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Full Length Research Paper

Assessing hydroaeroponic culture for the tripartite symbiosis of mungbean (*Vigna radiata* L.) with arbuscular mycorrhizal fungi and rhizobia

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Mungbean (*Vigna radiata* L.) has the potential to establish symbiosis with rhizobia that fix atmospheric dinitrogen (N_2), and arbuscular mycorrhizal fungi (AMF) that improve the uptake of low mobile soil nutrients such as phosphorus. Both rhizobial and mycorrhizal symbioses can benefit plants synergistically. The tripartite symbiosis of mungbean with rhizobia and AMF was assessed in hydroaeroponic culture under sufficient *versus* deficient P supplies (250 *versus* 75 µmol P plant^{-1} week^{-1}) by comparing the effects of three AMF species on the mycorrhizal root colonization, rhizobial nodulation, and plant growth. Although, *Glomus intraradices* colonized well the roots of mungbean in sand and hydroaeroponic cultures, *Gigaspora rosea* only established well under sand culture conditions, and *Acaulospora mellea* weakly colonized roots under both culture conditions. Though significant differences of mungbean growth were found with different AMF species in sand, only few differences were observed in hydroaeroponic cultures. It is argued that the later will probably be a valuable tool for scrutinizing the interactions among the three symbionts, as well as plant physiology, and nutrient partitioning within the symbiotic system.

Key words: *Acaulospora mellea*, arbuscular mycorrhizal, *Bradyrhizobium*, *Gigaspora rosea*, *Glomus intraradices*, mungbean, phosphorus, symbiotic nitrogen fixation.

INTRODUCTION

Mungbean (*Vigna radiata* L.) is a crop widely grown in the tropics as a green manure or grain legume, usually in rotation with cereals. Like other species of the *Phaseolea* tribe, mungbean can establish symbiosis with rhizobia fixing atmospheric dinitrogen (N_2) thus, supplying the plant with nitrogen. Inoculation of mungbean with specific rhizobia has been reported to increase yield (Singh and Choubey, 1971) or subsequent crop in case the mungbean is used as green manure (Sharma et al., 1995), with efficiency of N_2 fixation depending upon the plant cultivar, rhizobial strains, and environmental conditions (Giller, 2001).

Mungbean can also establish symbiosis with arbuscular mycorrhizal fungi (AMF) (Clarkson, 1985; Singh and Kapoor, 1998) by improving plant uptake for soil nutrients with low mobility such as phosphorus (Jakobsen et al., 2002). Both rhizobial and mycorrhizal symbioses share some signalling pathways, indicating intimate interactions between all three partners during co-evolution (Guinel and Geil, 2002; Harrison, 1998). They can act synergistically on promoting plant growth and fitness (Jia et al., 2004; Requena et al., 1997), although, the effects of tripartite symbiosis on legume growth and yield were shown to range from slightly negative to strongly positive as compared to asymbiotic plants or plants harbouring only a single symbiont such as *Cicer arietinum* L.

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Abbreviations: AMF, Arbuscular mycorrhizal fungi.

(Nautiyal et al., 2010).

More P is required by nodulated than non-nodulated legumes, with up to 20% of total plant P being allocated to legume nodules (Ribet and Drevon, 1995). However, P requirements for growth and N_2 fixation differ widely among legume species (Sanginga et al., 1996; Araújo et al., 1997), and among genotypes within a species such as common bean (Vadez and Drevon, 2001). Carbon is also required by both symbioses and need to be partitioned for achieving optimal symbiotic outcome. However, the regulation of this partitioning is hindered by relatively complicated nature of the system. Aysan and Demir (2009) reported that the information on the mechanisms controlling interactions of bacteria with AM fungi and plant roots in the mycorrhizospher 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.

