0.008	<0.001	NS

Note: Abbreviations as follows: RP, rock phosphate; AMF, arbuscular mycorrhizal fungus (*Glomus fistulosum*); PSF, phosphate-solubilizing fungus; SDM, shoot dry mass; SPC, shoot P content; MC, mycorrhizal colonization; PSFR, presence of *Mortierella* sp. in the roots; NS, not significant.

ascorbic acid, and 0.83 mol/L H_2SO_4 and then diluting with 10 mL of deionized water (Habte and Osorio 2001).

Mycorrhizal colonization of roots was determined after clearing roots with 10% KOH and staining them with 0.15% acid fuchsin in lactic acid (Habte et al. 1987). Then, the proportion of root length colonized by AMF was determined by the gridline intersection method (Giovanneti and Mosse 1980). The presence of *Mortierella* sp. in the roots was evaluated in 20 root fragments of 1 cm length, which were randomly taken from each plant (Hallman et al. 2006). The root fragments were transferred into Petri-dishes that contained 15 mL of a selective medium (Osorio, 2008). This medium contained (per liter) KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.1 g), mannitol (10 g), yeast extract (1 g), agar (15 g), streptomycin sulfate (500 μg), benomyl (75 μg), and cycloheximide (100 μg). Three days later, we counted in how many of the 20 root fragments the fungal growth was observed and this was expressed as a percentage. Also, the presence of *Mortierella* sp.was examined under a scanning electron microscope (SEM) (JEOL, JSM-5910 V model, Japan). For this purpose, root samples were oven-dried (60 °C, 72 h) and then coated with gold particles (6–8 nm) under vacuum conditions before being examined by SEM at 15 kV.

Experimental design and data analysis

The treatments consisted of a factorial combination of four levels of RP, two levels of mycorrhizal inoculation, and two levels of PSF inoculation (4 × 2 × 2) in a completely randomized design; each treatment had four replicates. A replicate consisted of a plant growing in a potted soil with the respective treatment. Data were subjected to analysis of variance (ANOVA) and the Duncan multiple range test was employed for mean separation ($P \leq 0.05$). The statistical package used was Statgraphics Plus version 4.0 (Statpoint, Inc., Herdon, Virginia, USA).

Results

The P content of pinnules of leucaena was significantly influenced by treatments (Table 1). In plants grown in uninoculated soil (regardless the level of P added as RP), pinnule P content decreased drastically Figs. and typical symptoms of P deficiency, such as chlorosis and defoliation of pinnules of lower leaves (Habte and Manjunath 1987; Smith et al. 1992), were detected 25 days after planting. Plants inoculated only with *Mortierella* sp. (+PSF) exhibited similar symptoms, the P content of their pinnules was similar to that of plants grown in uninoculated soil (Fig. 1).

In the unfertilized soil, the inoculation with *G. fistulosum* (+AMF) soil pinnule P content of plants declined progressively over time (Fig. 1*a*) and P deficiency symptoms were also observed. However, when the soil was fertilized with RP the pinnule P content initially decreased but then increased beginning on day 25 and continued to increase until harvest (Fig. 1*b*).

Plants inoculated with both microorganisms (+AMF and +PSF) exhibited a pattern similar to the one described above for mycorrhizal plants (+AMF), but had significantly higher pinnule P content at some sampling periods (37 and 47 days after transplanting) if soil was amended with RP Figs. At harvest, dual inoculation increased pinnule P content by 31%, 43%, and 38% over those of plants grown in soil only inoculated with *G. fistulosum* alone at the rates of *P* addition of 150, 300, and 600 mg·kg^{-1} (Fig. 2).

