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Dependency, colonization, and growth in *Gmelina arborea* inoculated with five strains of Arbuscular Mycorrhizal Fungi

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Dependencia, colonización y crecimiento en *Gmelina arborea* inoculada con cinco cepas de Hongos Micorrízicos Arbusculares

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ABSTRACT

Gmelina arborea is a forest species of increasing use in the establishment of commercial plantations in Colombia. The areas where it is currently planted are deficient in nutrients, so the use of Arbuscular Mycorrhizal Fungi (AMF) can be an alternative to improve phosphoric fertilization. The aim of this work was to determine the mycorrhizal dependency, colonization, and growth of *G. arborea* when it is inoculated with *Rhizoglomus fasciculatum, Rhizoglomus aggregatum, Rhizoglomus irregulare, Glomus fstulosum*, and *Entrophospora colombiana*, under different concentrations of phosphorus (P) in a soil solution. A completely randomized design was used with a 6×3 factorial arrangement, five AFM strains + control (uninoculated) and three P doses (0.002, 0.02, and 0.2 mg L^{-1}) with five replicates per each treatment and twice through time. Mycorrhizal colonization and dependency, foliar concentration of P, dry biomass, leaf area, and height were evaluated. A moderate mycorrhizal dependency was obtained under a P concentration of 0.002 and 0.02 mg L^{-1} and inoculation with *R. fasciculatum*, *R. aggregatum*, and *R. irregulare* while inoculation with *G. fstulosum* and *E. colombiana* produced a marginal dependency. It was found a negative effect on *G. arborea* inoculated with all AMF strains under 0.2 mg L^{-1} of P. Mycorrhizal colonization presented values between 62.5 - 2.5% for all the AMF evaluated, influenced by AFM strains and P concentration. Plants inoculated with *R. fasciculatum*, *R. aggregatum,* and *R. irregulare* showed a signifcant increase ($P<0.05$) in their growth. Mycorrhizal dependency and colonization in *G. arborea* and its growth were highly influenced by species of AMF and amount of P.

Palabras clave:
Bosques comerciales
Fertilización P
Rhizoglomus spp.

RESUMEN

Gmelina arborea es una especie forestal de uso creciente en el establecimiento de plantaciones comerciales en Colombia. Las zonas donde es actualmente plantada son deficientes en nutrientes, por lo que el uso de Hongos Micorrízicos Arbusculares (HMA) puede ser una alternativa para mejorar la fertilización fosfórica. El objetivo de este trabajo fue determinar la dependencia y colonización micorrizal, el crecimiento de *G. arborea* inoculada con *Rhizoglomus fasciculatum*, *Rhizoglomus aggregatum*, *Rhizoglomus irregulare*, *Glomus fistulosum* y *Entrophospora colombiana*, bajo diferentes concentraciones de fósforo (P) en la solución de suelo. Se utilizó un diseño completamente al azar, con un arreglo factorial 6×3, cinco cepas de HMA + un control (sin inocular) y tres dosis de P (0,002, 0,02 y 0,2 mg L^{-1}), con cinco replicas por tratamiento y dos a través del tiempo. La colonización y dependencia micorrizal, la concentración foliar de P, la biomasa seca, el área foliar y la altura fueron evaluadas. Una dependencia micorrizal moderada se obtuvo bajo una concentración de P de 0,002 y 0,02 mg L^{-1} y la inoculación con *R. fasciculatum, R. aggregatum y R. irregulare* mientras que la inoculación con *G. fistulosum* y *E. colombiana* produjo una dependencia marginal. Se encontró un efecto negativo en *G. arborea* inoculada con los HMA bajo una concentración de 0,2 mg L^{-1} de P. La colonización micorrizal presentó valores entre 62,5 - 2,5% para todos los HMA evaluados, influenciados por las cepas de HMA y el nivel de P. Las plantas inoculadas con *R. fasciculatum, R. aggregatum* y *R. irregulare*, mostraron un aumento significativo ($P<0.05$) en su crecimiento. La dependencia y la colonización micorrizal en *G. arborea* y su crecimiento estuvo altamente influenciado por las especies de HMA y la cantidad de P.

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Gmelina arborea Roxb. ex Sm is one of the most promising forest species known worldwide in the use of different reforestation programs due to its rapid growth, it is a secure source of raw wood in a short time. This plant behaves as an opportunistic in rainforests, and it is classified as a pioneer (Moya, 2004; Rojas *et al*., 2004; Hernández and Salas, 2008). This species is currently being used in commercial forest plantations, and it is commonly used on the Atlantic coast with around 11,000 planted hectares in Colombia (Cadena and Guauque 2009; Romero, 2004). The growing importance that this species is having is due to relative homogeneity and stability of its wood which makes it adequate for obtaining cellulose as raw material for paper making (Dvorak, 2004). Other uses are furniture manufacturing (Moya, 2004; Romero, 2004), living fences, windbreaker curtains and timber boundaries (Rojas *et al*., 2004).

