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TITLE PAGE

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ABSTRACT

An experiment was conducted to evaluate the effects of individual and dual with the phosphate solubilizing fungus *Mortierella* sp. and the arbuscular mycorrhizal fungus *Rhizoglomus fasciculatum* on plant phosphate uptake and growth of avocado (*Persea americana* Mill.) plantlets cv. Hass at the nursery. A completely randomized design was used. Treatments consisted of individual and combined inoculations with *R. fasciculatum* and *Mortierella* sp. at two concentrations (10^6 and 10^8 CFU mL^{-1}) and an uninoculated control. The plant height, shoot dry weight, and shoot phosphate uptake were significantly higher with the co-inoculation with both fungi than with individual inoculation or uninoculated control plants. The colonization of fine roots with both fungi decreased when both were coinoculated in comparison to when they were individually inoculated, which suggest that these fungi compete for root space. Despite this competition, the dual inoculation showed that both fungi had additive effects on plant performance. Thus, shoot phosphate in mycorrhizal plantlets was significantly higher when *Mortierella* sp. was co-inoculated at both concentrations as compared to individual and uninoculated control plants (mycorrhizal-free).

Keywords:

Biofertilization, *Mortierella* sp., *Rhizoglomus fasciculatum*, Andisol

INTRODUCTION

Avocado (*Persea americana* Mill.) cv. Hass is the most common commercial avocado cultivar in the world (Dreher and Davenport 2013). In 2013, the annual harvest reached 3.9 million tons, more than 86% of that obtained in developing countries (FAO 2013). Latin America and the Caribbean area are expected to become the main producers of this crop (Schaffer et al. 2013; Bost et al. 2013).

The cultivar Hass is particularly grown in the high mountains of Colombia where volcanic-ash soils (Andisols) dominate this area, but these soils exhibit low concentrations of soil available phosphate (P) that impairs plant growth, development, and productivity (Batti and Yamar 2010; Singh and Reddy 2011). In these soils, P ions are either strongly adsorbed onto soil minerals (e.g., allophane and Fe-oxides) or precipitated with Al and Fe ions forming insoluble compounds (Barber 1995; Trolove et al. 2003; Do Carmo Harta and Torrent 2007). As a result of that, only a small fraction of soil P (<1%) is available for plant roots (Barber 1995). To overcome this situation, the conventional approach consists of applying high doses of soluble P fertilizers (Narsian and Patel, 2000; Reddy et al. 2002), but only 5 to 10% of it remains available for plant uptake, the rest is insolubilized (adsorbed or precipitated) (Vassileva et al. 2000; Osorio and Habte 2009). For this reason, this practice is very expensive and might increase risks of water pollution (Arcand and Schneider 2006; Shigaki et al. 2006).

In recent years, there is an increasing interest in testing the capacity of both phosphate solubilizing microorganisms (PSM) and arbuscular mycorrhizal fungi (AMF) to enhance plant phosphate uptake and growth (Whitelaw 2000; Vassilev and Vassileva 2003; Jayasinghearachchi and Seneviratne 2006; Mittal et al. 2008; Relwani et al. 2008; Singh and Reddy 2011; Osorio and Habte 2014). Whereas the PSM can dissolve rock phosphate via organic acid production (Kim et al. 1998; Guppy et al. 2005; Gyaneshwar et al. 2002; Pandey et al. 2006; Xiao et al. 2008), the AMF increase the capacity of plant roots to uptake water and nutrient (particularly P ions). Although this biotechnological approach is a very attractive strategy for sustainable crop management, there is no information on the effects of the dual inoculation with the two types of microorganisms on avocado plants. We hypothesize that dual inoculation with AMF and PSM can increase avocado plant P uptake and growth over individual inoculations and uninoculated plants. The objective of this study was to evaluate the effects of individual and dual inoculation with the fungi *Mortierella* sp. (a PSM) and

Rhizoglomus fasciculatum (an AMF) on plant P uptake and growth of avocado seedlings cv. Hass in a nursery setting.

