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Phosphorus use efficiency in common bean (*Phaseolus vulgaris* L.) as related to compatibility of association among arbuscular mycorrhizal fungi and rhizobia

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The tripartite symbiosis of common bean (*Phaseolus vulgaris* L.) recombinant inbred line (RIL) 147 with rhizobia and arbuscular mycorrhizal fungi (AMF) was assessed in sand culture by comparing the effects of three AMF species on the mycorrhizal root colonization, rhizobial nodulation, plant growth and phosphorus use efficiency for symbiotic nitrogen fixation. Although *Glomus intraradices* well colonized roots of RIL147, *Gigaspora rosea* and *Acaulospora mellea* weakly colonized roots-plants. Higher colonization by *Glomus* was also obtained by contact with mycorrhized *Stylosanthes guianensis*. Significant differences among colonization and nodulation of the roots and growth were found between AMF species. Although the results showed that the double inoculation, especially with *Glomus*, induced a significant increase in all parameters whatever the AMF treatments in comparison to the control. In addition, the combined inoculation of *Glomus* and CIAT899 strains resulted in significantly higher nitrogen and phosphorus accumulation of common bean plants and improved phosphorus use efficiency compared with their controls which were not dually inoculated. It was concluded that phosphorus use efficiency for plant growth and nitrogen fixation could be improved by compatibility with arbuscular mycorrhizal fungi, rhizobial strain and common bean genotype, same under P limited.

Key words: *Acaulospora mellea*, arbuscular mycorrhizal fungi, *Glomus intraradices*, *Gigaspora rosea*, nitrogen fixation, *Phaseolus vulgaris*, phosphorus, rhizobia, *Stylosanthes guianensis*, symbiosis.

INTRODUCTION

Beans play an important role in the enhancement of the level of nourishment due to the fact that this plant is rich in elements like potassium, calcium, iron and phosphorus (P) and it contains 18 to 32% of protein in every grain averagely (Numan and Nuri, 2005). The plant is also rich in A, B and D vitamins in developing countries. Despite this interest of the food legume nutritional, several conditions limit the growth and production of this legume, such as phosphorus and nitrogen which constitute the most limited nutriment for vegetative growth (Vance et al., 2003; Tajini et al., 2009, 2011). However, nitrogen (N) and P availability is generally low in arable soils, thus

making them major limiting factors for crop growth and yield, especially in tropical and subtropical areas (Hardarson and Atkins, 2003). Xiurong et al. (2011) reported that during the past 50 years, the widespread use of chemical fertilizers to supply N and P has had a substantial impact on food production, and has become a major input in crop production around the world. However, further increases in N and P application are unlikely to be as effective at increasing yields.

Today, only 30 to 50% of applied N fertilizer and 10 to 45% of P fertilizer are taken up by crops (Adesemoye and Kloeppel, 2009). In addition, the heavy use of chemical fertilizers in agriculture causes frequent deleterious environmental consequences, and has become a global concern (Tilman et al., 2002). Therefore, developing nutrient-efficient varieties will likely play a major role in increasing crop yield and would be in a

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more sustainable and economical approach to agriculture (Xiurong et al., 2011). The capacity of plant to acquire and utilize nutrients has encouraged researchers to study how nutrient efficiency is influenced by both nutrient absorption in roots and utilization in plants (Sepehr et al., 2009). In order to assess the capacity of plant to acquire nutrients, arbuscular mycorrhizal fungi (AMF) and rhizobia are two of the most important plant symbionts. They play a key role in natural ecosystems and influence plant productivity, plant nutrition and improved inhibition of fungal plant pathogens (Demir and Akköpru, 2007; Wehner et al., 2010; Abohatem et al., 2011). Mycorrhiza benefits the host through mobilization of phosphorus from non-labile sources, whereas rhizobia fix N₂ (Scheublin and Vander Heijden, 2006). Previous works on the tripartite symbiosis legume-mycorrhiza-rhizobia have shown stimulatory (Demir and Akköpru, 2007; Xiao et al., 2010) or inhibitory (Scheublin and Vander Heijden 2006; Franzini et al., 2010) effects on each other or on the growth of plants.