Uninoculated plants had very low shoot dry mass (SDM), although they responded significantly to RP applications of 600 mg P·kg^{-1} (Fig. 3), they grew poorly. When soil was inoculated only with *Mortierella* sp. they exhibited a similar pattern and SDM was not significantly different from that of plants grown in the uninoculated soil. Inoculation with *G. fistulosum* significantly increased SDM if soil was fertilized with RP (Fig. 3). The SDM of plants grown in soil inoculated with both fungi (AMF and PSF) followed the same pattern as that of plants grown in soil inoculated with AMF alone. However, at the levels of added P of 150 and 300 mg·kg^{-1}, SDM of plants grown in dually inoculated soil was significantly higher (by 15% and 16%) than those grown in soil inoculated with AMF alone; at 600 mg·kg^{-1} the increase was not significant.

Uninoculated plants exhibited a very low shoot P content (SPC) (<0.6 mg/shoot) regardless the level of RP applied (Fig. 4). Inoculation with *Mortierella* sp. alone did not increase SPC of plants at any of the levels of RP addition. On the other hand, inoculation with *G. fistulosum* alone significantly increased SPC of plants grown in soil fertilized with RP, but not in unfertilized soil. At 600 mg·kg^{-1} of added P, the SPC of mycorrhizal plants reached a value of 2.3 (mg/plant), which was significantly higher than values observed at other levels of RP (Fig. 4). In the unfertilized soil, dual inoculation did not have any effect on the variables studied. However, if RP was 150 and 300 mg·kg^{-1} of added P, dual inoculation significantly increased SPC (by 40% and 66%, respectively) beyond that obtained with mycorrhizal inoculation alone.

Mycorrhizal colonization was only detected in the roots of plants inoculated with *G. fistulosum* (+AMF). However, the extent of mycorrhizal colonization was significantly increased by the addition of RP. In the unfertilized soil, the level of mycorrhizal colonization observed was 38%, but the value increased significantly to 58%, 62%, and 64% if RP was added at 150, 300, and 600 mg P·kg^{-1}, respectively. Inoculation with *Mortierella* sp. slightly reduced the extent of mycorrhizal colonization.

Mortierella sp. was detected only in root samples of plants grown in soil inoculated with the fungus (+PSF) (Fig. 5). Regardless of the level of added P, inoculation with *G. fistulosum* decreased the presence of *Mortierella* sp. in the roots.

Discussion

The results clearly indicate that there was a synergistic interaction of *Mortierella* sp. and *G. fistulosum* in enhancing leucaena growth and Pi uptake. However, the effect depended on the level of RP added into the soil. When RP was applied at 150 and 300 mg·kg^{-1}, the combination of both fungi had synergistic effects on the host plant, but it did not occur in the unfertilized soil or when P was added at 600 mg·kg^{-1}. The poor growth of leucaena seedlings and the lack of

Fig. 1. Pinnule P content of *Leucaena leucocephala* in soil amended with rock phosphate (RP): (*a*) 0 mg·kg^{-1}; (*b*) 150 mg·kg^{-1}; (*c*) 300 mg·kg^{-1}; (*d*): 600 mg·kg^{-1}. Uninoculated or inoculated with *Glomus fistulosum* (+AMF) and *Mortierella* sp. (+PSF) at different sampling dates after transplanting. The bars represent the standard error.