Gmelina arborea is a perennial plant, which makes it a key step in nursery seedling production that determines the success of the future plantation, making necessary to look for alternatives that improve the development and fertilization at this stage of the production cycle. This is how the use of Arbuscular Mycorrhizal Fungi (AMF) becomes a viable option to fulfill this objective because of these microorganisms support the growth, survival and seedling development in different vegetable species under tropical conditions (Osorio, 2011; Ramírez-Gil *et al*., 2013; Ramírez-Gil *et al*., 2014; Ramírez-Gil *et al*., 2015).

AMF improves the growth of its host, especially in soils with low levels of phosphorus and under stress due to lack of water (Yano and Takaki, 2005; Osorio, 2011; Osorio *et al*., 2017). This symbiosis also helps the absorption of other nutrients, improves the response to the attack of pathogenic microorganisms, among other benefits (Osorio, 2011; Ramírez-Gil *et al*., 2014; Osorio *et al*., 2017). It is expected that inoculation with AMF in *G. arborea* improves the productivity of this species in Colombia since most areas planted are arid zones and have soils with low natural fertility (Cadena and Guauque, 2009). Besides they are an economical and friendly alternative to protect the environment by reducing fertilizer applications on soil, especially with phosphorus (P) fertilizers (Osorio, 2011; Osorio *et al*., 2017).

The first step for an adequate use of AMF in agriculture consists in determining the relationship between microorganism strains with the host species, which can be quantified through mycorrhizal colonization and dependency (Osorio, 2011; Ramírez-Gil *et al*., 2013; Ramírez-Gil *et al*., 2014; Ramírez-Gil *et al*., 2015; Osorio *et al*., 2017). Given the need to understand the symbiotic relationship between *G. arborea* and different AMF species, this work aimed to determine the mycorrhizal dependency and colonization, so as the plant development when was inoculated with five AMF species (*R. fasciculatum, R. aggregatum, R. irregulares, G. fistulosum,* and *E. colombiana*) in three different P levels in a soil solution (0.002, 0.02, and 0.2 mg L^{-1}).

MATERIAL AND METHODS

Location

The experiment was performed between 2012 and 2013, in the Environmental Microbiology and Tropical Phytotecnia laboratories and greenhouse at the Universidad Nacional de Colombia, in Medellín (6°15'51.7"N, 75°34'40.1"W, and 1495 m of altitude). The greenhouse environmental conditions were: temperature at 18-22 °C, relative humidity in a range of 75-95%, and photosynthetically active radiation of 650-1920 µmol photons m^{-2} s^{-1}.

Obtaining soil and seedlings

The soil used in these assays was collected from the subsurface horizon (Bt) in Volador hill, Medellín, Antioquia, Colombia (6°15'56.4"N, 75°34'57.1"W). The soil sample was collected under 30-60 cm of deep to reduce the influence of native organic P available on the soil (Ramirez-Gil *et al*., 2015). The soil sample was analyzed based on protocols used in the Soil Fertility Laboratory at Universidad Nacional de Colombia-Sede Medellín. Soils results were: sand: 65%, silt: 15%, clay: 20% (Bouyoucos); pH 4.8 (water 1:2, v:v); Al: 0.7 $cmol_c$ kg^{-1} (KCl 1 M); Ca, Mg, K: 1.2, 0.5, 0.02 $cmol_c$ kg^{-1} (1 M ammonium acetate); Fe, Mn, Cu, Zn: 37, 3, 1, 2 mg kg^{-1} (Olsen-EDTA), B: 0.1 mg kg^{-1} (hot water); S: 9 mg kg^{-1} (0.008 M Calcium phosphate); NO_3^-: 2 mg kg^{-1} (0.025 M ammonium sulfate); NH_4^+: 2 mg kg^{-1} (KCl 1M); P: 2 mg kg^{-1} (Bray II); P soluble: 0.001 mg L^{-1} (0.01 M of $CaCl_2$.) and organic material: 2% (Walkley and Black method).

The soil was sterilized following the recommendations reported by Ramírez-Gil *et al*. (2015) to eliminate

microorganism interferences. Using an autoclave at 0.1 MPa and 121 °C, for two cycles of one hour each. Besides, the aim to improve the AMF ecological condition (Osorio *et al*., 2017), lime was added to adjust soil pH at 5.6, based on lime incubation method (Uchida and Hue, 2000). On the other hand, KH_2PO_4 was added to achieve a soil solution of P concentration of 0.002 0.02 and 0.2 mg L^{-1}, based on P sorption isotherm (Fox and Kamprath, 1970), which is considered optimal for the studied mycorrhizal dependence and activity (Habte and Manjunath, 1991; Osorio 2011; Osorio *et al*., 2017).