A few studies have shown that some bacterial species respond to the presence of certain AMF (Andrade et al., 1997; Artursson et al., 2006), suggesting a high degree of specificity between bacteria associated with AMF. Thus, the specific bacteria together with AMF may create a more indirect synergism for plant growth (Barea, 1997); including nutrient acquisition (Barea et al., 2002) and enhancement of root branching (Gamalero et al., 2004). In addition, the AMF themselves have also been shown to have an impact on the composition of bacterial communities in their mycelium environment (Artursson et al., 2006). The rhizobia-bean symbiosis when in association with arbuscular mycorrhizal fungi (AMF) is known to benefit from a better supply of phosphorus (Sanginga et al., 2000). The AMF is also able to acquire phosphorus in organic form that is not directly assimilated by plants (Bucher et al., 2001). The mechanisms affecting the efficiency of absorption and utilization of phosphorus in plants are related to colonization by mycorrhizae (Jia et al., 2004). Furthermore, Jin et al. (2010) found that dual inoculation with AMF and rhizobia decreased the harmful influence of sulphate salinity on plant growth and nutrient accumulation (P, N and proline) in *Lathyrus sativus*, compared with the control treatments.

Both symbioses share parts of signalling pathways, indicating intimate interactions between all three partners during co-evolution (Demir and Akköpru, 2007; Xiao et al., 2010). On the other hand Aysan and Demir (2009) reported that the information on the mechanisms controlling interactions of bacteria with AMF and plant roots in the mycorrhizosphere and their activities are very difficult to generalize because the interactions involving arbuscular mycorrhiza, root rot fungi and *Rhizobium* vary with the microbial species and plant cultivars. In order to assess the effect of inoculation with arbuscular mycorrhizal fungi or by contact with mycorrhized *Stylosanthes guianensis* on phosphorus use efficiency for

plant growth and to investigate whether sensitivity of symbiotic nitrogen fixation to phosphorus deficiency was restored by symbiosis with arbuscular mycorrhizal fungi, common bean genotype from the cross of BAT477 by DOR364 was grown in a glasshouse with both symbionts.

MATERIALS AND METHODS

Two cases of tripartite symbiosis were realized in this study. In the first, the common bean genotype was inoculated by mycorrhizal inoculant with AMF spp. diversity; but in the second, the common bean genotype was inoculated by contact with mycorrhized *Stylosanthes guianensis* or not (control). In all cases received similar rhizobial inoculation. The experimental design consisted of randomized block with 3 replications. The SAS software (1997) was used to perform the statistical analyses, results were submitted to ANOVA, and comparison of means was achieved by the Duncan's multiple range tests ($P \leq 0.05$).

Biological material

The common bean genotype used in this study was recombinant inbred line (RIL) 147, from the cross of BAT477 and DOR364 (CIAT-INRA cooperation). Seeds were surface-sterilized with 1.3% calcium hypochlorite for 15 min with constant stirring, and then washed with sterile distilled water. They were germinated on 0.8% sterile agar plates for 3 days at 28°C in the dark, and had a germination rate of 80%. Rhizobial inoculation was performed by soaking the seedlings for 45 min in a freshly prepared suspension of *Rhizobium tropici* CIAT899 containing 10⁸ bacteria ml⁻¹. Seedlings were grown in 1000 ml pots filled with an autoclaved sand-soil mixture (9:1 v:v) recolonized with soil bacteria according to Jansa et al. (2002). Fifty gram (50 g) of one of three AMF inoculums, namely, *Glomus intraradices* BEG157 (Schenck and Smith), *Gigaspora rosea* BEG9 (Nicolson and Schenck) or *Acaulospora mellea* NM54 (Spain and Schenck), were placed below the seedlings. The inoculum used for the pots consisted of chopped roots of pot cultures planted with leek (*Allium porrum*) and grown for 18 months in a glasshouse. Fifty gram (50 g) of AMF inoculum is approximately equal to 1000 spores of the AMF specie contained at least 20 infective propagules of AMF per gram of chopped root. In order to assess the mycorrhization by contact, also some surface-sterile seeds were sown and grown in soil-sand culture in contact with mycorrhized *Stylosanthes guianensis* by *Glomus intraradices* BEG157. The amount of mycorrhizae substrate was characterized by low available N (0.007%) and P (0.001%). In non mycorrhizal treatments, each pot filled with same amount of mycorrhizae free substrate.

Growth conditions

Trials were performed in a temperature-controlled glasshouse with night/day temperatures of 25/35 °C, and a 16 h photoperiod with complementary illumination of 400 μmol photons m⁻² s⁻¹. Inoculated seedlings with *R. tropici* CIAT899 and AMF inoculants or by contact with mycorrhized *Stylosanthes* or without AMF were grown in soil-sand substrate. Pots were watered with distilled water every 2 days until harvest, and received once a week using the Vadez et al. (1996) nutrient solution: macroelements: K₂SO₄ (1.25 mM), MgSO₄·7H₂O (2.05 mM), CaCl₂ (3.3 mM); microelements: Fe EDDHA (8.5 μM Fe as sequestrene), H₃BO₃ (4.0 μM), MnSO₄ (6.0 μM), ZnSO₄ (0.9 μM), CuSO₄ (1.0 μM), NaMoO₄ (0.1 μM). The nutrient solution was supplemented with 2 mmol urea plant⁻¹ during the first two weeks, 1 mmol urea per plant during the next two

weeks and no more urea during the last two weeks. The pots were distributed in a complete randomized block design with 3 replications and one plant only per pot.

