Presence of mycorrhizal fungi and a fluorescent *Pseudomonas* sp. in the rhizosphere of cacao in two agroecosystems and their effects on cacao seedling growth

Presencia de hongos micorrízales y una *Pseudomonas* sp. fluorescente en la rizosfera de cacao en agroecosistemas y sus efectos en el crecimiento de plántulas de cacao

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ABSTRACT

In recent years the cultivation of cacao (*Theobroma cacao* L.*)* in Colombia has been growing up, resulting in the need to develop a sustainable production system. In this regard, beneficial soil microorganisms are an alternative for improving plant productivity, but this requires knowledge of their ecology and functioning. This study had the objective of identify and quantify arbuscular mycorrhizal fungi (AMF) and fluorescent *Pseudomonas* sp. associated with the soil and rhizosphere of cacao plants in two agroecosystems, one of them was in a tropical dry forest (TDF) and the other in a tropical moist forest (TMF). In a second stage of the study, native strains of *Glomus* sp., *Acaulospora* sp., and fluorescent *Pseudomonas* sp. were selected and multiplied in the lab. Subsequently, the effectiveness of these strains to promote cacao seedling growth was tested in a greenhouse experiment. The results indicate that there was a significant ($P \leq 0.05$) greater mycorrhizal colonization and diversity associated to the roots of cacao growing in agroecosystems of the tropical moist forest. However, not significant differences were detected regarding the presence of fluorescent *Pseudomonas* sp. in the two agroecosystems. Otherwise, in the greenhouse experiments, the inoculation with the mycorrhizal fungus *Glomus* sp. was the only treatment that promoted the cacao seedling growth.

Key words: tropical dry forest, tropical moist forest, *Glomus* sp.

RESUMEN

En los últimos años, el cultivo de cacao (*Theobroma cacao* L.*)* en Colombia viene creciendo, esto hace necesario desarrollar un sistema de producción sostenible. Es así, como los microorganismos benéficos del suelo son una excelente alternativa para mejorar la productividad de la planta, pero es importante conocer su funcionamiento y ecología. Este trabajo tuvo como objetivo identificar y cuantificar hongos micorrízicos arbusculares (HMA) y *Pseudomonas* sp. fluorescente associados al suelo y la rizosfera de plantas de cacao en dos agroecosistemas, bosque seco tropical (BST) y bosque húmedo tropical (BHT). En una segunda etapa del trabajo se seleccionaron y multiplicaron cepas nativas de *Glomus* sp., *Acaulospora* sp.*,* y *Pseudomonas* sp. fluorescente en el laboratorio. Asi la efectividad de estas cepas para promover el crecimiento de plántulas de cacao bajo condiciones de invernadero fue evaluada. Los resultados indican que hubo una diferencia significativamente ($P \leq 0.5$) mayor en la colonización micorrizal y diversidad asociada a raíces de cacao creciendo en el agroeosistema asociado al bosque húmedo tropical. Sin embargo, no se encontraron diferencias significativas associados a la presencia a *Pseudomonas* sp. fluorescente associados a los dos agroecosistemas. Por otro parte en los experimentos en invernadero, la inoculación con el hongo micorrízico *Glomus* sp. fue el único tratamiento que promovió el crecimiento de plántulas de cacao.

Palabras claves: bosque húmedo tropical, bosque seco tropical, *Glomus* sp.

Introduction

Cacao (*Theobroma cacao* L.) is a neotropical plant species originated from the humid tropics of America, and thus presents great diversity in this zone (Enríquez, 2004; Motamayor *et al.* 2008). In Colombia, the cacao is cultivated in soils with different biological, physical, and chemical characteristics (Suárez *et al.*, 2010). Most of the soils in the tropical areas present low nutrient availability, which results in low crop productivity (Brady and Weil, 2008; Meason *et al.*, 2009; Osorio, 2011). This condition leads to add high doses of chemical, mineral, and organic fertilizers in order to improve yields, but the excessive use of these supplies can increase productions costs and generate negative environmental impacts (Brady and Weil, 2008; Xiao *et al.*, 2008).