Seeds of *G. arborea* were collected from healthy trees in Santa Fe de Antioquia at the Cotove Agriculture Experimental Station (6°31'55.2"N, 75°49'33.8"W) of Universidad Nacional de Colombia Sede Medellín. The process of extraction, cleaning, disinfection, and germination was developed according to the indications reported by Ramírez-Gil (2017). The plants were maintained in autoclaved quartz until the experiment was assembled.

Obtaining microorganisms and their inoculation

The AMF strains used were *Rhizoglomus fasciculatum, Rhizoglomus aggregatum,* and *Rhizoglomus irregulare*. Originally provided by Dr. M. Habte from University of Hawaii (Honolulu, USA). They were subsequently multiplied following the indication of Environmental Microbiology Laboratory at the Universidad Nacional de Colombia Sede Medellín, using roots of sorghum plants. *Glomus fistulosum* and *Entrophospora colombiana* strains were supplied by a private laboratory and multiplied based on the indication reported before.

The inoculation in soil was developed based on methodology reported by Ramírez-Gil *et al*. (2015) using plastic pots containing 2 kg of the chosen soil. In each, 35 g of crude inoculum per kg of soil was applied. The inoculum presented a concentration of 45-50 infective mycorrhizal propagules per g of soil (Table 1), determined according to the most probable number method (Porter, 1979). In controls, an autoclaved (120 °C, 0.1 MPa, for 60 min) crude inoculum (35 g kg^{-1}) and 10 mL of a suspension (10%) obtained from inoculant filtered through a 10 µm filter paper were used (Ramírez-Gil *et al*., 2015). Then two seedlings were transplanted into plastic pots, a month later one of the plants was removed. The plants were kept for three months in net-house conditions under 50-60% of the soil's maximum water retention capacity. Every week 50 mL of a P-free Hoagland solution was added (Ramírez-Gil *et al*., 2015).

Table 1. Inoculum content of each Arbuscular Mycorrhizal Fungi (AMF) species used for inoculation of *Gmelina arborea.*

Species of AMF	Infective mycorrhizal propagules per g of soil
Rhizoglomus fasciculatum	45±1.5
Rhizoglomus aggregatum	48±1.8
Rhizoglomus irregulare	46±2.0
Glomus fistulosum	48±2.3
Entrophospora colombiana	50±2.5

± standard deviation.

Colonization and mycorrhizal dependence and development of *Gmelina arborea* to five species of AFM

A completely randomized design was used, with a factorial arrangement 6×3, five strains of AMF + control (uninoculated plans), and three doses of phosphorus in the soil solution, with five replicate per each treatment and twice through time. Experimental units consisted of three pots with one plant each.

Ninety days after the treatment started, biometrics and symbiotic variables were evaluated. The height (cm) was measured using a Mitutoyo Digimatic Caliper®, dry biomass (g) was quantified in by Binder® stove at 60 °C for 72 h, leaf area (cm^2) was measured using a LI-Cor® LI 3000A foliar area meter, and foliar phosphate concentration (%) was determined by non-destructive samples using the blue molybdate method (Murphy and Riley, 1962;

Aziz and Habte, 1987). Mycorrhizal Colonization (MC) was evaluated based on the following steps (i) roots were discolored using KOH (10%) for 24 h (Phillips and Hayman, 1970), (ii) coloration of roots by fuchsine acid staining (0.15%) for 48 h (Kormanik *et al*., 1980), (iii) excess of fuchsine on roots were removed using glycerin-lactic acid-water (0.7-0.2-0.1 v:v), and (iv) intensity of mycorrhizal colonization using the interception lines method (Giovannetti and Mosse, 1980). Mycorrhizal dependency (MD) was calculated based on total dry material value, using equation 1 proposed by Plenchette *et al.* (1983). MD classification was performed following the indications reported by Habte and Manjunath (1991), MD values of 75% or higher, 50-75%, 25-50%, less than 25% and 0% represents very highly, highly, moderately, marginally, and independent mycorrhizal dependency, respectively.

$$MD = \frac{Dry\ mass\ inoculated\ plants - Dry\ mass\ uninoculated\ plants}{Dry\ mass\ inoculated\ plants} x100 \quad (1)$$

Statistics analysis of data

For each variable evaluated, data homoscedasticity and normality were determined by Levene and Kolmogorov Smirnov methods. Once homoscedasticity and normality were proved, an ANOVA test was performed. Means were compared with the Tukey test (P<0.05). The evaluation was performed using the R software (R Core Team, 2013).