MATERIALS AND METHODS

Site and soil

This study was conducted at the Agricultural Research Center "La Selva" (06º08´01"N, 75º25´05"W) (Rionegro, Antioquia, Colombia), located at an altitude of 2120 m, mean temperature 17 ºC and annual rainfall 1850 mm. In the study, a soil surface sample (A horizon, 0-20 cm) was collected from an Andisol (Typic Melanudand) at this center and was used for root growth substrate. Soil testing was carried out at the Plant and Soil Laboratory of the Universidad Nacional de Colombia at Medellin and yielding the following data: soil pH 5.7 (water, 1:2), soil organic matter content 159 g kg^{-1} (Walkley and Black), P149 mg kg^{-1} (Bray II), Ca, Mg, K, and CEC 13.5, 2.1, 0.4, and 16.0 $cmol_c$ kg^{-1}, respectively (1M ammonium acetate), S 41 mg kg^{-1} (0.008 M calcium phosphate); Fe, Mn, Cu, and Zn 49, 3, 2, and 10 mg kg^{-1}, respectively (Olsen-EDTA), and B 0.75 mg kg^{-1} (hot water). The soil was not amended with fertilizers, but was disinfected with Basamid (a.i.: Dazomet, tetrahydro-3,5,-dimethyl-2H-1,3,5-thiadiazine-2-thione) at a rate of 250 g m^{-3}, covered with plastic for 10 days, and aerated for 5 days, and solarized for 45 days. After that, the soil was transferred to plastic bags (30x20 cm), 5 kg per bag (dry-basis).

Experimental design

The experimental design was completely randomized. Six treatments were used in a 2x3 factorial combination, which consists of two levels of mycorrhizal inoculation (uninoculated and inoculated) and three levels of PSM inoculation (uninoculated and inoculated with a *Mortierella* sp. at two concentrations 10^6 and 10^8 CFU per mL -designated as M6 y M8, respectively-). Each treatment was applied to 11 seedlings.

Fungal inocula

A crude mycorrhizal inoculum of *R. fasciculatum* (Sieverding et al. 2014) was used, which consisted of spores (250 per g), hyphae, and root fragments of maize plants all suspended in a soil: sand (4:1) matrix (previously disinfected with Basamid). With the most probable number technique (Porter 1979) we detected 50 mycorrhizal propagules per g of inoculum.

The second inoculum was the fungus *Mortierella* sp. (strain HI-27), a known PSM capable of dissolving rock phosphate through oxalic acid production. This fungus was originally isolated at the University of Hawaii at Manoa from an Andisol by Osorio and Habte (2001). It was multiplied in potato-dextrose-agar medium at 25ºC and stored at 4 ºC for its experimental use. For this study, the fungus was grown for 5 days and then suspended in sterile water to obtain two concentrations (10^6 and 10^8 CFU mL^{-1}) determined by plate counting in serial dilutions. Both fungi were obtained from the collection of the Laboratory of Ecology and Environmental Conservation of the Universidad Nacional de Colombia.

Plant material

Sixty-six avocado seeds cv. Hass weighing 40-50 g were collected from fruits of healthy trees in a commercial orchard at Jerico (Antioquia, Colombia). Seeds were surface-disinfected with sodium hypochlorite (3%, v:v) for a minute, then rinsed with water and treated with hot water (48-50º C for 30 minutes). Then, in order to accelerate and homogenize germination, the seed coat was removed and cut at both extremities 2-4 mm at the bottom and 10-20 mm on the top. Seeds were left to germinate for 2 months in a sterilized sand-substrate. Seedlings were transferred into the plastic bags with the prepared soil substrate, one seedling per bag.

Treatments

The treatments consisted of a factorial combination 2x3: two levels of mycorrhizal inoculation (either uninoculated or inoculated with 50 g of *R. fasciculatum* inoculum) combined with three levels of inoculation with *Mortierella* sp. (either uninoculated or inoculated with 10 mL of a suspension that contained 10^6 or 10^8

CFU per mL). Each treatment had 11 replicates. The mycorrhizal inoculum was thoroughly mixed with the upper half of the substrate, then the inoculum of *Mortierella* sp. (10 mL) was injected also in the upper half of the substrate. The plants grew in a shadehouse for 8 months and were watered as needed to maintain 50 to 60% of the maximal holding water capacity. The treated plants were distributed randomly in the shadehouse.