Arbuscular mycorrhizal fungi (AMF) are considered to be of great importance for adequate plant development, since they can improve water and nutrient uptake, particularly phosphorus (P) (Osorio, 2011; Ramírez *et al.*, 2013; Ramírez *et al.*, 2014; Ramírez *et al.*, 2015). This is relevant because in the tropics approximately 71% of plant species form arbuscular mycorrhizal association, 16% form other types of mycorrhizal associations, and only 13.4% are non-mycorrhizal (Coyne, 2000; Barea and Azcon, 2002). Among the first ones, it is well known the ability of cacao plants to form mycorrhizal associations and increase plant growth (Azizah-Chulan and Martin, 1992).

On the other hand, some bacteria from the *Pseudomonas* genus are known as plant growth promoters. They can colonize plant root systems and promote plant growth by (i) nutrient solubilization (e.g., P, Fe, among others), (ii) synthesizing hormonal regulators such as auxins and gibberellins (Gunes *et al.*, 2015; Pii *et al.*, 2015) and (iii) decrease phytopathogens populations in the soil and thus prevent plant diseases (Hallmann *et al.*, 1997; Van Veen *et al.*, 1997; Lugtenberg and Kamilova, 2009).

However, few researches exist about the presence of both types of these microorganisms in cacao plantations in contrasting environmental conditions, as those imposed by the TDF and TMF life zones, which certainly limits the application of these microorganisms at a commercial scale (Cuadros *et al.*, 2011). For this reason, the present study had two objectives (i) identifying and quantifying AMF and strains of fluorescent *Pseudomonas* sp. in the rhizosphere of cacao plants in two agroecosystems of contrasting life zones: tropical dry forest (TDF) and tropical moist forest (TMF); and (ii) to evaluate the effectiveness of inoculation with these two groups of microorganisms on cacao plant growth in greenhouse.

Materials and methods

Sampling sites

Soil samples were collected from two sites: (i) Santa Fe de Antioquia at the Cotove Agriculture Experimental Station

(altitude 540 m a.s.l., annual rainfall 900 mm, mean air temperature 27°C and relative humidity 75.5%) located at 6º32´55''N and 75º50´3´´W, which corresponds the TDF ecological life zone according to Holdridge (1967); (ii) Maceo at the Theobroma farm (altitude 1,050 m a.s.l., annual rainfall 2,500 mm, mean air temperature 20°C, relative humidity 93%) located at 6º32´43''N and 74º47´27´´W, which represent the TMF ecological life zone. Samples were transported to the Soil Laboratory of the Universidad Nacional de Colombia at Medellin for microbial processing. Greenhouse experiments were carried out in the same university (6º15´47''N and 74º34´40´´W).

Soil sampling and testing

In each location four plots were selected and five trees of the IMC-67 (rootstock) and CCN-51 (scion) clones were randomized and chosen for sampling their rhizosphere. Surface soil (0-30 cm) and root fragments were taken 1 m away from the tree stem; from each plot 1 kg of soil and 50 g of roots were collected. Soil sampling was conducted at the Soil Fertility Laboratory at the same university; the results are shown in table 1.

AMF isolation, counting, identification, and root colonization

AMF spores were isolated using the wet sieving and decanting method described by Gerdemman and Nicholson (1963). Briefly, it consists of passing 20 g of soil sample through a set of sieves (425, 90, and 25 µm). The portion retained in each sieve was suspended with 50 mL of water and then centrifuged for two min at 2,000 rpm. The precipitate was suspended in 50 mL of a 50% sucrose solution and centrifuged again for five min at 4,000 rpm. The supernatant obtained was passed through a filter paper for spore counting using a microscope (Nikon Eclipse E200). AMF identification was based in taxonomic keys (Clap *et al.*, 1995; Heijden *et al.*, 2004; Peterson *et al.*, 2004). The Shannon diversity index was constructed using the PAST program, version 2.16 (Hammer *et al.*, 2001).

Root mycorrhizal colonization was conducted after clearing root fragments (1 cm length) with 10% KOH for 24 h

TABLE 1. Soil fertility parameters from each cacao agroecosystem.