Assessment of AMF colonization

The plants growing in pots were harvested at 50 days after sowing (DAS), corresponding to flowering stage, half of the root system was used for estimation of the extent of root colonization by the AMF as follows: roots were cleared in KOH 10% (w:v) at 80°C for 30 min followed by rinsing with water and two rinses with 1% HCl of 1 h each. Thereafter, the roots were immersed at 80 °C for 1.5 h in a staining solution consisting of lactic acid: glycerol: water (1:1:1 v:v:v) and 0.1% of each Trypan Blue and Methylene Blue. After washing away the stain, the roots were de-stained in tap water for 30 min at room temperature. The roots were examined under a compound microscope for quantitative colonization assessment by magnified-intersection (McGonigle et al., 1990).

Biomass and percentages of P and N at harvest

At harvest, shoot, nodules and roots were separated and dried at 70°C for 2 days, and dry weight of each fraction was calculated. The percentage of P was measured in samples of ground tissues following wet digestion with nitric-perchloric acid (6:1, v:v) at 250°C for 6 h, using the phosphovanado-molybdate method (Tausky and Shorr, 1953). The P use efficiency (PUE) was calculated as the ratio of biomass (shoot + root) g⁻¹/mean plant P content mg⁻¹. Total nitrogen percentage (TN) was measured by the Kjeldahl procedure on plants harvested for biomass determinations. Because no significant difference in most of the results of *Gigaspora* and *Acaulospora* and control plants, only plants inoculated either with *Glomus* or by contact with mycorrhized *Stylosanthes* were analyzed for this parameter.

RESULTS

Mycorrhizal colonization

Data in Figure 1A show that the parameters of root-colonization were affected by AMF treatments ($P \leq 0.01$). The AMF effect was predominant in plants inoculated by *Glomus*, and significant difference between both treatments (by contact and by mycorrhizal inoculant). Thus, rates of colonization in plants inoculated by *Glomus* were nearly two-fold higher for all structures in comparison to that in plant grown with mycorrhized *Stylosanthes*. On the other hand, higher vesicles and hyphae levels were found in plants inoculated with *Glomus* than *Gigaspora*, and arbuscules were only encountered in roots of plants inoculated with *Glomus* by mycorrhizal inoculant or by contact with mycorrhized *Stylosanthes*, but colonization remained very low with *Acaulospora* (Figure 1A). Figure 1B shows root colonization by vesicles and hyphae of *Glomus*.

Nodulation

Nodulation was strongly affected by AMF treatments

($P \leq 0.01$), significantly more nodules were encountered for plants inoculated by *Glomus* than other AMF species (Figure 2A). In comparison to control plants, inoculation with AMF induced a significant increase in nodule number of 86 and 77% in plants inoculated with *Glomus* and for plants grown in contact with mycorrhized *Stylosanthes*, respectively, but no significant difference between plants inoculated with other AMF species and controls plants (Figure 2A). On the other hand, plants inoculated with *Gigaspora* and *Acaulospora* show low nodule number in this experiment (Figure 2A). Also, AMF affected significantly the nodule mass per plant ($P \leq 0.05$). Data in Figure 2B show higher biomass of nodules in plants grown in contact with mycorrhized *Stylosanthes* than other AMF treatments. Thus, in comparison with control plants, AMF induced a significant increase of 85, 78, 52 and 27%, respectively by contact with mycorrhized *Stylosanthes* and with *Glomus*, *Gigaspora* and *Acaulospora* inoculants (Figure 2B).

Shoot and root growth

Plant growth of RIL147 was significantly increased by AMF treatments ($P \leq 0.05$) since higher dry weight was observed for plants inoculated with AMF than for control plants whatever the AMF treatments, and the highest shoot dry weight was observed for plants inoculated with *Glomus* than other treatments (Figure 3A). AMF treatments induced a significant increase in shoot dry weight of 46, 21 and 26%, in plants inoculated with *Glomus*, *Gigaspora* and for plants grown in contact with mycorrhized *Stylosanthes*, respectively in comparison to that in the control plants. But, plants inoculated with *Acaulospora* show low shoot dry weight without significant difference with the control plants (Figure 3A). Root biomass was also affected by inoculation with AMF ($P = 0.043$) (Figure 3B). In comparison to the control plants, AMF treatments induced a significant increase in root-growth of 48 and 32% in plants inoculated with *Glomus* and for plants grown in contact with mycorrhized *Stylosanthes*, respectively. But no significant difference between root-growth of plants inoculated with *Gigaspora*, *Acaulospora* and control plants (Figure 3B).