Most of the fungi also require soil-based cultivation system which is particularly unsuitable to study signalling and nutrient exchanges by hi-tech analytic equipment and by noninvasive tools such as Nuclear Magnetic Resonance. Soil-less cultivation system such as hydroponics or aeroponics would be much more suitable for fast progress, but many attempts have not been successful so far. Hydroaeroponic cultures were proved to be useful for studying rhizobial symbiosis of legumes, establishing for example the relation of P requirement with the uptake of O_2 for nitrogenase activity (Vadez et al., 1996; Drevon and Hartwig, 1997; Jebara et al., 2004). Therefore, the purpose of this study was to assess the suitability of hydroaeroponic culture to establish a tripartite symbiosis between different AMF species, rhizobia and mungbean.

MATERIALS AND METHODS

The experiments concerned in this study are genotype of Mungbean, 3 AMF and 2 levels of P supply (75 and 250 µmol P plant^{-1} week^{-1}). Plants were inoculated with one of AMF and in all cases received similar rhizobial inoculation; plants were grown in hydroaeroponic after their transfer from sand culture. Thus, the experimental design consisted of randomized complete block with 3 replications.

Biological material

Mungbean (*V. radiata* L.) cv. CN72 was supplied by Chainat Field Crops Research Center (Amphoe Sunpaya, Chai Nat, Thailand). Seeds were surface-sterilized in 1.3% calcium hypochlorite for 15 min with constant stirring, then rinsed with sterile distilled water. They were germinated on 0.8% sterile water agar plates for 3 days at 28°C in the dark, where germination rate of 80% was achieved. Rhizobial inoculation was performed by soaking the mungbean seedlings for 15 min in a freshly prepared suspension of *Bradyrhizobium* sp. *vigna* CB756 (10^8 bacterial cells ml^{-1}). Following the inoculation with rhizobia, the seedlings were transferred into 1000 ml pots filled with autoclaved quartz sand-soil (Orthic Luvisol) mixture (9:1 v:v) recolonized with soil bacteria according to Jansa et al. (2002). This mixture, referred as our substrate, was inoculated with AM inoculum containing either *G. rosea* BEG9 (Nicolson & Schenck), *G. intraradices* BEG157 (Schenck & Smith), or *A. mellea* NM54 (Spain and Schenck) isolated from Kenyan Ferralsol (Mathimaran et al., 2007). The inoculum consisted of potting substrate of previous pot cultures planted with leek (*Allium porrum*), chopped leek roots, and AMF spores. Fifty grams of AMF inoculum was thoroughly mixed into each pot that received approximately 1000 spores of the respective AMF species. Three mungbean seedlings were planted per pot and three pots per AMF species were established.

Growth conditions

After transfer of inoculated seedlings into each pot of sand-soil culture, the plants were grown in a temperature controlled glass-house with night/day temperatures of 25/35°C, and a 16 h photoperiod with complementary illumination of 400 mmol photons m^{-2} s^{-1}. After 2 weeks, one plant only was left in the soil-sand substrate. Pots were watered with distilled water every 2 days until harvest, and received once a week the Vadez et al. (1996) nutrient solution: macroelements: K_2SO_4 (1.25 mM), $MgSO_4.7H_2O$ (2.05 mM), $CaCl_2$ (3.3 mM); microelements: Fe EDDHA (8.5 mM Fe as sequestrene), H_3BO_3 (4.0 mM), $MnSO_4$ (6.0 mM), $ZnSO_4$ (0.9 mM), $CuSO_4$ (1.0 mM), $NaMoO_4$ (0.1 mM).

After 2 weeks, seedlings from sand culture were transferred into 45 l plastic vats by gently passing them through the hole of a rubber stopper with cotton wool fixed at the hypocotyl level. Each vat contains 20 plants and 20 l of the above nutrient solution, and complemented to 45 1 with sterile distilled water. Plants were grown in vats and received once a week either 75 or 250 µmol KH_2PO_4 plant^{-1} week^{-1} in addition to the earlier mentioned nutrient solution. This solution was replaced every two weeks. It was supplemented with 2 mmol urea plant^{-1} during first two weeks, 1 mmol urea plant^{-1} during the next two weeks and no more urea during the last two weeks. The nutrient solution was constantly aerated at a flow of 400 ml plant^{-1} min^{-1}. The pH was buffered close to 7 with $CaCO_3$ (1 g l^{-1}).