Agroecosystem	Life zone	Sand	Silt	Clay	pH	SOM	Al	Ca	Mg	K	P	Solution P
		------- g kg^{-1} -----				g kg^{-1}	------- cmol$_c$ kg^{-1} -------				mg kg^{-1}	mg L^{-1}
Santa Fe	TDF	400	280	320	5.5	59	0.3	4.55	1.5	0.5	29	0.190
Maceo	TMF	400	140	460	4.8	44	2.1	1.50	0.6	0.1	3	0.014

Methods: texture by Bouyoucos; soil pH measured in water (2:1, V:V); soil organic matter (SOM) measured by Walkley and Black; Al extracted by 1 *M* KCl; Ca, Mg, and K extracted by 1 *M* ammonium acetate; P extracted by Bray II; soil solution P measured in 0.01 *M* CaCl$_2$.

(Phillips and Hayman, 1970) and then settled down in an alkaline solution (0.5% NH_4OH and 0.5% H_2O_2) for 30 minutes (Brundrett and Abbott, 1995). Root fragments were washed with tap water and then acidified with 10% HCl for five min. After that, roots fragments were stained with 0.025% trypan, blue dissolved in lacto-glycerol (Kormanik *et al.*, 1980) and the extension of roots colonized by the AMF was measured by the line-intercept method (Giovannetti and Mosse, 1980).

Fluorescent *Pseudomonas* sp.

Rhizosphere samples were diluted with sterile water up to 10^{-4} serial dilution; 100 µL of the 10^{-3} and 10^{-4} serial dilutions were transferred onto King B (KB) culture medium in Petri dishes (King *et al.*, 1954). The grouth medium was supplemented with ampicillin (50 mg L^{-1}) and chloramphenicol (12.5 mg L^{-1}) (Simon and Ridge, 1974). Petri dishes were incubated for 48 h at 28°C. Positive colonies were considered those capable of producing fluorescent pigment under ultraviolet light (260 nm) (Ramírez, 2005).

Greenhouse experiments

Soil

Surface soil sample (0-30 cm) was collected from the Carimagua Experimental Station (USDA Soil Taxonomy Oxisol, Haplustox). It was analyzed in the Soil Fertility Laboratory at the same university and with the same methods. Soils results were: sand 480, silt 200, and clay 320 g kg^{-1} (Bouyoucos), pH 4.8 (water, 2:1), SOM 60 g kg^{-1}, Al 0.9 $cmol_c$ kg^{-1} ($1M$ KCl); Ca, Mg, and K 1.1, 0.4, and 0.2 (1 M ammonium acetate).

In order to avoid microbial interference, the soil was sterilized in an autoclave at 0.1 MPa and 121°C, for two cycles of one hour each. Lime was added to adjust soil pH at 5.6, based on the lime incubation method (Uchida and Hue 2000). Based on a P sorption isotherm (Fox and Kamprath, 1970), KH_2PO_4 was added to achieve a soil solution P concentration of 0.02 mgL^{-1}, which is considered optimal for mycorrhizal activity.

Cacao plants

At 50 d seeds of the IMC-67 clone germinated in peat until developing 5 leaves. Then two seedlings were transplanted into plastic pots containing 2 kg of the soil. One month later, one plant was removed. The plants were kept for other four months in greenhouse conditions and were frequently watered to maintain soil moisture content between 50-60% of the soil's maximum water retention capacity. Every week 50 mL of a P-free Hoagland solution was added.

Microorganisms

For this experiment, we used two mycorrhizal inocula, one inoculum contained spores of the genus *Glomus* sp. and the other spores of the genus *Acaulospora* sp., it is also called *Rhizoglomus* (Sieverding *et al.*, 2015) and *Rhizofagus* (Schüßler and Walker, 2010). These were selected since both genera were the most abundantly in the plots evaluated in the field. Both AMF were multiplied in corn roots until reaching a minimum concentration of 45 infective propagules per g of inoculum (Porter, 1979).

Several isolates of fluorescent *Pseudomonas* sp. were tested for their capability to produce indoleacetic acid using Salkowski indicator solution (Gordon and Weber, 1951). The solution was modified for use in bacterial culture supernatants (Patten and Glick, 2002). Additionally, the bacteria *Ralstonia solanacearum* was used as a control strain (C). This was conducted to select the isolate with the highest production of indole-acetic acid. The isolate selected (designed as P10) was multiplied in KB medium for 48 h at 28°C and suspended in sterile water; the bacterial suspension contained $1x10^6$ colony forming units (CFU) per mL (Jena, 2012).