P accumulation and P use efficiency

Data in Figure 4A show that shoot phosphorus content varied significantly with AMF treatments ($P = 0.04$). The highest phosphorus percentage was observed in plants inoculated with both treatment of *Glomus* (by mycorrhizal inoculant and by contact), and the lowest phosphorus content was obtained in plants inoculated with *Acaulospora* without significant difference with the control plants (Figure 4A). With AMF, the percentage phosphorus content increased significantly of 30, 14 and

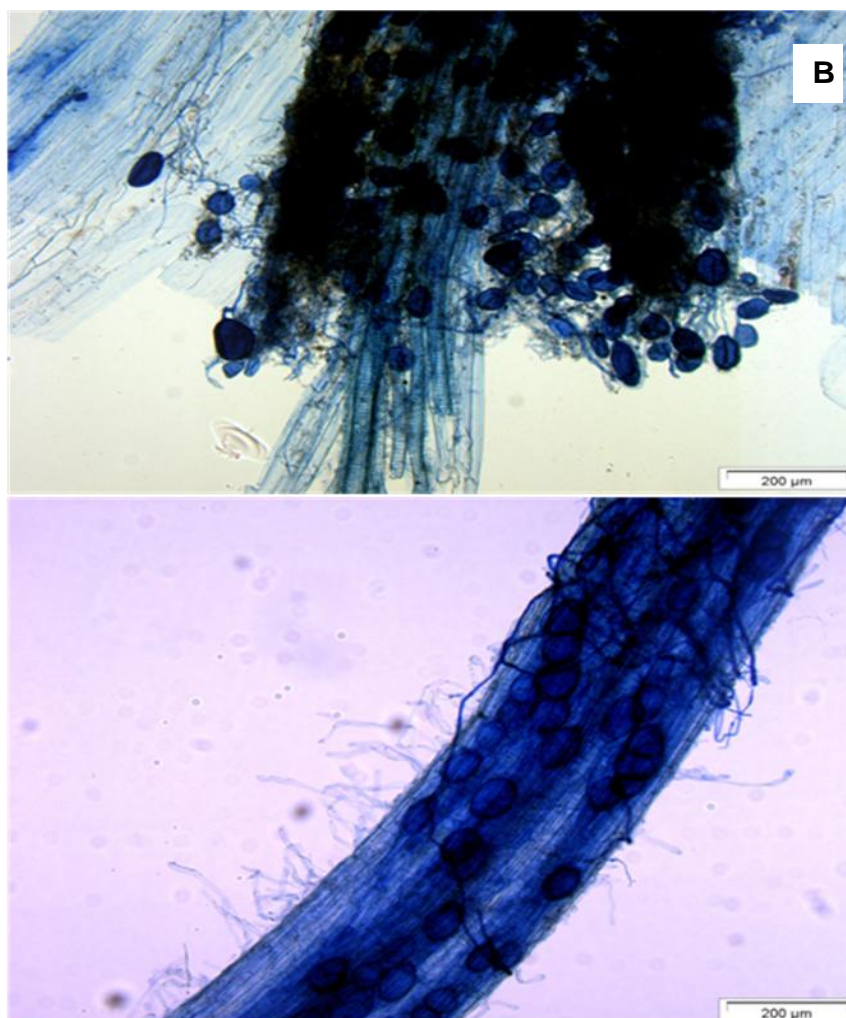
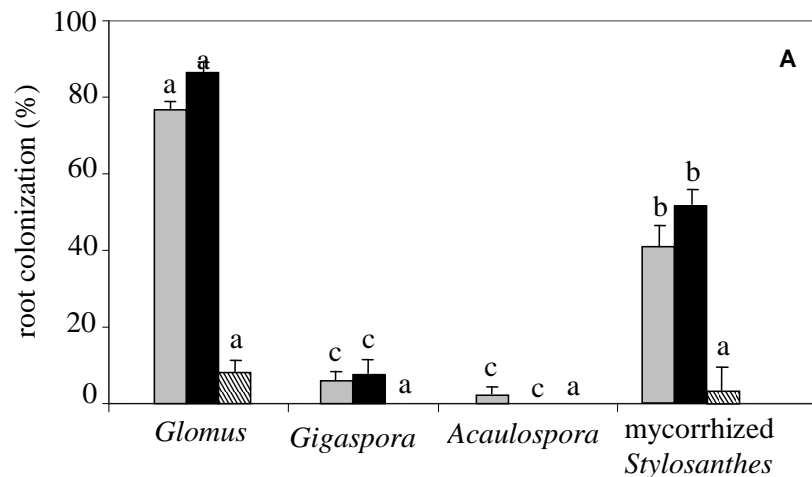