RESULTS AND DISCUSSION

Mycorrhizal colonization and dependency of *Gmelina arborea* with five AMF species

All plants of *G. arborea* inoculated with *R. fasciculatum*, *R. aggregatum*, *R. irregulare*, *G. fistulosum*, and *E. colombiana* presented mycorrhizal colonization, which was evidenced by the presence of hyphae in these fungi, particularly within intracellular spaces in the root. In uninoculated plants, no associated structures of AMF were found (Figure 1A and B).

Most mycorrhizal colonization (P<0.05) in *G. arborea* was found with inoculation of *R. fasciculatum* and *R. aggregatum* under 0.002 and 0.02 mg L^{-1} of P in the soil solution. These values were 62.5-55.5% and 55.2-54.5%, respectively. In decreasing order and without statistical differences within them (P>0.05), were found the inoculation *R. irregulare*, and *G. fistulosum* under 0.02 and 0.002 mg L^{-1} of P in the soil solution, with values of 38.3-36.2% and 35.3-34.8%, respectively. *E. colombiana* inoculation showed the lowest values (P<0.05) concerning other AMF species that were evaluated with a 22.3 and 26.2% of colonization under 0.02 and 0.002 mg L^{-1} of P in the soil solution, respectively. Also, the mycorrhizal colonization of these five AMF species under 0.2 mg L^{-1} of P did not exceed 5.9% (Figure 2A).

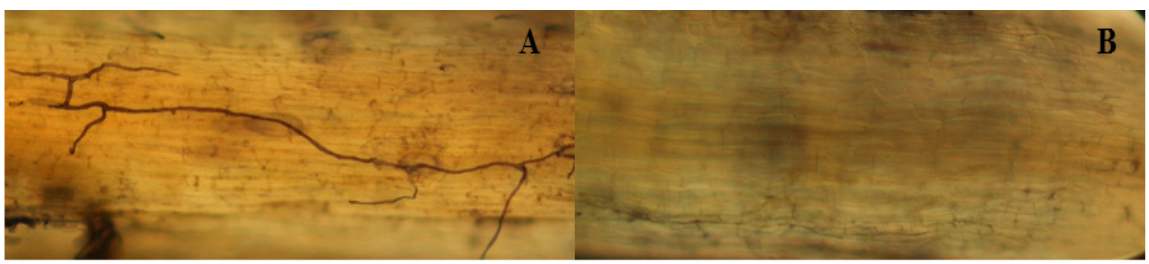

Figure 1. Presence and absence of arbuscular mycorrhizal fungi in roots of *Gmelina arborea*. A. root inoculated with an Arbuscular Mycorrhizal Fungi (AMF) (*Rhizoglomus fasciculatum*) (40x); B. root uninoculated (40x).

Based on Plenchete *et al.* (1983) formula (equation 1), and determined according to the classification criteria of Habte and Manjunath (1991), the mycorrhizal dependency (MD) in *G. arborea*, showed that this species presents a moderate dependency with respect to *R. fasciculatum*, *R. aggregatum*, and *R. irregulare* under P soil solution of 0.02 and 0.002 mg L^{-1} with values of 42.3-35.1%, 34.5-26.2%, and 28.3-26.2%, respectively (Figure 2B). Meanwhile,

G. fistulosum and *E. colombiana* MD was classified as marginally dependent for P levels of 0.02 and 0.002 mg L^{-1} with values of 14.8-15.3 and 10.2-12.3%, respectively. In this sense the *G. arborea* presented the highest MD to species of AMF *R. fasciculatum* (*P*<0.05) (Figure 2B). For the AMF species evaluated and with a P level of 0.2 mg L^{-1} in the soil solution, the MD values in *G. arborea* were negative, indicating that under these conditions of P in the soil the symbiotic relation becomes adverse for the plant (Figure 2B).