Treatments and variables

The treatments evaluated were: (i) inoculation with *Acaulospora* sp. (designated as "A") at a rate of 70 g of inoculum per 2 kg of soil and mixed throughout, (ii) inoculation with *Glomus* sp. (designated as "G") at a same rate; (iii) inoculation with fluorescent *Pseudomonas* sp. (P10) at a rate of 5 mL per plant; (iv) inoculation with *Acaulospora* sp. + fluorescent *Pseudomonas* sp. (A+P10) at the same rates described above; (v) inoculation with *Glomus* sp. + fluorescent *Pseudomonas* sp. (G+P10); (vi) an not inoculated control (control) was included as a reference. All inocula were added at the transplanting time. Each treatment had five replicates.

The response variables were: plant dry weight after ovendry a 60°C for 72 h, foliar P concentration using the nondestructive method developed by Aziz and Habte (1987), and root mycorrhizal colonization as described above. Additionally, the fluorescent *Pseudomonas* sp. bacterium was recovered from the rhizosphere in order to confirm its presence.

Statistical analysis

In each experiment (sampling and greenhouse) a completely randomized design was used. Levene and Kolmogorov-Smirnov criteria were used to confirm data homoscedasticity and normality ($P<0.05$), respectively. The

data were subjected to analysis of variance and the Tukey test for mean separation both with a significance level $P \leq 0.05$. Statistical analysis was performed with the software R (R version 3.2.3 (2015-12-10) "Wooden Christmas-Tree" Copyright (C) 2015 The R Foundation for Statistical Computing Platform: i386-w64-mingw32/i386 (32-bit).

Results and discussion

The two agroecosystems exhibited a significant difference in soil parameters; for instance, soil pH in Maceo was extremely acidic (4.7), whereas in Santa Fe the soil pH was neutral (6.7) (Tab. 2). Consistently, the soil solution P in Maceo was very low (0.011 mg L^{-1}) and in Santa Fe was high (0.240 mg L^{-1}). Despite of these contrasts, not significant difference was detected in the foliar P concentration of cacao plants. Similarly, there was not significant difference in the CFU of *Pseudomonas* spp.

The soil test results are consistent with the typical soil fertility parameters commonly found in these two life zones. For instance, Maceo soils are very acidic and poor in available P as a result of leaching out of bases (Ca, Mg, K, Na) produced by a high rainfall regime and is rich in Al as a result of high weathering rates, which generate oxides of Al and Fe that held P in unavailable forms for plant roots. By contrast, Santa Fe soil has a neutral soil reaction and is rich in available P, this associated with low weathering, low rainfall and the consequent dominance of 2:1 clays (e.g.,

montmorillonite, vermiculite) that retain high amounts of exchangeable bases (Zapata, 2002).

Mycorrhizal colonization was significant different between both agroecosystems, in Maceo was 12.1% and in Santa Fe was 3.7%. Accordingly, the number of AMF spores was significantly higher in Maceo (16.2) than in Santa Fe (12.8).

Both agroecosystems showed similar AMF genera, but their relative distribution was different (Fig. 1). Although, *Glomus* genus is more abundant than the other genera, in Maceo it represented almost half AMF population, while in Santa Fe it was three quarters of the AMF population. *Acaulospora* genus was the second in abundance in both agroecosystems (9-24%), followed by *Gigaspora* (8-11%) and by the other two genera (*Scutellospora*, *Entrophospora*). These differences were satisfactorily reflected in Shanon Index (Tab. 2), which was significantly higher in Maceo (1.82) than in Santa Fe (1.30).

The bacterium that was used as control *R. solanacearum* did not produce indole-acetic acid. In contrast, all *Pseudomonas* isolates did produce it, but the concentration produce was variable among them (Fig. 2). The isolate P10 was the most active in this regard (79.9 µg mL^{-1}) followed by P9, P3 and P8 (48.9-59.3 µg mL^{-1}), and then by P1, P2, and P3 (29.5-38.9 µg mL^{-1}). The less effective isolate were P4, P5 and P6 (20.2-25.0 µg mL^{-1}).

TABLE 2. Variables evaluated in the two agroecosystems.