Figure 1. A- Effect of *Glomus*, *Gigaspora* and *Acaulospora* by mycorrhizal inoculant, and effect of *Glomus* by contact with mycorrhized *Stylosanthes guianensis* on root colonization by vesicles (grey bars), hyphae (black bars), and arbuscules (hatched bars) of common bean genotype RIL147 grown in sand culture. Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing. For each structure, different letters indicate significant differences between treatment means according to Duncan's multiple range test ($P \leq 0.05$). B- root colonization of common bean genotype RIL147 by vesicles and hyphae of *Glomus intraradices*.

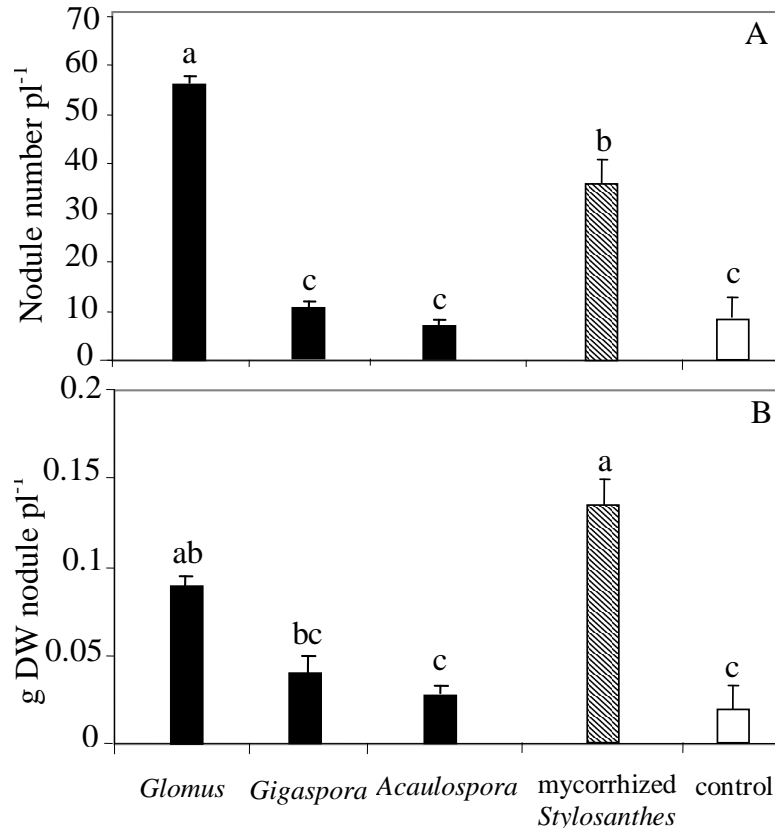


Figure 2. Effect of AMF on number (A) and dry weight (B) of nodules of common bean genotype RIL147, grown in sand culture, after inoculation with *R. tropici* CIAT899, and with *Glomus*, *Gigaspora* and *Acaulospora* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* with *Glomus* (hatched bars) or not as control (open bars). Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($P \leq 0.05$).

23% in plants inoculated with *Glomus*, *Gigaspora* and for plants grown in contact with mycorrhized *Stylosanthes*, respectively in comparison to that in the control plants (Figure 4A). P use efficiency (PUE) was also affected by AMF treatments ($P \leq 0.03$) (Figure 4B). The highest P use efficiency was observed in plants inoculated with *Glomus* or by contact with mycorrhized *Stylosanthes* with means of 0.10 ± 0.014 and 0.12 ± 0.028 g DW mg⁻¹ P, respectively. But the lowest P utilization was obtained in plants inoculated with *Acaulospora* with a mean of 0.06 ± 0.016 g DW mg⁻¹ P. The inoculation with AMF increased significantly the P utilization of 40, 15 and 50% in plants inoculated with *Glomus*, *Gigaspora* and in plants grown in contact with mycorrhized *Stylosanthes*, respectively in comparison to control plants (Figure 4B).