Variables

At the end of the growth period (240 days), the plantlets were harvested and the shoot height (SH) and the the shoot dry weight (SDW) were measured after oven-drying (60º C for 72 h). Also, the foliar P content (FPC) was also measured. Leaf disk-samples (0.6 cm of diameter) were collected using a non-destructive method developed by Habte et al. (1987). Leaf-disks were taken from the fourth fully mature leaf (counting from up to down) in the center of the leaf near the central vein. The samples were weighed after oven-drying (60ºC, 12 h) and then combusted in a muffle-furnace at 500°C for 3 h. After that, the ashes were dissolved with 10 mL of distilled water. The P concentration was measured using the blue-molybdate method (Murphy and Riley, 1962) at 890 nm in a spectrophotometer (Genesys 20, Thermo Spectronic). The shoot P content (SPC) was estimated by multiplying SDW with the foliar P concentration.

In addition, the extent of the mycorrhizal colonization (MC) in fine roots was measured following the protocol described by Habte and Osorio (2001). Likewise, the root colonization by the fungus *Mortierella* sp. (PSMC) was measured with the method developed by Osorio and Habte (2013).

Data analysis

Data were subjected to analysis of variance (F-test) and the multiple range comparison Duncan test (t-test). In both cases, a level of significance (*P*-value) ≤0.05 was used. The tests were performed with the software Statgraphics Centurion version XVI.

RESULTS

Significant differences in all variables were detected as a function of treatments. Avocado plants exhibited a significantly ($P < 0.05$) faster growth when both fungi were coinoculated. The uninoculated plants had a mean value for SH of 51 cm; those individually inoculated with *R. fasciculatum* and *Mortierella* sp. were not significantly different (55 and 46 cm, respectively). However, when both fungi were coinoculated (AMF+M8) the shoot height significantly increased up to 65 cm, which represents an increase of 27.4% compared with control plants (Fig. 1A). Treatment with the dual inoculation but with the lowest *Mortierella* sp. concentration (AMF+M6) had no significant effect.

Uninoculated plants (control) exhibited a SDW of 37.8 g/plant (leaves: 19.99 g and stem 17.80 g). Only the dual inoculation (*R. fasciculatum* + *Mortierella* sp.) increased the SDW significantly by 61%, but there were not significant differences with the two concentrations of *Mortierella* sp. (AMF+M6 and AMF+M8) (Fig. 1B).

Uninoculated plants exhibited a mean SPC of 86.2 mg/plant. Individual inoculations did not increase the value of SPC and only the dual inoculation (at both concentrations of *Mortierella* sp.) (AMF+M6 and AMF+M8) significantly ($P \leq 0.05$) increased the value of this variable reaching 170.4 and 172.5 mg of P/plant, which represents a two-fold increase compared to control plants (Fig. 1C).

There were significant ($P \leq 0.05$) differences in the MC as a function of treatments (Figs. 1D). The uninoculated (control) plants had a very low MC (1.2%), those inoculated with *Mortierella* sp. also had low values of MC (1.2-2.3%), which did not differ from control plants. By contrast, the plants inoculated only with *R. fasciculatum* (AMF) had significantly higher MC values than the other treatments (30.9%). When both fungi where coinoculated, the MC was significantly lower (AMF+M6= 14.9% and AMF+M8= and 19.9%); these values were significantly higher than those in control plants (Fig. 1D).

The colonization of fine roots by *Mortierella* sp. (PSMC) was significantly ($P \leq 0.05$) affected by treatments (Fig. 1E). Non-inoculated treatments were void of *Mortierella* sp. By contrast, in those inoculated with the fungus, the values were 72.9% at a concentration of 10^8 CFU mL^{-1} (M8) and 34.7% (M6) at the lowest concentration of 10^6 CFU mL^{-1}. When both fungi where coinoculated, the colonization of fine roots by *Mortierella* sp. was 36.1 and 41.3% at 10^6 and 10^8 CFU mL^{-1}, respectively (Fig. 1E).