Agroecosystem	Foliar P (g kg^{-1})	Soil pH	Soil solution P (mg L^{-1})	MC[1] (%)	*Pseudomona* spp. (CFU g^{-1})	Shanon index	AMF spores[2]
Maceo	5.6 a	4.7 b	0.011 b	12.1 a	4.1 x 10^2 a	1.82 a	16.2 a
Santa Fe	6.6 a	6.7 a	0.240 a	3.7 b	4.2x 10^2 a	1.30 b	12.8 b

Means followed by different letters indicate significant differences according to the Tukey test ($P \leq 0.05$).
[1] MC: mycorrhizal colonization; [2] Spores per g of soil.
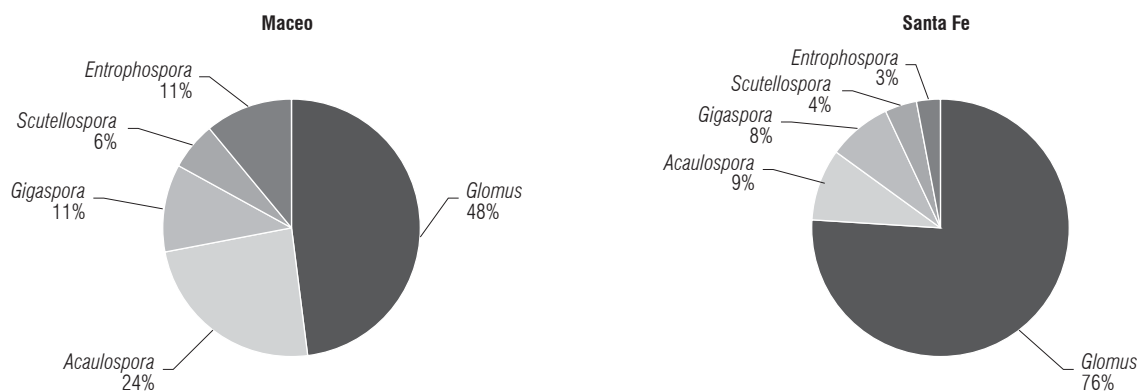

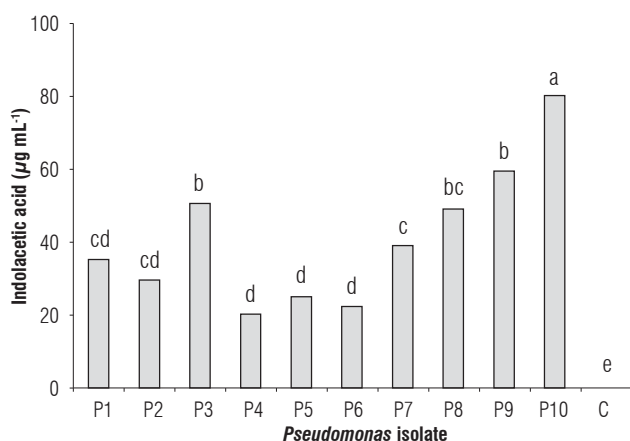**FIGURE 1.** Relative distribution of AMF genera in two agroecosystems of cacao.

FIGURE 2. Indoleacetic acid produced by different isolates of fluorescent *Pseudomonas* sp. isolated from cacao rhizospheres; C = control inoculated with *R. solanacearum*. T1-T10 = number of isolated strains. Means with different letters indicate significant differences according to the Tukey test (*P*≤0.05).

The findings of this study coincide with those of Prieto *et al.* (2012) who found AMF species of *Glomus*, *Scutellospora*, *Acaulospora* and *Gigaspora* in cacao agroforestry systems placed in a TDF in Ecuador. However, these authors did not report *Entrophospora* genus. In a study by Cuenca and Meneses (1996) in Venezuela the genus most associated with cacao was *Glomus*, being *G. etunicatum* particularly prominent. According to Sieverding (1991), *Glomus* is one of the most competitive and effective AMF genus in with different botanical groups and different soil conditions. In this study, clearly *Glomus* is dominant in both agroecosystems and in the case of any inoculation with AMF, species of *Glomus* should be considered. This was confirmed with the greenhouse results, in which *Glomus* was consistently effective to promote plant P uptake and growth (Zandavalli *et al.*, 2004; Osorio 2011; Ramírez *et al.*, 2013; Ramírez *et al.*, 2014; Ramírez *et al.*, 2015).