Assessment of AMF colonization

At harvest, half of the root systems were used for estimation of AMF root colonization as follows : roots were first cleared in 10% KOH (w:v) at 80°C for 30 min followed by rinsing with water and two rinses with 1% HCl for one hour. Then, roots were immersed in a staining solution of lactic acid: glycerol: water (1:1:1, v:v.v) and 0.1% of each trypan and methylene blue. Then root samples were incubated at 80°C for 90 min, followed by several rinses with water, and finally destained in tap water for 30 min at room temperature. The roots were examined under a compound microscope and AMF colonization was assessed by the magnified intersection method according to McGonigle et al. (1990). The rates of root colonization by hyphae, arbuscules and vesicles were recorded.

Biomass and percentages of P at harvest

The plants growing in both pots and hydroaeroponic containers were harvested at 50 days after sowing (DAS), corresponding to flowering stage, and the shoots, roots, and nodules were separated and dried at 70°C for 2 days, and dry mass of each fraction was calculated.

Concentrations of P were measured from ground tissue samples following wet digestion with nitric-perchloric acids (6:1, v:v) at 250°C for 6 h, using the phosphovanado-molybdate method

Table 1. F-ratios from two-way ANOVAs are shown with accompanying measures of statistical significance. Influence of cultivation system and AMF species identity on percentage of mungbean roots colonized by hyphae (H%), arbuscules (A%) and vesicles (V%).

Colonization parameter (%)	Cultivation system (C)	AMF species (S)	C×S
H	17.0 ***	20.9 ***	5.41 **
A	11.44 ***	29.4 ***	3.86 *
V	5.05 *	22.75 ***	5.05 **

Ns: p≥0.05; * 0.01≤p<0.05; ** 0.001≤p<0.01; *** p<0.001.

Table 2. F-ratios from one-way ANOVAs with accompanying measure of statistical significance and treatment means are shown. Different letters indicate significant differences between treatment means in one row. Influence of AMF species identity used for inoculation on percentage of mungbean roots colonized by AMF hyphae (H%), arbuscules (A%) and vesicles (V%) in the different cultivation systems. Data are means of 3 replicate plants harvested at 50 days after sowing.

Cultivation system	Colonization parameter (%)	F-ratio	*Glomus*	*Gigaspora*	*Acaulospora*
Hydroaeroponic 250 µmol KH_2PO_4	H	1.13 ns	16.0 a	8.0 a	1.3 a
	A	3.88 ns	3.3 a	0 a	0 a
	V	3.7 ns	2.0 a	0 a	0 a
Hydroaeroponic 75 µmol KH_2PO_4	H	19.2 **	47.3 a	4.7 b	2.0 b
	A	9.91 *	12.0 a	1.3 b	0 b
	V	1.0 ns	3.3 a	0 a	0 a
Sand	H	19.3 **	64.7 a	71.3 a	4.0 b
	A	30.9 ***	13.3 a	10.0 a	0 b
	V	71.7 ***	16.0 a	0 b	0 b

Ns: p≥0.05; * 0.01≤p<0.05; ** 0.001≤p<0.01; *** p<0.001.

(Hanson, 1950). Only shoot and root samples of plants inoculated either with *Glomus* or *Gigaspora* from the sand and hydroaeroponic culture were analyzed.

Calculations and statistical analyses

The dry mass and root to shoot ratio from each plant were determined. Also, the ratio of nodule to shoot biomass was calculated. Colonization parameters (percentages of root length colonized by AMF hyphae, arbuscules and vesicles) were recorded and the P use efficiency was calculated as the ratio of plant dry mass (g) and plant P content.

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 test ($p \leq 0.05$).