DISCUSSION

The results of this study support our hypothesis that dual inoculation with AMF and PSM can increase avocado plant P uptake and growth. These results agree with those reported by Montañez (2009) who found that inoculation with arbuscular mycorrhizal fungi *Glomus* sp. and *Acaulospora* sp. favored the SDW of avocado plants at the nursery stage in comparison to uninoculated control plants. In that study, increases in plant growth were 50% for a local cultivar, 48.9% for cv. Santana, and 46.2% for cv. Lorena. Furthermore, Osorio et al. (2012) found that the inoculation with the *G. fasciculatum* increased the SDW of avocado plants cv. Villagrande by 37%. Similarly, inoculation with *G. fasciculatum* and *G. deserticola* enhanced the growth of both root and shoot, and increased the shoot: root ratio (Azcón-Aguilar et al. 1992; Vidal et al. 1992). In a study in India with plants of *Cassia siamea,* the inoculation with *G. fasciculatum* improved plant growth in the juvenile stage (Bhoopander et al. 2005). Similar effects with mycorrhizal inoculation have been amply reported by several authors in diverse plant species particularly in soil with low levels of available P (Kim et al. 1998; Osorio and Habte 2009; Ramaekers et al. 2010; Zhang et al. 2014).

Hernandez (2001) found that inoculation of avocado cv. Mexicola with *Glomus* sp. significantly increased plant height, stem diameter, number of leaves, and shoot dry weight compared with uninoculated plants. Furthermore, Alarcon et al. (2001) found that *G. fasciculatum* favored the growth of *Vitis vinifera* L. seedlings (higher foliar dry matter and leaf area) despite of low values of mycorrhizal colonization in the roots. Osorio and Habte (2001, 2009), in a series of experiments, found additive effects when *Mortierella* sp. and *G. fasciculatum* were coinoculated in *Leucaena leucocephala* plants in different soils. Similarly, Zaidi et al. (2009) found that with a multiple inoculation with *Bradyrhizobium* sp., *Bacillus subtilis*, *Aspergillus awamori*, and *G. fasciculatum* plant growth increased in mung bean (*Vigna radiata* L. Wilczek) in both a unfertilized P deficient soil as well as in a fertilized soil with rock phosphate. These results are consistent with those made by Londoño (2010), who in a greenhouse study found that the coinoculation with *Mortierella* sp. and *G. fasciculatum* significantly increased *L. leucocephala* P uptake and growth compared to individual inoculation with *G. fasciculatum*. A positive response was also achieved in *Kostelelzkya virginica* with the dual inoculation with *Mortierella* sp. and *G. mosseae* (Zang et al. 2011).

In soils with high P sorption capacity (e.g., Andisols and Oxisols) the dual inoculation AMF+PSM is often necessary to obtain an increase in the plant P uptake, but in less weathered soils (e.g., Mollisols, sandy soils),

typically with low P sorption capacity, individual inoculation with PSM is effective to increase P even in non-mycorrhizal plants (Whitelaw 2000; Peix et al. 2001; Zhang et al. 2014). The mechanisms of P solubilization by PSM are associated with the production of organic acids (Goenadi et al. 2000; Hameeda et al. 2006; Bojinova et al. 2008; Marschner 2008), proton excretion due to assimilation of NH_4^+ by microorganisms as well as P desorption from adsorption sites (He and Zhu 1998; Osorio and Habte 2012). It is known that the P holding strength by soil minerals is lower as the P-sorbing sites are saturated by P (Do Carmo Harta and Torrent 2007). Osorio and Habte (2014) reported that the capacity of the fungus *Mortierella* sp. to desorb P depends on soil type and the level of saturation of P sorbing sites). In this way, PSM inoculation of soils with a long history of P fertilization may desorb enough P to increase P availability and plant P uptake (Osorio and Habte 2014). The mechanism for P solubilization by *Mortierella* sp. is the production and release of oxalic acid, which acts as a exchange ligand that removes P from sorbing sites (Jara et al. 2006; Sato and Comerford 2006; Welch et al. 2002; Ramirez and Osorio 2005).

Davis et al. (1978) affirmed that the effects of AMF inoculation on avocado plants were detected after six months. In the present study, it was necessary to wait nine months to detect the effects of dual inoculation. On the other hand, Londoño (2010) found the dual inoculation (AMF+PSM) improved plant growth and P uptake of *L. leucocephala* plantlets grown in an Oxisol of Colombia, a high P sorbing soil, which is similar to our findings. The results obtained in this study showed that the content of foliar P and plant growth were significantly improved by the coinoculation AMF+PSM despite the high P sorption capacity of the soils used. Thus, these results support that the biotechnological approach proposed here is highly promising to remediate plant P deficiency in avocado plantations.