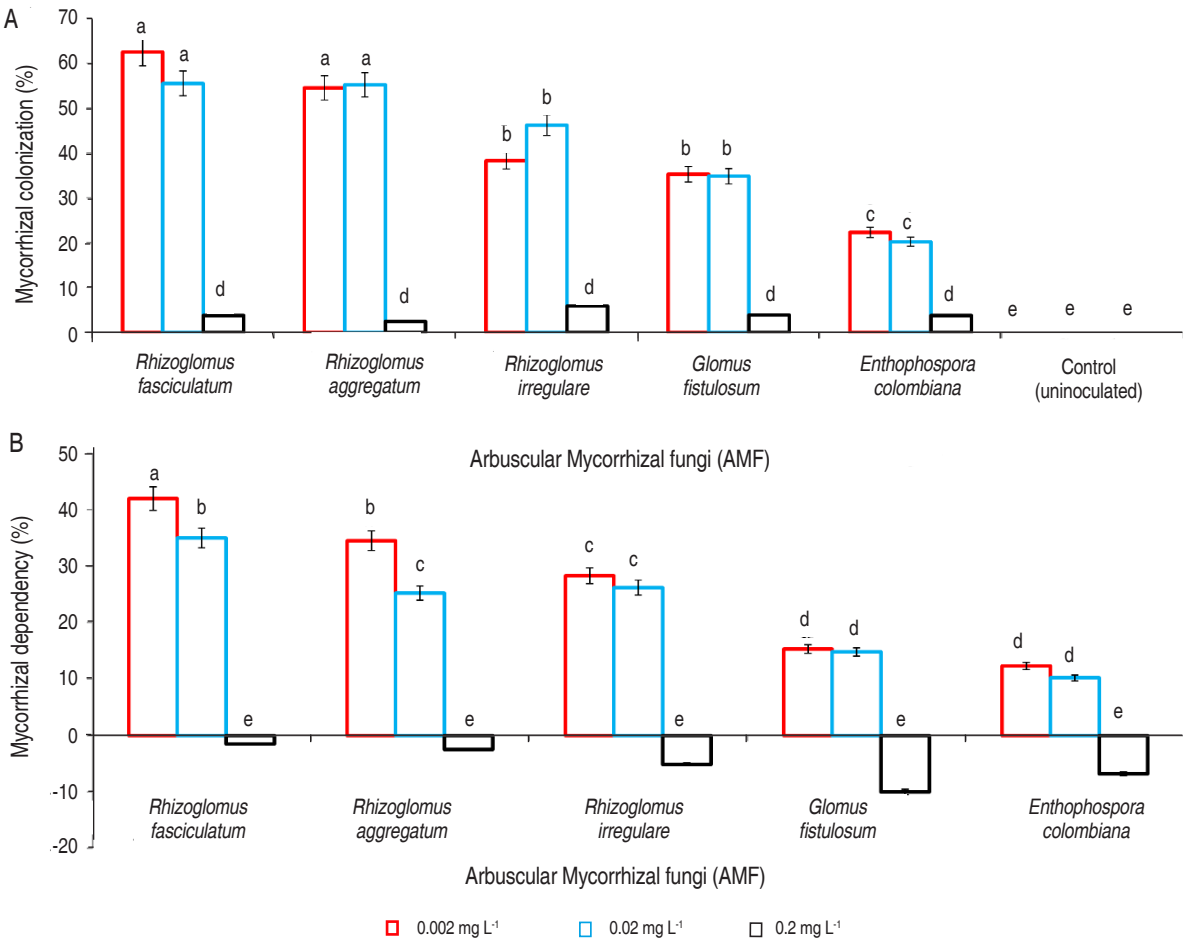

Figure 2. A. Mycorrhizal colonization; B. Mycorrhizal dependence in *Gmelina arborea* inoculated with five species of Arbuscular Mycorrhizal Fungi (AMF). P levels evaluated in the soil solution were 0.002, 0.02 and 0.2 mg L^{-1}. Statistical analyzes were performed for each level of P and each AMF species evaluated. Equal letters on the bars represent no significant differences with a significance level of 95%. Lines on the bars represent standard error of the mean.

The high MC and medium MD found in *G. arborea* show that this species needs positive symbiotic relations with AMF to achieve better development and survival, resulting in an improvement of the plant development under these circumstances. This relation was highly conditioned by the AMF species and the content of P in the soil solution, the best results were associated with P levels of 0.002 and 0.02 mg L^{-1} and *R. fasciculatum* inoculation, followed by other associated species of the *Glomus* genera. These results coincide with other works in different species, where inoculation with this same strain (*R. fasciculatum*) and under these soil conditions (P dose, 0.02 mg L^{-1}) presented high values of mycorrhizal colonization and dependency (Ramírez-Gil *et al*., 2013; Ramírez-Gil *et al*., 2014; Ramírez-Gil *et al*., 2015). According to Sieverding (1991), *Rhizoglomus*

is one of the most aggressive and effective generous for the establishment of symbiotic relations with different taxonomic plant groups under natural conditions.

The best development from symbiotic relation of *G. arborea* and the AMF species was found in a soil P dose of 0.02 mg L^{-1}. It takes place, thanks to the fact that under this condition the symbiotic relationship is favored, leading to a closer interaction and dependency where the plant through radial exudates supplies an energy source to the fungus, which increases exploration surface in the plant root system and improves nutrient absorption, especially of P (Osorio, 2011; Osorio *et al.*, 2017). The P levels in soil solution and foliar tissue in the host are so important that they can activate or deactivate P transporters in mycorrhizal hyphae, directly affecting P plant absorption by this mechanism (Zandavalli *et al.* 2004; Osorio, 2011; Osorio *et al.*, 2017).