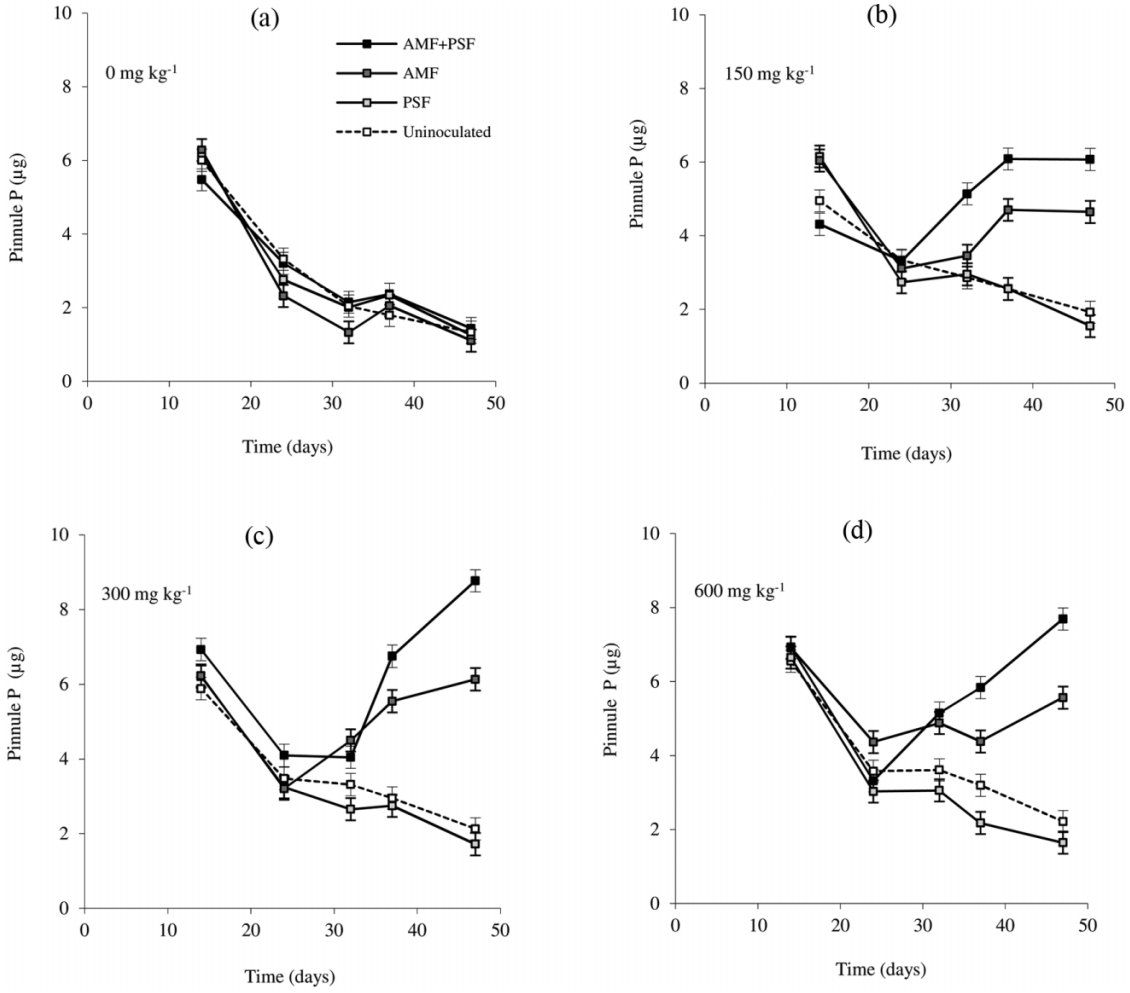
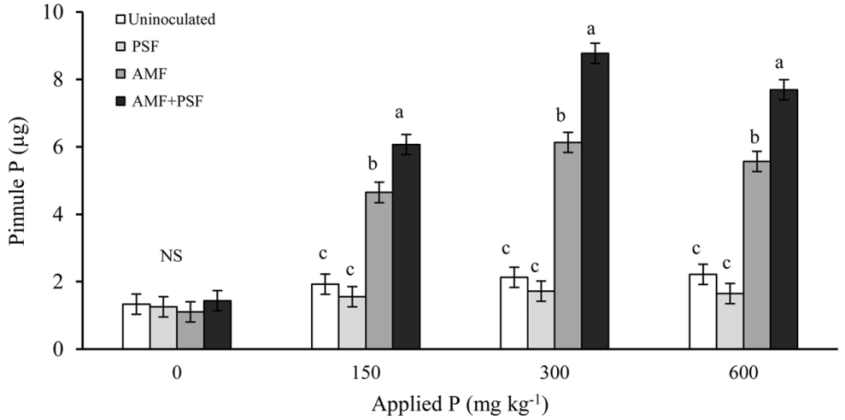