RESULTS

Root colonization by AM fungi

All parameters of root colonization by AMF were affected by both cultivation system and AMF species, and their interactions (Table 1). The AMF effect was predominant in plants grown in sand culture than in hydroaeroponics (Table 2). Vesicles were only encountered in roots of plants inoculated with *Glomus* either in hydroaeroponics or sand culture (Table 2). The arbuscular level in roots was the highest in plants grown in sand, followed by plants in hydroaeroponic culture under P deficiency (75P). The plants under P sufficiency (250P) had the lowest arbuscular root colonization. As interactions were significant between the two main factors (Table 1), one-way ANOVAs were carried to scrutinize the effect of AMF species on root colonization parameters in both cultivation systems (Table 2). In hydroaeroponic culture, under P deficiency, higher hyphal and arbuscular levels were found in plants inoculated with *Glomus* than *Gigaspora*. Overall, root colonization was limited under P sufficiency for all AMF species and higher in sand than in hydroaeroponics. It remained very low with *Acaulospora* (Table 2).

Nodulation

The number of root nodules (Figure 1(A)) was significantly affected by both the cultivation system (p =0.018) and AMF species (p<0.001), without any interaction (p = 0.097). Whatever the cultivation system, *Acaulospora*-inoculated plants did not establish any nodule, whereas

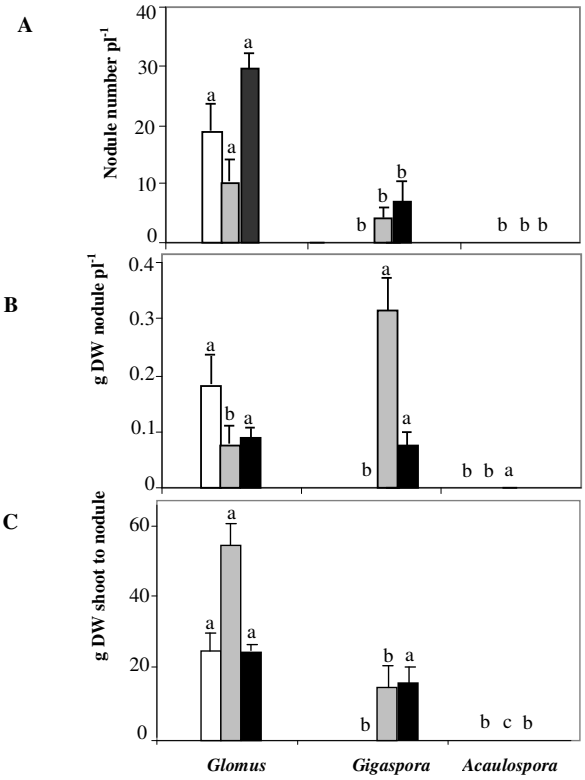

Figure 1. Effect of *Glomus, Gigaspora* and *Acaulospora* on number (A) and dry mass (B) of nodules and shoots produced per unit dry mass of nodules (C) of mungbean grown in hydroaeroponic culture under P sufficiency (open bars) *versus* P deficiency (grey bars) or in sand culture (black bars), Data are means ± SD (n = 3). For each cultivation system and for each treatment of P, different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0.05$).