CONCLUSIONS

Dual inoculation with *R. fasciculatum* and *Mortierella* sp. significantly increased plant growth and plant P uptake compared to individual inoculations and uninoculated control plants. However, the mycorrhizal colonization and PSM colonization of fine roots decreased when both were coinoculated compared to when they were individually inoculated, which suggest that the two different fungi compete for root space. Despite this competition, the dual inoculation showed that they had additive effects.

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Conflict of Interests. The authors declare that there is no conflict of interests regarding the publication of this paper.

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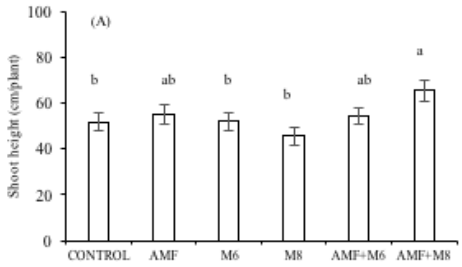
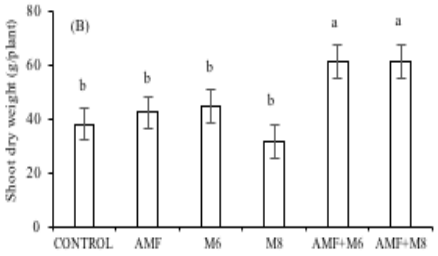
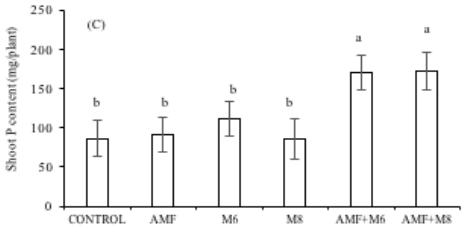
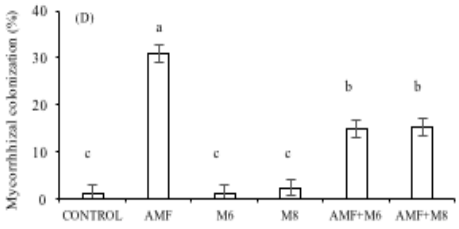
Figure caption

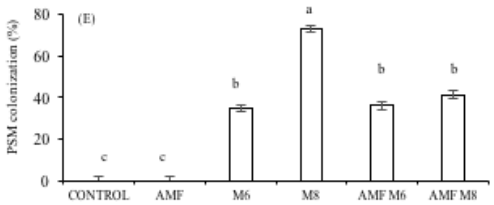
Fig. 1. Shoot height (A), shoot dry weight (B), shoot P content (C), mycorrhizal colonization of roots (D), and *Mortierella* sp. colonization of roots (E) of avocado plantlets cv. Hass as a function of treatments with individual and dual inoculation with *R. fasciculatum* sp. (AMF) and *Mortierella* sp. (M) at two concentrations 10^6 and 10^8 CFU mL^{-1}, 240 days after transplanting at nursery. Columns with different letters exhibit significant differences ($P \leq 0.05$) according to the multiple range test of Duncan.


(A)
Shoot height (cm/plant)
100
80
60
40
20
0
b
ab
b
b
ab
a
CONTROL
AMF
M6
M8
AMF+M6
AMF+M8


(B)
Shoot dry weight (g/plant)
80
60
40
20
0
b
b
b
b
a
a
CONTROL
AMF
M6
M8
AMF+M6
AMF+M8


(C)
Shoot P content (mg/plant)
250
200
150
100
50
0
b
b
b
b
a
a
CONTROL
AMF
M6
M8
AMF+M6
AMF+M8


(D)
Mycorrhizal colonization (%)
40
30
20
10
0
c
a
c
c
b
b
CONTROL
AMF
M6
M8
AMF+M6
AMF+M8


(E)
PSM colonization (%)
80
60
40
20
0
c
c
b
a
b
b
CONTROL
AMF
M6
M8
AMF M6
AMF M8