Growth and development of *Gmelina arborea* inoculated with five strains of AMF in P levels in soil solution

The height (P<0.05) of *G. arborea* was found in treatments with inoculation of *R. fasciculatum*, followed by *R. aggregatum*, *R. irregulare*, *G. fistulosum*, and *E. colombiana*, for P levels in a soil solution of 0.02 and 0.002 mg L^{-1}, respectively. Meanwhile, for 0.2 mg L^{-1} of P in soil solution and control (no-inoculated plant), the values were not statistically different (P>0.05) and presented the lowest height (P<0.05) (Figure 3A).

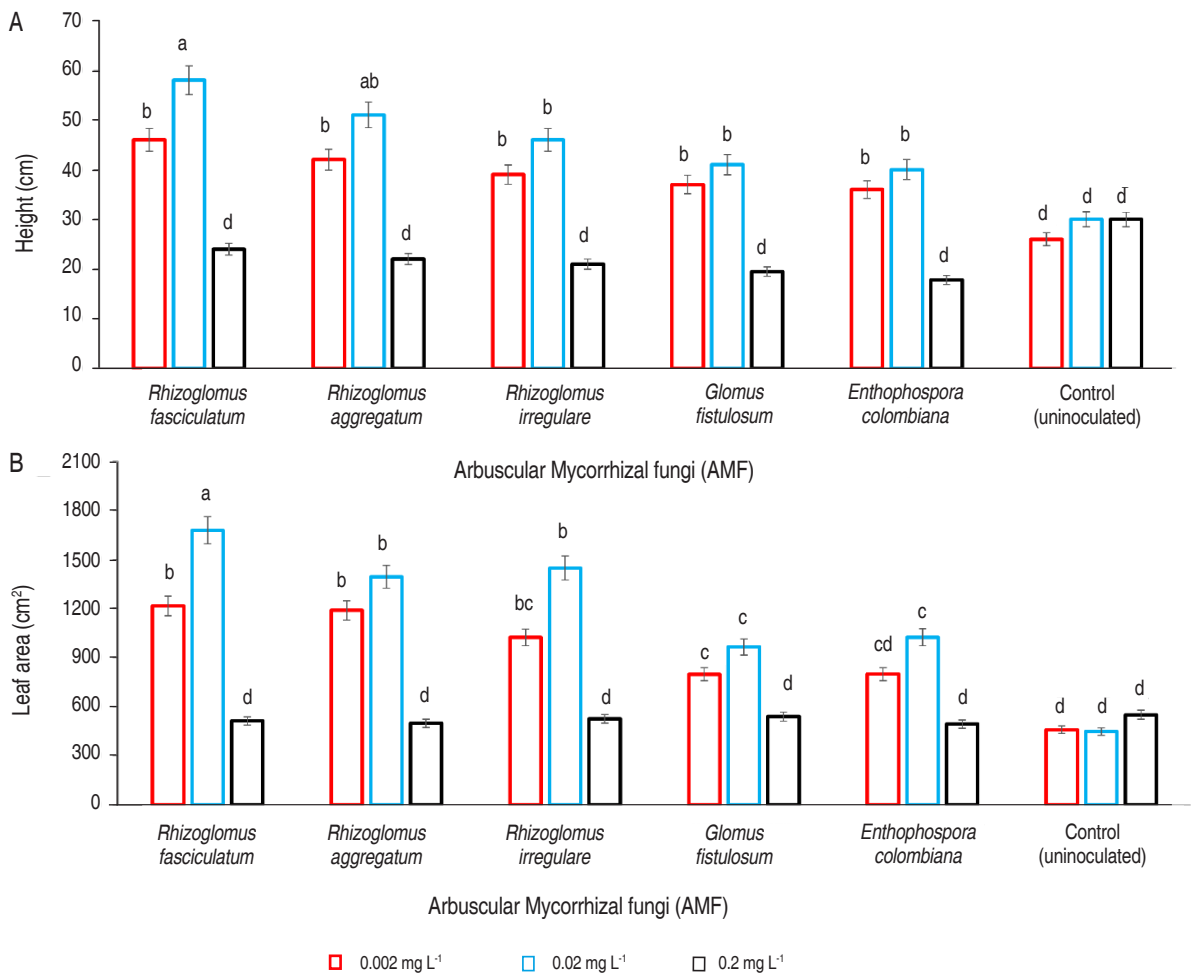

Figure 3. A. Height; B. leaf area of *Gmelina arborea* inoculated with five species of arbuscular mycorrhizal fungi. P levels evaluated in the soil solution were 0.002, 0.02 and 0.2 mg L^{-1}. Statistical analyzes were performed for each level of P and each AMF species evaluated. Equal letters on the bars represent no significant differences with a significance level of 95%. Lines on the bars represent standard error of the mean.