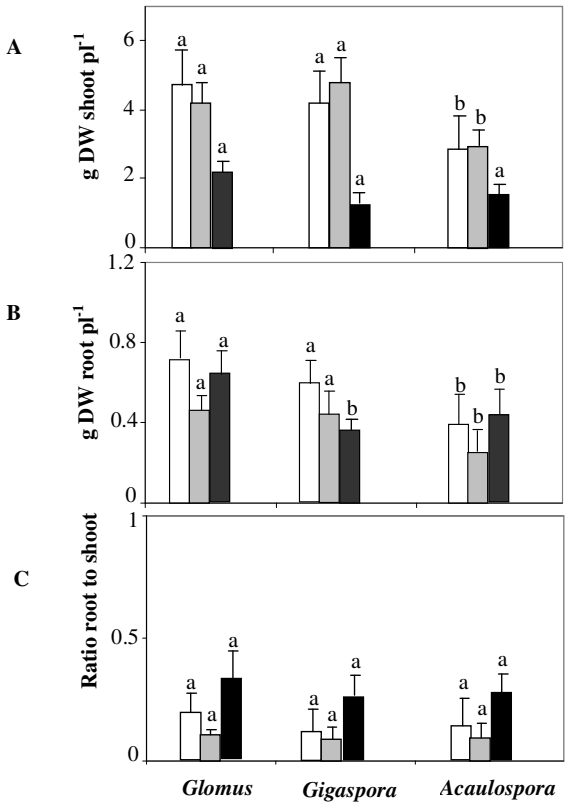

Figure 2. Effect of *Glomus, Gigaspora* and *Acaulospora* on dry mass of shoot (A) and root (B) and ratio: root to shoots (C) of mungbean grown in hydroaeroponic culture under P sufficiency (open bars) *versus* P deficiency (grey bars) or in sand culture (black bars), Data are means ± SD (n = 3). For each cultivation system and for each treatment of P, different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0.05$).

more nodules were present on plants inoculated with *Glomus* (29.6±4.06 nodule plant^{-1}) than with *Gigaspora* (7±1.32) in sand culture (Figure 1(A)). Thus, in hydroaeroponic culture with sufficient P, nodulation was found only with *Glomus*, and under P deficiency, higher nodule number was found with *Glomus* than *Gigaspora*.

Nodule biomass (Figure 1(B)) was affected by the cultivation system ($p<0.005$) and AMF species ($p = 0.03$), without any significant interaction ($p = 0.56$). In hydroaeroponic culture under P deficiency, higher dry mass of nodules was obtained with *Gigaspora* (0.316 ± 0.054 g plant^{-1}) than *Glomus* (0.076±0.038). But in sand culture, no significant difference was found between AMF species on nodule biomass (Figure 1(B)). Dry mass of shoots produced per unit dry mass of nodules (Figure 1(C)) varied significantly among the AMF species ($p<0.001$) and cultivation systems ($p = 0.04$), and their value under P deficiency, though values varied between interaction ($p = 0.023$). Plants inoculated with *Glomus* produced 35.2±7.9 g shoot g^{-1} nodules with the highest 55.3±11.2, 25.3±3.0 and 25.1±7.12 at 75 P, 250 P and in sand culture, respectively (Figure 1C). This was significantly higher than with *Gigaspora* (10.5± 3.0), without any significant differences with hydroaeroponic culture at 75 P (15.2±5.7) and sand (16.4±6.9) (Figure 1C).

Plant biomass

Shoot dry mass of mungbean plants (Figure 2(A)) was significantly affected by the cultivation system ($p = 0.002$), AMF species ($p = 0.014$) and their interaction ($p = 0.023$). Higher shoot dry mass was observed in hydroaeroponic than sand culture (Figure 2(A)). Under P sufficiency, shoot dry mass was higher with *Glomus* and *Gigaspora* (4.67 ± 0.87 and 4.53 ± 0.38g DM plant^{-1}, respectively) than *Acaulospora* (2.91±0.54).