Fig. 2. Pinnule P content of *Leucaena leucocephala* at 47 days after transplanting in a soil amended with rock phosphate and not inoculated (–) or inoculated (+) with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF). The bars represent the standard error. Columns with different letters are significantly different within each level of P applied, according to the Duncan's multiple range test ($P \leq 0.05$).



response to microbial inoculation in the unfertilized soil suggest that low soil Pi content was a limiting factor for beneficial soil–plant–microbe interactions. It is known that the extremely low availability of soluble Pi in this soil (0.003 mg P·L^{-1}) can impair leucaena performance and certainly it is suboptimal for mycorrhizal activity (Habte and Manjunath 1987) and likely for RP dissolution activity of *Mortierella* sp. (Londoño 2010). However, this limitation of a Pi source seemed to be overcome with the addition of RP.

The magnitude of the increase in Pi uptake by mycorrhizal plants due to inoculation with the PSF ranged between 40% and

Fig. 3. Shoot dry mass (SDM) of *Leucaena leucocephala* at 47 days after transplanting in a soil amended with rock phosphate and not inoculated (–) or inoculated (+) with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF). The bars represent the standard error. Columns with different letters are significantly different within each level of P applied, according to the Duncan's multiple range test.

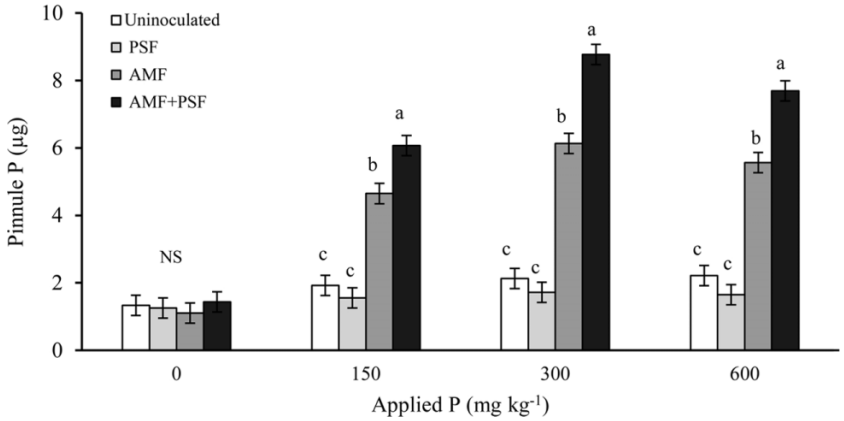


Fig. 4. Shoot P content (SPC) of *Leucaena leucocephala* at 47 days after transplanting in a soil amended with rock phosphate and not inoculated (–) or inoculated (+) with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF). The bars represent the standard error. Columns with different letters are significantly different within each level of P applied, according to the Duncan's multiple range test.