Leaf area presented same behavior that height, where the greatest values (P<0.05) were for P level of 0.02 mg L^{-1} in soil solution, and inoculated with *R. fasciculatum* (1668 cm^2), followed by *R. aggregatum* (1394.4 cm^2) and *R. irregulare* (1447.4 cm^2). No statistical differences were found (P>0.05) among *G. fistulosum* (964.2 cm^2) and *E. colombiana* (1024.4 cm^2), and the control (447.3 cm^2) presented the lowest values (P<0.05) (Figure 3B). Total biomass was significantly greater (P<0.05) when *G. arborea* was inoculated with AMF species in the soil that contained 0.02 mg L^{-1} of P, followed by P dose of 0.002 mg L^{-1} and with less value when P concentration was 0.2 mg L^{-1} (Figure 4A). Concerning the AMF strains evaluated, the biggest biomass (P<0.05) was obtained when plants were inoculated with *R. fasciculatum*, followed by inoculation of *R. aggregatum*, *R. irregulare*, and *G. fistulosum*. Meanwhile, treatment with *E. colombiana* did not show a statistical difference (P>0.05) with *G. fistulosum*, with these two strains the biomass was inferior (P<0.05) to the other three *Rhizoglomus* species, but superior (P<0.05) to the uninoculated plants (control) (Figure 4A).