The MC values reported here coincide with those from Bolívar *et al.* (2009), who found MC between 2.8 and 13.3% under natural conditions. The former value might be considered low, since Cuenca *et al.* (1991) reported MC values up to 69%. Meanwhile, in agroforestry systems in a TMF life zone in Ecuador, Prieto *et al.* (2012) reported that MC did not surpass 3.5%, while values were as low as 0.9%. Perhaps, the high levels of soil available P found in Santa Fe agroecosytems may restrict the extent of mycorrhizal colonization (Peters and Habte, 2001), while the low levels of this in Maceo may promote a high mycorrhizal colonization. Cuenca *et al.* (1991) reported that in soils with low nutrient availability, cacao plants are highly dependent on AMF. This is because under these conditions, the plant requires this positive symbiosis for the uptake of this nutrient (Bonfante and Genre, 2010; Barea and Pozo, 2013).

Cuadros *et al.* (2011) reported that in field conditions MC depends largely on soil P content. López *et al.* (2007), Toro *et al.* (2008), and Azizah-Chulan and Martin (1992) reported that the recommended commercial dose of P fertilizers for cacao had negative effect on native AMF populations. They recommended adjusting the P fertilization management in order to promote the AMF symbiosis. On the other hand, Tena (2002) affirms that high levels of Ca and Mg (as those found in Santa Fe) may affect the AMF spores density and thus the symbiotic relationship with the host plant.

Despite the presence of fluorescent *Pseudomonas* sp., we did not find any promoting effects on cacao plants in the greenhouse experiment. It is not clear if in the two agroecosystems they had a role on plant nutrition or growth, it has been reported that they are biocontrol agents and plant growth promoters (Hallmann *et al.*, 1997; Van Veen *et al.*, 1997; Lugtenberg and Kamilova, 2009). Notice that in cacao plants at Maceo the foliar P was as good as in Santa Fe, in despite of that the former one was grown in a P deficient soil. It may be explained by the high levels of MC, the role of this bacterium in those systems should be investigated.

Uninoculated plants (control) showed a plant dry weigh of 150.9 g, which was significantly increased (+36%) only by *Glomus* inoculation. Other inoculation did not increase significantly the plant growth of cacao plant, included the co-inoculation G+P10 (Fig. 3A).

Control plants exhibited a foliar P concentration of 0.43%, this was significantly higher when the mycorrhizal fungi were inoculated (Fig. 3B). Inoculated plants with *Acaulospora* had foliar P concentration of 0.67% and those inoculated with *Glomus* had a concentration even higher 0.92%, which represented relative increases of 56 and 114%, respectively. The inoculation with *Pseudomonas* sp. (P10) was ineffective to promote foliar P (0.51%). Moreover, the co-inoculation with P10 was not effective to increase the foliar P beyond that obtained with the AMF inoculation only. In this way, the foliar P concentration of plants treated with A+P10 was 0.73% (similar to A-inoculation) and with G+P10 was 0.86% (similar to G-inoculation).