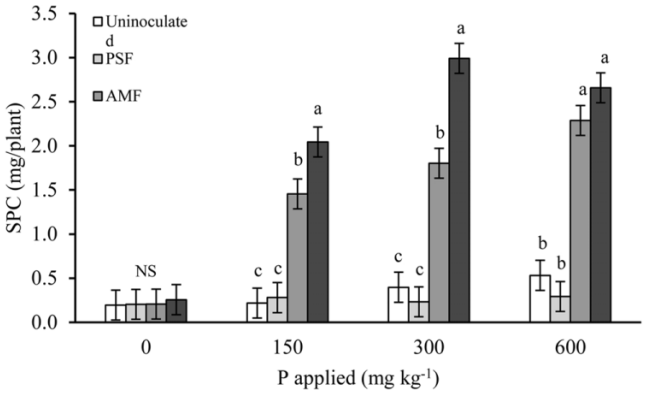
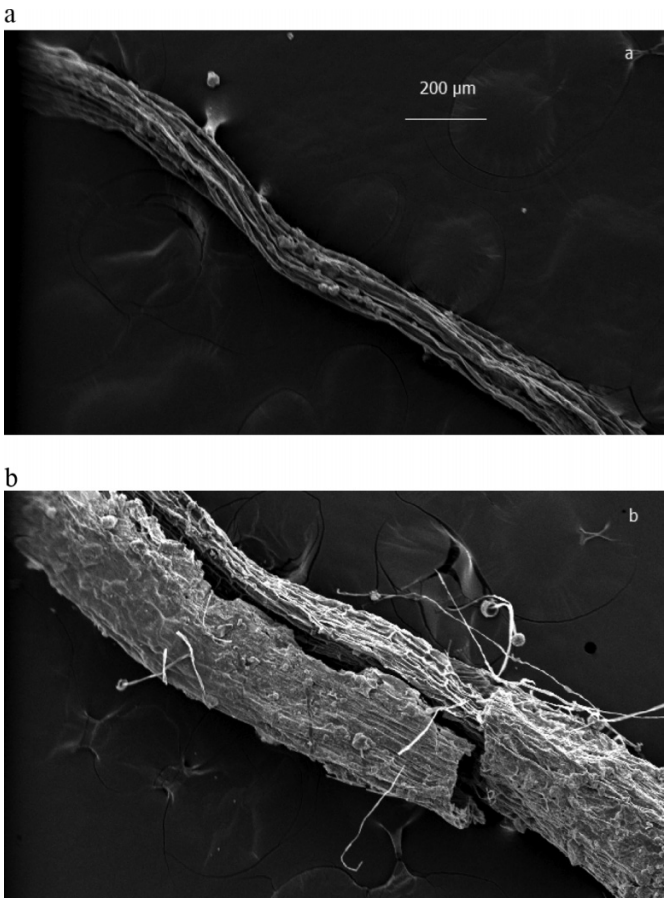


Fig. 5. Scanning electron microscope images of *Leucaena leucocephala* roots showing (*a*) absence or (*b*) presence of *Mortierella* sp. on the rhizoplane (47 days after transplanting).



60%. The presence of mycorrhizal hyphae ensures that at least part of the Pi freshly released due to *Mortierella* activity is intercepted by AMF hyphae before it is reabsorbed by soil constituents. This phenomenon is particularly true for plant species such as *L. leucocephala* that are inefficient in taking up Pi from soil because of the few and short root hairs they have on the surface of their roots (Manjunath and Habte 1991).

Synergistic effects between AMF and PSM have been observed in different plant species including sunflower (Gururaj and Mallikarjunaiah 1995), cotton (Prathibha et al. 1995), rice (Mohod et al. 1991), chili (Sreenivasa and Krishnaraj 1992), wheat (Gaur et al. 1990), alfalfa (Toro et al. 1998), tomato (Kim et al. 1998), and leucaena (Young et al. 1990; Osorio and Habte 2001).

Data on the effectiveness of PSM to enhance nonmycorrhized plant Pi uptake in subtropical–tropical acidic soils is particularly rare and varied, with reported increases ranging from 8% to 25% (Young et al. 1990; Whitelaw et al. 1997; Osorio and Habte 2001). Differences in the Pi sorption capacities of the soils used probably explain the different values reported and the differences between what was observed in the current investigation and what is reported in the literature. In lowly weathered soils characterized by low P sorbing capacities, PSM has been shown to increase plant P uptake of nonmycorrhizal plants (Kucey 1983, 1987, 1988; Asea et al. 1988; Kucey and Leggett 1989; Gleddie 1993; Omar 1998; Peix et al. 2001; Osorio and Habte 2009). Most of the soils used by these authors were Mollisols, calcareous soils, or sandy soils, which are characterized by a low P sorption capacity and relatively high inherent Ca–Pi content (Crews et al. 1995; Cross and Schlesinger 1995). In these types of soils, Pi released by PSM can remain in the soil solution long enough to be absorbed by roots (Osorio and Habte 2009). Although the benefit of mycorrhizal fungi to plants in these soils can be very substantial (Muthkumar et al. 2001; Toro et al. 1996; Barea et al. 2002), the presence of the fungi in highly weathered soils of the tropics appears to be mandatory if Pi re-

Table 2. Phosphate use efficiency of leucaena fertilized with rock phosphate as affected by individual and dual inoculation with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF).