Also, under P deficiency, shoot dry mass was higher

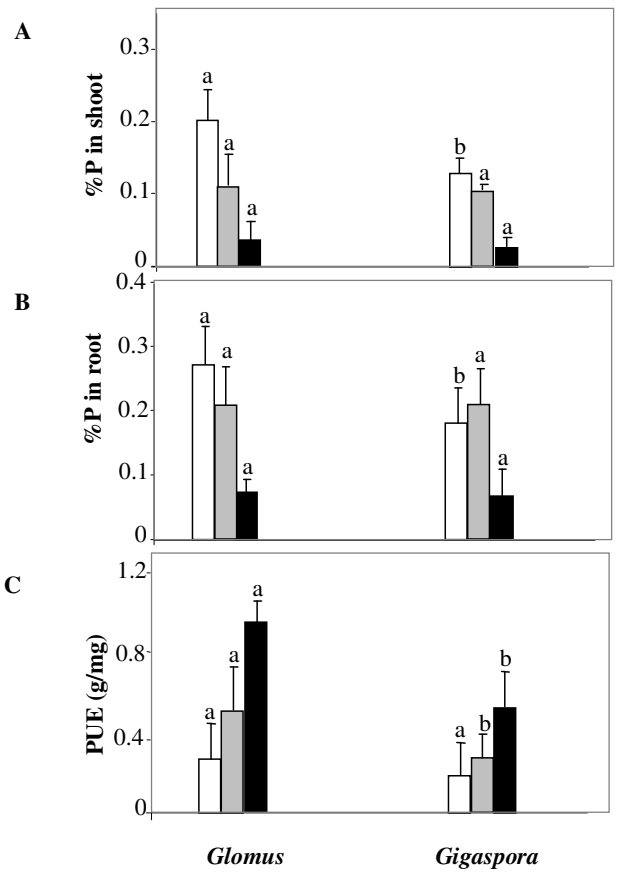

Figure 3. Effect of *Glomus* and *Gigaspora* on shoot (A) and root (B) phosphorus content (%) and phosphorus use efficiency (C) of mungbean grown in hydroaeroponic culture under P sufficiency (open bars) *versus* P deficiency (grey bars) or in sand culture (black bars), Data are means ± SD (n=3). For each cultivation system and for each treatment of P, different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0.05$).

with *Glomus* and *Gigaspora* (4.20±0.23 and 4.79±0.51 g DM plant^{-1}, respectively) than *Acaulospora* (2.94±0.13) (Figure 2A). No significant difference between AMF treatments was observed in sand culture where plants had lower dry mass with the three AMF species (Figure 2(A)).

Root dry mass was significantly affected by the cultivation system (p = 0.03), AMF species (p = 0.004) and their interaction (p = 0.032). Root dry mass with *Acaulospora* was lower than the other AMF species (Figure 2(B)) in both cultivation systems. In hydro-aeroponic culture, P deficiency induced a significant decrease on root dry mass in comparison to P sufficiency with all AMF treatments. But, in sand culture, root dry mass was higher with *Glomus* and *Acaulospora* than in hydro-aeroponic culture under P deficiency (Figure 2(B)). Biomass partitioning between roots and shoots was significantly affected by the cultivation system (p<0.001), but not by AMF species (p = 0.76), nor their interaction (p = 0.53). Whatever the AMF species, the root to shoot ratio was lower in hydroaeroponic culture (0.16±0.04 and 0.11±0.05) under P sufficiency and P deficiency, than in sand (0.31±0.05) (Figure 2(C)). Regardless of the cultivation system, the inoculation with *Glomus* resulted in higher plant dry mass (4.75±0.47 g plant^{-1}) than the other two AMF species.

Phosphorus content and P use efficiency (PUE)

Phosphorus content in shoots and roots (Figure 3 (A and B) was affected by the cultivation system (p<0.001), and AMF species (p = 0.042 and p = 0.05, respectively), without any significant interactions (p = 0.23 and p = 0.64, respectively). P content in shoot and root was nearly four-fold higher and three-fold higher in hydro-aeroponic under P sufficiency than in sand with *Glomus* and *Gigaspora*, respectively. But under P deficiency, P content in the whole plant was nearly three-fold higher than in sand with both AMF species. Under P sufficiency, P plant content was higher with *Glomus* than *Gigaspora*, without any significant interactions (Figure 3 (A and B)).

Similarly, the P use efficiency (Figure 3(C)) was affected by the cultivation system (p<0.001), and the interaction with AMF (p = 0.034). With *Glomus*, the P use efficiency was more than three-fold lower in hydro-aeroponic under P sufficiency (0.32±0.02 g DM mg^{-1} P) and almost two-fold lower under P deficiency (0.57±0.023 g DM mg^{-1} P) than in sand (0.97±0.07 g DM mg^{-1} P). But for *Gigaspora*, the P use efficiency was almost three-fold lower in hydroaeroponic under P sufficiency (0.21±0.02 g DW mg^{-1} P) than in sand (0.59±0.028 g DM mg^{-1} P) (Figure 3(C)). P use efficiency was higher with *Glomus* than *Gigaspora* under P deficiency (0.57±0.023 and 0.32±0.02 g DM mg^{-1} P, respectively) and in sand culture (0.97±0.07 and 0.59±0.028 g DM mg^{-1} P, respectively), but no significant difference under P sufficiency between AMF treatments was detected.