N accumulation

Data in Figure 5 show that shoot nitrogen content was

increased by AMF treatments ($P \leq 0.05$). Thus, *Glomus*, in combination with CIAT899, induced a significant increase in shoot nitrogen percentage in comparison to control plants, whereas there was no significant difference between both AMF treatments (by mycorrhizal inoculant and by contact). With AMF, the percentage of nitrogen increased significantly of 45 and 38% in plants inoculated with *Glomus* and by contact with mycorrhized *Stylosanthes* respectively, in comparison to that in the control plants (Figure 5).

DISCUSSION

In this study, we established a tripartite symbiosis of common bean with rhizobia and AMF by mycorrhizal inoculant or by contact with mycorrhized *Stylosanthes*. The interesting finding of this work is the significant use efficiency of phosphorus for growth benefit of the tripartite symbiosis with AMF treatments. The highest phosphorus

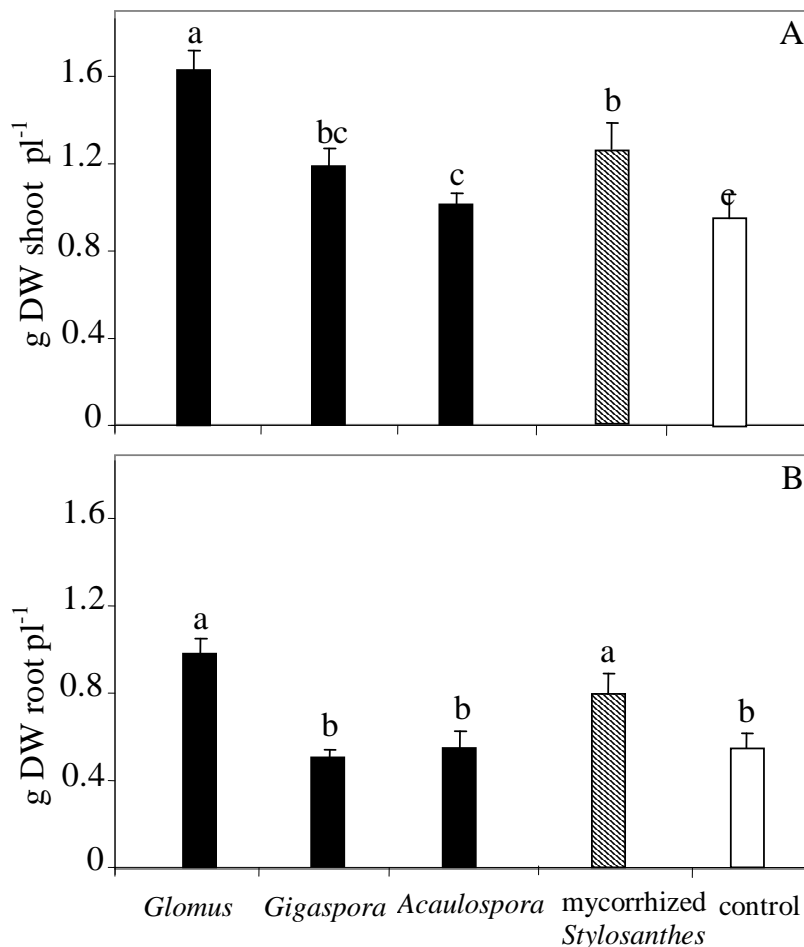


Figure 3. Effect of AMF on dry weight of shoot (A) and root (B) of common bean genotype RIL147, grown in sand culture, after inoculation with *R. tropici* CIAT899, and with *Glomus*, *Gigaspora* and *Acaulospora* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* with *Glomus* (hatched bars) or not as the control (open bars). Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($P \leq 0.05$).

use efficiency in the present work was obtained in plants inoculated by *Glomus* or in plants grown with mycorrhized *Stylosanthes* (Figure 4) demonstrates that the mechanisms affecting the efficiency of absorption and utilization of phosphorus in plants are related to colonization by AMF (Bucher et al., 2001; Jia et al., 2004). Also nitrogen accumulation was higher with *Glomus* under both treatments in comparison to that in the control plants (Figure 5), illustrates the relationship between P use efficiency and symbiotic nitrogen fixation (Tang et al., 2001; Tajini et al., 2011a) and it was also indicated that the shoot growth and N₂ fixation were determined mainly by the efficiency in P utilization (Rodino et al., 2009; Bargaz et al., 2011a, b).

In sand-soil culture where P limited conditions are more pronounced, the expression of mycorrhizal benefits was more obvious in plants with both treatments of *Glomus*

(Figure 1), it is believed that mycorrhizae especially benefit plants grown in soils where P is likely to limit plant growth by increasing the soil volume explored by AM hyphae relative to that of root hairs of non-AM plants. This would agree with previous studies showing highest mycorrhizal benefits to plant growth under moderate P deficiency (Mathimaran et al., 2005; Tajini et al., 2009, 2011b; Faghire et al., 2010; Xiurong et al., 2011), especially with leguminous plants harbouring a coarser root system with less extension of root hairs than graminaceous (Isobe and Tsuboki, 1998).