RP level (mg P·kg^{-1})	Uninoculated	PSF	AMF	AMF + PSF
0	—	—	—	—
150	0.30	0.43 (1.4)	3.38 (11.3)	3.78 (12.6)
300	0.34	0.23 (0.7)	2.08 (6.1)	2.37 (6.9)
600	0.44	0.23 (0.5)	1.35 (3.0)	1.36 (3.1)

Note: RP, rock phosphate; phosphate use efficiency (PUE), (mg of biomass per mg of P added) = [(SDW of fertilized plants − SDW of unfertilized plants)/P level added]. The relative value with respect to the uninoculated treatment is given in parentheses.

leased from the dissolution of RP by PSM is to be prevented from readsorption by highly active Pi adsorbing surfaces in these soils.

The positive interactions between RP application and mycorrhizal activity in tropical soils has been discussed extensively elsewhere (Manjunath et al. 1989; Ba and Guissou 1996; Lange and Vleck 2000; Takacs et al. 2006). It is clear that the RP addition alone was ineffective to enhance plant Pi uptake and growth and that *Mortierella* sp. inoculation alone did not improve its efficiency as Pi fertilizer. By contrast, *G. fistulosum* inoculation did enhance the efficiency of RP. For instance, at 150 mg·kg^{-1}, the P use efficiency (PUE, production of plant biomass per unit of P added (Satter et al. 2006)), was 11.3 times higher in mycorrhizal plants than in mycorrhiza-free plants (Table 2). The proportion declined at 6.1 and 3.0 as RP level increased at 300 and 600 mg P·kg^{-1}. Further increases in PUE were observed when both fungi were concomitantly inoculated, particularly at the levels of 150 and 300 mg P·kg^{-1} (Table 2).

From a practical point of view, the increase of RP effectiveness with the co-inoculation with AMF and PSF may help to reduce the level of RP needed as fertilizer. Note that the SDM of leucaena plants at 300 mg P·kg^{-1} + AMF inoculation (0.828 g/plant) was similar to that at 150 mg P·kg^{-1} with both inoculations (AMF+PSF) (0.835 g/plant). The same situation occurred at 600 mg P·kg^{-1} + AMF inoculation (1.02 g/plant) and 300 mg P·kg^{-1} + AMF + PSF (0.97 g/plant). Thus, the use of RP and co-inoculation with both AMF and PSF can be an effective and environmental friendly approach, reducing production costs and preventing soil and water pollution (Entry and Sojka 2007; Syers et al. 2008).

Although the effect of AMF in enhancing plant P uptake is commonly associated with the higher efficiency of the extensive network of mycorrhizal hyphae, RP dissolution may be triggered by removing the Pi that is released into the soil solution, which follows the solubility product principle. However, in these Pi-sorbing soils, further removal of Pi from the soil solution will not have a dramatic effect on the dissolution rate of RP, as RP is probably already far from saturation (Lindsay 2001; Bashan et al. 2012).

On the other hand, the dissolution of RP by PSM is carried out by the following two direct mechanisms: production of H^+ and the formation of Ca^{2+}-complex by oxalic acid – oxalate released by *Mortierella* sp. as reported with other microorganisms (Whitelaw 2000; Welch et al. 2002; Osorio and Habte 2009).