DISCUSSION

In this study, we established a tripartite symbiosis of mungbean with rhizobia and AMF under hydroaeroponic conditions that we previously developed from a bipartite rhizobial symbiosis with *Vigna* spp. (Drevon et al., 1987), soybean (Drevon et al., 1988) or common bean (Hernandez and Drevon, 1991). Among other systems without solid support, aeroponic cultivation was suitable only for very few symbiotic microorganisms such as *Glomus intraradices*, using precultivation in sand or perlite like in our study (Dugassa et al., 1995; Gryndler et al., 1992; Jarstfer and Sylvia, 1995; Vantilburg and Cook, 1995; Hawkins and George, 1997; Weber et al., 2005). The lower colonization of *V. radiata* by *G. intraradices* in hydroaeroponics than in sand was found

to agree with that of Dugassa et al. (1995) who showed similar results with *Linum*. Also, this result agrees with the previous observation of slower colonization of *Phaseolus vulgaris* by *G. intraradices* in same cultivation systems (Tajini et al., 2009). This lower colonization of *V. radiata* by *G. intraradices* in our hydroaeroponic cultivation could be explained by higher P supply than in sand. Thus, P is known to inhibit AMF colonization, either directly by limitation of fungal growth or indirectly by better plant P status (Grant et al., 2005). Our results are supported by higher limitation in colonization under P suficiency than P deficiency in hydroaeroponic, and by higher mungbean growth in hydroaeroponics with proportionally less biomass allocated to roots than in sand where less P was supplied. Moreover the P supply in hydroaeroponics was regular throughout the entire cultivation period, preventing any mycorrhizal involvement in the delivery of P from remote zones beyond the root P depletion zone like shown in a solid substrate (Smith et al., 2001) in particular for legumes that have coarse root-system and limited extension of root hairs (Abbott et al., 1995; Isobe and Tsuboki, 1998). However, the interaction with P supply may not explain differences in colonization among mycorrhizal species in hydroaeroponic under P sufficiency. The stability that *Gigaspora* requires for successful initiation and development of root colonization (Antunes et al., 2006a; Klironomos and Hart, 2002) may be altered by solution movement in hydroaeroponics. Alternatively, *Gigaspora* is known to require large quantities of photosynthates from the plants during its colonization establishment, sometimes leading to plant growth decrease (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 dilution of signals involved in communication between the plant and the AMF could be another cause of lower mycorrhization in hydroaeroponic culture, and explain the differences between AMF species. The later may also explain the very low detection of colonization by *Acaulospora* in hydroaeroponics, 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., 2006b).

Different compatibilities between mycorrhizal and rhizobial species may explain the differences in nodulation of mungbean upon inoculation with different AMF species. Although, low levels of nodulation obser-ved in our study suggest suboptimal matching of *V. radiata* and *Bradyrhizobium* CB756 in sand, and low aptitude of mungbean roots to be infected by rhizobia in hydroaeroponic under both P treatments (75P and 250P).

They result in differences in mungbean growth in hydroaeroponics. They may explain the differences in P use efficiency for *Glomus*- compared to *Gigaspora* -inoculated plants. In sand, the higher nodulation with *Glomus* than with the other AMF species is most likely to improved P nutrition of the plants since *G. 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, even in hydroaeroponic where the amount of P is fully available but limiting. 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.

Conclusion

Successful establishment of mycorrhizal symbiosis in hydroaeroponic culture reported in this study opens possibilities for production of particularly clean material and *in situ* non-destructive studies of (i) signaling between plants and symbiotic fungi, and the relation with rhizobial signaling for legumes, (ii) energetic balance in terms of carbon and oxygen (respiration) requirements, (iii) metabolic monitoring (NMR), and (iv) molecular analyses (*in situ* hybridization and PCR). Nevertheless more work is needed to establish the optimal P supply for mungbean mycorrhization in hydroaeroponics.

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