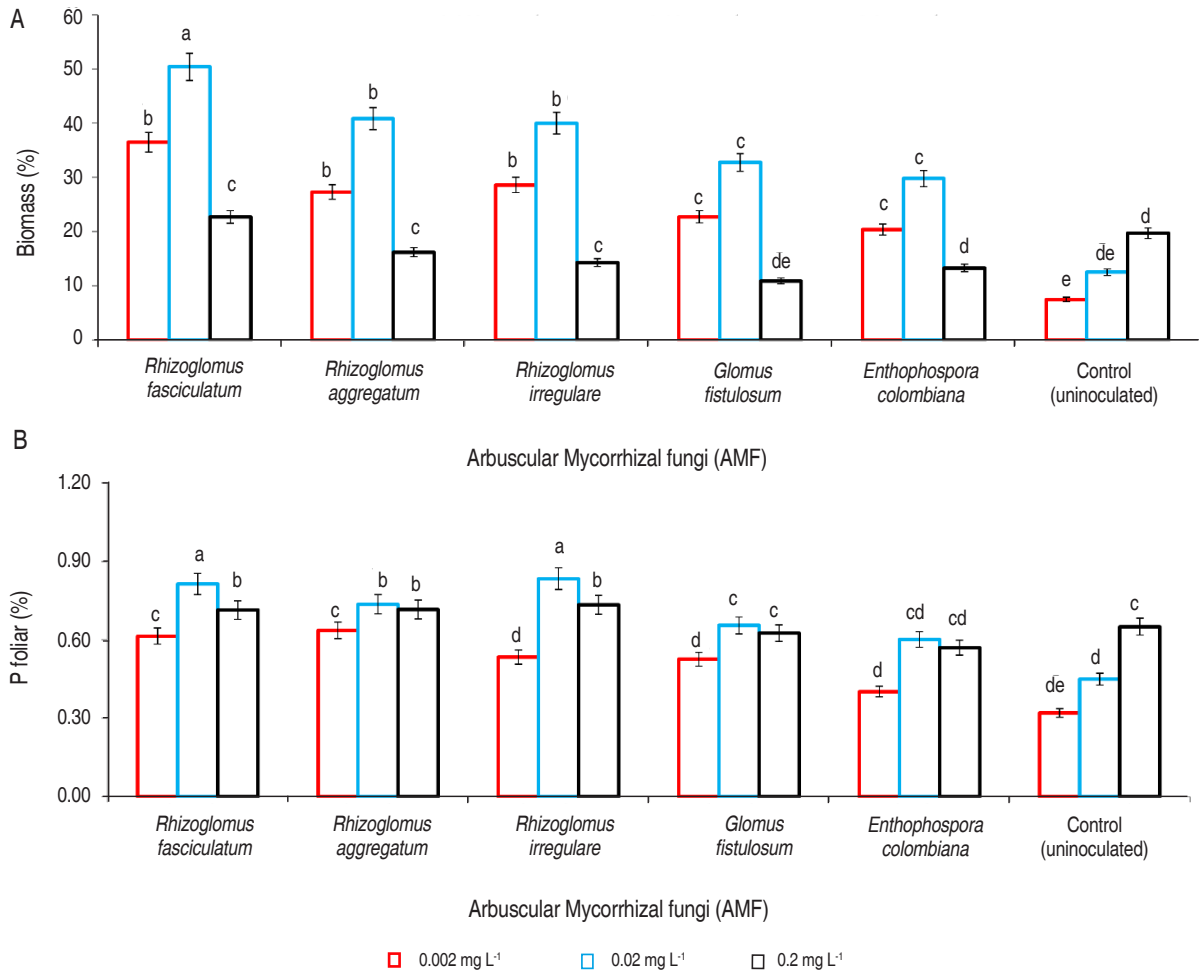

Figure 4. A. Biomass; B. foliar P of *Gmelina arborea* inoculated with five species of arbuscular mycorrhizal fungi. P levels evaluated in the soil solution were 0.002, 0.02 and 0.2 mg L^{-1}. Statistical analyzes were performed for each level of P and each AMF species evaluated. Equal letters on the bars represent no significant differences with a significance level of 95%. Lines on the bars represent standard error of the mean.

In the case of foliar P (Figure 4B) the highest concentration (P<0.05) was found when the soil had a P level of 0.02 mg L^{-1} and the plants were inoculated with *R. fasciculatum* and *R. irregulare*. They were followed by treatments associated with the same P concentration and the other inoculated AFM strains. Control and

plants inoculated with all strains under phosphorus level of 0.2 mg L^{-1} did not show statistical differences (P>0.05) to the group above mentioned. All treatments associated with AMF inoculation and a P soil level of 0.002 mg L^{-1} were classified as a third group. The lowest P amount (P<0.05) were presented in the control with a P concentration of 0.002 and 0.02 mg L^{-1} (Figure 4B).

Same to MC and MD, growth and development of *G. arborea* was associated with P concentration in soil solution and specific AMF species inoculated. The highest values were obtained under 0.02 mg L^{-1} of P in the soil solution and *R. fasciculatum* presence. On the other hand, plant growth and development improved with all AMF treatments, and a higher P concentration in plant leaves was found concerning the plants that were not inoculated. These results agree with Hernández and Salas (2008) findings, indicating that inoculation of *G. arborea* with *R. fasciculatum* presented an increase in height, diameter, foliage weight, and roots of 10, 6.2, 6.7 and 44.7%, respectively. It also showed an improvement in inoculated seedlings behavior at nursery stage when transplanted to field.

Many comparative advantages were found in *G. arborea* when it established a positive symbiotic relation with AMF. Given these interactions, the plant improves its ability to increase the P absorption among other beneficial effects not evaluated in this study, especially under low P levels in soil solution (Osorio, 2011, Osorio *et al*., 2017). This effect has been evaluated in many other species of economic importance for the Colombian tropics, in which all show better growth and development when the symbiotic relationship is successfully generated (Osorio, 2011; Osorio *et al*., 2017; Ramírez-Gil *et al*., 2013; Ramírez-Gil *et al*., 2014; Ramírez-Gil *et al*., 2015).

This work is a pioneer in reporting a detailed study of the interactions between *G. arborea* and five AMF species, it is a fundamental part of the search process for sustainable alternatives for fertilization, where the use of AMF may compensate the low nutrient levels in tropical soil and systems, especially P (Randhawa *et al*., 2006; Osorio, 2011; Osorio *et al*., 2017). The use of this biotechnology may help to decrease the use of chemical fertilizers with many economic and environmental benefits since the frequent use of these compounds increase production cost and could have adverse effects on the environment (Brady and Weil, 1999).

The inoculation of the strains *R. fasciculatum*, *R. aggregatum*, and *R. irregulare* is recommended during nursery stage which may increase the yield of the productive systems with this species since its cultivation is carried out in Colombia with low levels of natural fertility (Cadena and Guauque, 2009). Besides, it shows a high capacity of P retention, limiting phosphoric fertilization because the phosphate ion is rapidly precipitated and absorbed (Osorio, 2011; Osorio *et al*., 2017).

Moreover, understanding that the symbiotic relations with AMF show comparative advantages such as better adaptability to adverse conditions like drought, tolerance to disease attack among others (Yano and Takaki, 2005; Ramírez-Gil *et al*., 2014; Osorio *et al*., 2017). It could be a solution to face adverse effects of climate change, each time more frequent and intense, by alleviating the devastating effects on some vegetable species, especially those which are in tropical zones where climate variability is a problem that threatens the sustainability of different production systems.

CONCLUSIONS

The interaction of *G. arborea* and the five AMF species evaluated favored its development under low values of P (0.02 and 0.002 of mg L^{-1}). The best results were obtained with *R. fasciculatum*, *R. aggregatum* and *R. irregulare* strains. It is important to continue the research with the objective of improving AMF usage in forest plantation in order to improve the sustainability in tropical areas with infertile soils.

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