The ineffectiveness of *Mortierella* sp. in the unfertilized soil thus can be explained by the low content of soil Ca-Pi, which is a common feature that characterizes highly weathered soils (Sanchez 1976; Hedley et al. 1994; Crews et al. 1995; Maroko et al. 1999; Trolove et al. 1996, 2003). The lack of effectiveness in the unfertilized soil also represents indirect evidence that the *Mortierella* sp. cannot solubilize Fe-Pi even though it was present at relatively high concentrations (22.4 mg·kg^{-1}; 86.3% of total soil mineral P). Nevertheless, some authors have observed that PSM can solubilize Al-P and Fe-P compounds under in-vitro conditions (Illmer and Schinner 1995; Toro et al. 1996; Bashan et al. 2012). This is conceivable in the absence of soil minerals, but certainly not in highly weathered soils. The result of the earlier study of Bar-Yosef et al. (1999) lends to support this view. They noted that dissolution of RP in the presence of kaolinite by *Pseudomonas cepacia* decreased the pH of the growth medium to 3.0, which in turn favored the dissolution of kaolinite and the concomitant release of Al^{3+}. The presence of Al^{3+} promoted the formation of Al-P at the end of the incubation. This, in fact, is expected to occur in highly weathered soils (Mahmoud et al. 1999; Lindsay 2001). Thus, given the low solubility of Al-P and Fe-P under acidic conditions makes them poor candidates for solubilization by PSM (via acidification) in highly weathered soils.

Even though Pi desorption has been recognized as another mechanism of PSM (He et al. 2002), it probably did not play an important role in this study. The lack of Pi fertilization and the extremely low soil soluble Pi (0.003 mg·L^{-1}) suggests that there was a low saturation of Pi-sorbing sites and consequently Pi was held so strongly by soil minerals that it was hardly desorbed (Osorio and Habte 2012).

Both test microorganisms were established in the root cortex of leucaena as endophytes, they appeared to interact competitively for root colonization sites. Some species of *Mortierella* have been found growing as endophytes in healthy plant roots (Narisawa et al. 1998; Kageyama et al. 2008; JGI 2012). Despite this antagonistic interaction between them for root space, the effect they had on Pi uptake and growth was significantly better if they were present together than if only one or the other was present, at least in soil amended with RP. The complementary roles that these microorganisms have on soil Pi use (Pi availability and uptake) can offset the space competition between both and finally produce benefits on the host plants. Thus, *Mortierella* sp. increases soluble Pi via RP dissolution and *G. fistulosum* up-takes efficiently the Pi released, which is then delivered into the root cells. This microbial cooperation allows the host plant to acquire Pi, the most limiting factor in the system, and consequently grow better, which in turn favors the allocation of more carbon for both endophytes in the roots.

The fact that RP addition increased the mycorrhizal colonization, 38% in the unfertilized soil to 58%–64% when RP was added (150–600 mg P·kg^{-1}), establishes a limit to the idea that less P to the plant results in more mycorrhizal colonization. Habte and Manjunath (1987) found that the mycorrhizal colonization of *G. aggregatum* in leucaena roots increased from 62% up to 74% when the soil solution P concentration rose from 0.002 to 0.153 mg·L^{-1}; colonization levels were depressed only as the soil soluble P increased above this level reaching a level of 55% at 0.807 mg P·L^{-1}.

In summary, this study demonstrates the following: (*i*) the inability of mycorrhiza-free leucaena roots to take up P at low concentrations of soil soluble Pi; (*ii*) the ineffectiveness of RP alone to improve soil Pi availability; (*iii*) the effectiveness of dual inoculation with AMF and PSF to improve Pi use efficiency from RP, thus reducing the amount of RP required; and (*iv*) the increased plant Pi content with dual inoculation indicates a more efficient Pi uptake from the soil probably due to the extensive development of AMF hyphae and the increase in RP dissolution by the PSM. Further research should focus to predict PSM efficiency in tropical soils under field conditions.

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