Different compatibilities between mycorrhizal and rhizobial may explain the differences in colonization and nodulation of RIL147 upon inoculation with different AMF species. They may explain the differences in P use efficiency for *Glomus*- compared to *Gigaspora* or *Acaulospora*-inoculated plants. The higher nodulation

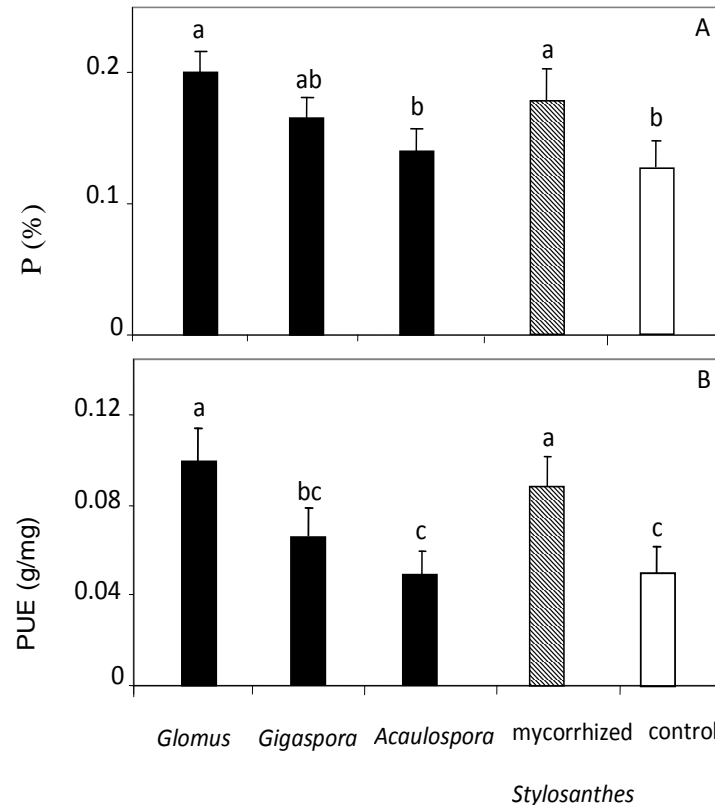


Figure 4. Effect of AMF on plant phosphorus percentage (A) and phosphorus use efficiency (B) of common bean genotype RIL147, grown in sand culture, after inoculation with *R. tropici* CIAT899, and with *Glomus*, *Gigaspora* and *Acaulospora* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* with *Glomus* (hatched bars) or not as control (open bars). Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($P \leq 0.05$).

with *Glomus* in both treatments (by mycorrhizal inoculant or by contact), than with the other AMF species is most likely due to improved P nutrition of the plants since *Glomus intraradices* is very efficient in transporting large quantities of P to the plants from remote zones (Jansa et al., 2003). Also, the colonization by *Glomus* was higher under low P. So that means that there may be signal coming from the plants, in relation to their P deficiency, that aim at increasing the level of mycorrhization. Alternatively, *Gigaspora* is known to require large quantities of photosynthates from the plants during its colonization establishment, sometimes leading to plant growth decrease (Figure 3B) (Smith and Smith, 1996). This high carbon requirement by *Gigaspora* might compete with nodulation under low availability of sugars in roots (O'Hara, 2001). The later may also explain the very low detection of colonization by *Acaulospora*, unless we failed to visualize the root colonization structures that are known to be difficult to stain (Boddington and Dodd, 2000). Both symbioses depend on signal exchange with

plants and share some part of the signalling pathway (Antunes et al., 2006).

Previous studies with common bean found that nitrogen fixation was significantly limited by P deficiency, and plants deficient in P show decreased nodule-number and biomass when grown in soil, sand and alkaline solution, or hydroaeroponics (Bargaz et al., 2011a, b; Mandri et al., 2011; Vadez and Drevon, 2001). This was not the case of our study (Figures 2 and 5) and this could be attributed to the effect of inoculation with both rhizobia and AMF, especially with *Glomus*, whatever the treatments (by mycorrhizal inoculant or by contact with mycorrhized *Stylosanthes*). In addition, AMF improve plant growth parameters and nutrient uptake. It has been demonstrated for increased N_2 fixation in mycorrhizal plants that when both nitrogen and phosphorus are limiting, AMF can improve phosphorus uptake by the plant which in turn would result in more energy available for nitrogen fixation by rhizobia (Fitter and Garbaye, 1994).