Uninoculated plants and those inoculated with P10 did not have mycorrhizal colonization (Fig. 3C). In contrast, the plants inoculated with *Acaulospora* (without and with P10) showed a mycorrhizal colonization that ranged between 15.8-13.4%, respectively, which did not differ to each other. Otherwise, the inoculation with *Glomus* had values
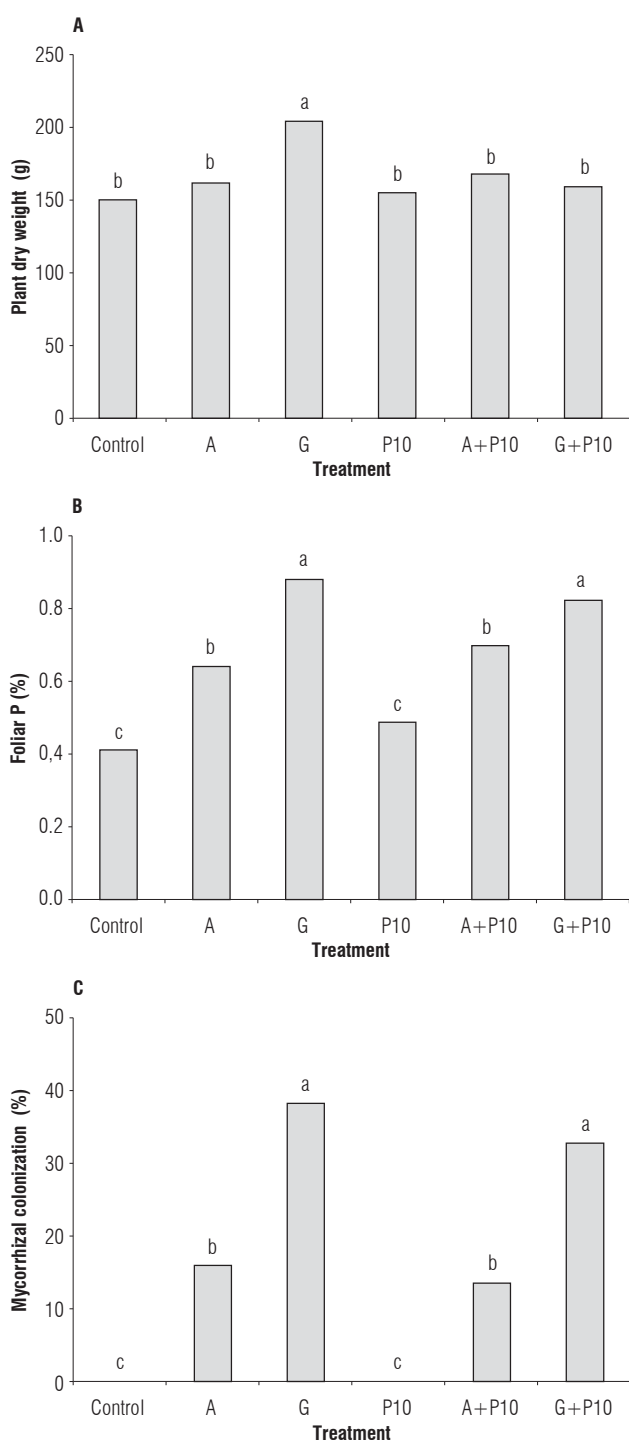
FIGURE 3. Variables evaluated in greenhouse conditions in cacao seedlings inoculated with an AMF. A: *Acaulospora* sp. G: *Glomus* sp. in combination with fluorescent *Pseudomonas* sp. (P10). Means with different letters indicate significant differences according to the Tukey test ($P \leq 0.05$).

of mycorrhizal colonization significantly higher than the other with values that fluctuated between 32.5-37.9 %, without and with P10, respectively.

Inoculation of the IMC-67 clone with *Glomus* sp. resulted in higher MC values than those reported by Ballesteros *et al.* (2004) (*ca.* 20%) with native *Glomus* and *Acaulospora*. Similarly, Cuadros *et al.* (2011) reported MC values of 84% with both AMF genera. These results indicate that the MC of cacao roots by AMF inoculated is variable. From our perspective, this variable (MC) may be secondary, and it is necessary to focus on the effectiveness of AMF inoculation on plant nutrient uptake and growth or even better on crop yield.

In this study it is clear that, at least for cacao seedlings, the use of AMF inoculation promote growth, as reported by Azizah-Chulan and Martin (1992). However, this effect seems to be species-dependent. Thus, species of *Glomus* must be included in a commercial inoculum intended to promote seedling growth and P uptake. The benefits obtained with *Glomus* inoculation are quite relevant since the P foliar was twice higher with this than in control plants. On the other hand, the effect of *Pseudomonas* sp. P10 was null alone or in combination with AMF.

Conclusions

In both cacao agroecosystems, AMF and *Pseudomonas* were detected. The same AMF genera was found but in different relative distribution. The AMF genus *Glomus* was predominant in both agroecosystems. On the other hand, the number of CFU of *Pseudomonas* spp. was similar in both agroecosystems. In the greenhouse, cacao seedlings had higher levels of foliar P when inoculated with AMF (*Acaulospora* and *Glomus*) than uninoculated plants, but the effect was higher with *Glomus* species. Plant growth was only significantly promoted by *Glomus* inoculation and not by *Acaulospora*. The inoculation with the isolate P10 of *Pseudomonas* did not promote plant growth and P uptake in mycorrhiza-free or mycorhizal plants.

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