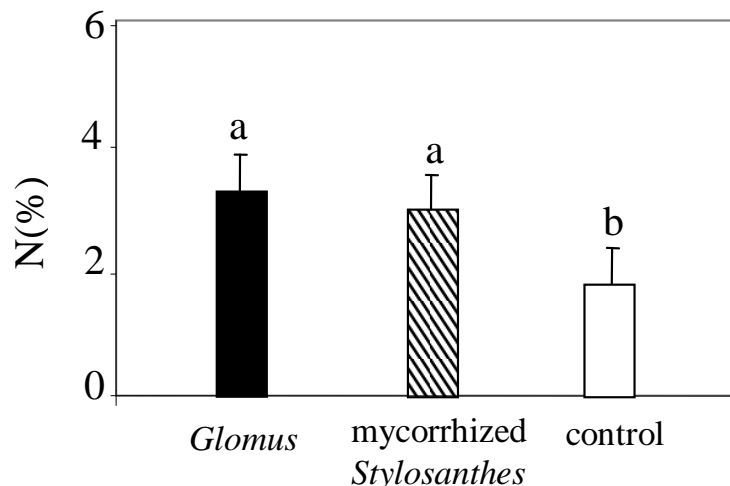


Figure 5. Effect of *Glomus* on shoot nitrogen percentage of common bean genotype RIL147 grown in sand culture, after inoculation with *R. tropici* CIAT899, and with *Glomus* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* (hatched bars) or not as control (open bars). Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($P \leq 0.05$).

In the same way and in order to ground one's argument for our hypothesis, Karandashov and Bucher (2005) found that the enhanced N_2 -fixing ability in mycorrhizal plants compared with non-mycorrhizal plants, usually disappears if the non-mycorrhizal plants are supplied with a readily available P source. The obtained results indicated that AMF treatments, especially with *Glomus*, significantly increased nitrogen accumulation in the shoot and increased the phosphorus content and phosphorus use efficiency in plants, compared with their controls which were not dually inoculated. These findings are in agreement with that of Aysan and Demir (2009), Askar and Rashad (2010) and Xiurong et al. (2011) with other AMF treatments, it is well known that AMF can improve the nutrient status of their host plants (Smith and Read, 2008; Tajini et al., 2009; Kim et al., 2010). It is also thought that the plant-rhizobium system benefits from the presence of AM fungi because the mycorrhizae ameliorate not only P deficiency but also any other nutrient deficiencies that might be limiting to rhizobium (Smith, 2002).

Similarly, Nautiyal et al. (2010) found that dual inoculation of *Cicer arietinum* L. with rhizobia and AMF significantly enhanced the number of nodules and the dry weight per plant. Kim et al. (2010) showed that the combined inoculation of *Methylobacterium oryzae* strains and AMF in soil culture increased plant growth, resulted in significantly higher nitrogen accumulation in roots as well as shoots, and increased the phosphorus content of red pepper plants compared with uninoculated controls. However, under drought stress inoculation with AMF and

rhizobial strains inhibited of nodule development and N_2 fixation, and caused a decrease in plant growth (Franzini et al., 2010, Ballesteros-Almanza et al., 2010). On the other hand, Lisette et al. (2003) reported that co-inoculation with rhizobia and compatible AMF could dramatically enhance pea growth, N and P uptake. Therefore, the AMF (*Glomus*) we used for the present study are compatible with the rhizobial strain and common bean genotype, which might have potential for agricultural application.

Conclusion

AM fungal species used in this study have different level of root colonization and nodulation, contribution to plant growth and nutrient uptake of common bean plants as compared to the control plants. The results indicate that inoculation with mycorrhizal species, especially with *Glomus*, had positive effects on the growth of common bean RIL147. Mycorrhizae inoculated plant had a higher yield (SDW and RDW) and increased P plant concentration and P utilization for symbiotic nitrogen fixation. Suitable combinations of AMF (by contact or by inoculation) and rhizobia and the compatibility between both symbionts, may decrease sensitivity of symbiotic nitrogen fixation to phosphorus deficiency. For the successful application of these results in biotechnology, multi- locational field trials are needed to determine the most efficient tri-partite symbioses. These results show an interesting practical application for agricultural

development in marginal lands that are often deficient in P. The present study opens possibilities for *in-situ* non-destructive studies of energy balance in terms of carbon and oxygen requirements for symbiotic respiration, metabolic monitoring (NMR), and molecular analyses with *in-situ* hybridization and real time polymerase chain reaction (RT-PCR) with particularly clean material.

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