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Interactions between *Glomus* species and *Rhizobium* strains affect the nutritional physiology of drought-stressed legume hosts

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ABSTRACT

The growth of legume plants is usually enhanced by the dual symbiosis of arbuscular mycorrhizal (AM) fungi and *Rhizobium* bacteria. However, most reports on this topic have been carried out under optimal water regime conditions. Here, four *Phaseolus vulgaris* varieties were single or dual inoculated with two different AM fungus and/or two different *Rhizobium* strains. All plants were grown under moderate drought conditions. Surprisingly, most of the biological treatments involving one fungus and one *Rhizobium* together caused a deleterious effect on plant growth. However, these negative effects were dependent on the *P. vulgaris* variety used as well as on the symbionts implicated. The results showed that AM symbiosis inhibited nodule development and N_2 fixation, causing diminution of plant growth. Therefore, under moderate drought conditions, the dual symbiosis formed by AM fungi and *Rhizobium* can be deleterious to *P. vulgaris* growth depending on the plant variety and the symbionts involved. Thus, under these common stress conditions, selection for the appropriated symbionts to each *P. vulgaris* variety is needed.

Introduction

In nature, many plants establish a symbiosis with soil microorganisms, including arbuscular mycorrhizal (AM) fungi or N_2-fixing bacteria like *Rhizobium* in legume (*Leguminosae*) plants. Both symbionts are known to improve plant growth under several environmental conditions (Azcón et al., 1988). In fact, it is common that *Rhizobium* and AM fungi in dual symbiosis enhance growth and yield of many legumes (Barea et al., 1992; Zahran, 1999). In general, legume plants are highly dependent on mycorrhiza to achieve their maximum growth. Mycorrhizal hyphae have access to a greater volume of soil and can absorb and transport fairly large amounts of low diffusing nutrients, such as P, to their host plants. The P requirements for nodule formation and the effectiveness of this element in the symbiotic system are well known (Barea et al., 1997; Zahran, 1999).

The plant growth response to dual symbiosis is influenced by factors such as microbial strains or host plant varieties, as well as by the compatibility of interactions among them (Azcón et al., 1991). Despite the lack of specificity in the AM symbiosis, AM fungi differ in their ability to enhance nutrient uptake by the host plant, even when the extent of AM-colonization is similar (Monzón and Azcón, 1996). Combinations of *Rhizobium* with different AM fungal strains or species are important since the compatibility of such interactions may be relevant to N_2 fixation and to nutrient and water uptake by the legume plants (Marulanda et al., 2006). In a previous study using chickpea plants, the symbiotic efficiency was found to be dependent on the particular combination of the Rhizobium strain and *Glomus* species, indicating selective and specific compatibilities between the bacterial strain and fungal isolate (Ruíz-Lozano and Azcón, 1993). Thus, legume growth responses to symbionts are influenced by microorganism strains and the compatibility of the interactions between them and the host plant (Azcón and Ocampo, 1981; Azcón et al., 1991; Redecker et al., 1997). At the same time, it is known that the interaction between the two symbionts also depends on the stage of development of both microorganisms inside the host roots (Mortimer et al., 2008).

Common bean (*Phaseolus vulgaris*) has become a valuable food resource worldwide, especially in tropical and subtropical regions (Fageria and Santos, 2008). Subtropical regions are characterized by marked drought seasons each year (Abrams and Hock, 2006) that limit bean growth and yield (Cuellar-Ortiz et al., 2008). This could be alleviated by AM and/or *Rhizobium* symbioses (Zahran, 1999; Aroca et al., 2007). Water limitation in the growing medium reduces diffusion, uptake by roots and transport of nutrients from roots to shoots due to restricted transpiration rate, impaired active transport, and altered membrane permeability (Sardans et al., 2008). It has been found that dual symbiosis of *Rhizobium*–AM fungi enhances drought tolerance of the legume host plant in

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terms of the total plant growth (Zahran, 1999; Valdenegro et al., 2001). To optimize bean growth under drought conditions, a selection of these symbionts is required, although their effectiveness may be greatly influenced by the bean variety involved (Daniels-Hylton and Ahmad, 1994; Redecker et al., 1997). How these different interactions could work under drought conditions is a question that has not yet been addressed.

In the present study, we compared the effect of two *Rhizobium leguminosarum* strains in interaction with two AM fungi (*Glomus mosseae* or *Glomus intraradices*) on the growth, nutrition, water relation parameters, and symbiotic developments in four different *P. vulgaris* varieties under moderate drought conditions. The aim of this study was to evaluate the response linked to the host variety and the effects associated with changes in AM-colonization and/or nodulation as a function of the symbiont involved.

Materials and methods

Biological material and experimental design

Seeds of *Phaseolus vulgaris* L. cvs [Efequince (L-1), Romano Bush (L-2), Contender (L-3) and Borlotto (L-4)] were washed for 3 min in pure ethanol and rinsed three times with distilled water. The seeds were then sown in wet sepiolite (a clay mineral) and after 7 d, seedlings were transferred to 1000 mL pots. Pots were filled with a sterilized mixture of soil/sand (1:1, v/v).

Treatments used were non-AM inoculated or inoculated with *G. mosseae* (M) or *G. intraradices* (I) and each AM fungus was assayed with Rh I (*Rhizobium* tropici 899) or Rh II (*Rhizobium* 912) *Rhizobium* strains. Non-inoculated controls or single *Rhizobium* (I or II) inoculated plants were also assayed in the four bean varieties, yielding 28 treatments with five replicates each.

The soil was collected from the field area in the Estación Experimental del Zaidín (Granada), sieved (2 mm) and autoclaved (100 °C for 1 h, three consecutive days). For details of soil characteristics, see Azcón et al. (1991).

Mycorrhizal inoculum from each fungus was multiplied in an open pot culture of maize and consisted of soil, spores, hyphae and AM root fragments. The AM fungal species used were *G. mosseae* (Nicol. And Gerd) (strain 121) and *G. intraradices* (Schenck and Smith) (strain 119). Both were from the collection of Estación Experimental del Zaidín. Ten grams of each mycorrhizal inoculum, having similar characteristics (an average of 40 spores g^{-1} soil and root fragments with 85% of colonized roots length), were placed below the seedlings. The inoculum was obtained from our own stock culture collection and kept in polyethylene bags in storage at 5 °C for 6 months. Pots without AM inoculum received 10 g of autoclaved AM inoculum in order to avoid differences in soil nutrient content linked to AM inoculum additions.

Two strains of *Rhizobium leguminosarum* were used: Rh I (tropici 899) and Rh II (912). Both were grown in TY medium (5 g L^{-1} tryptone, 3 g L^{-1} yeast extract, 9 mM $CaCl_2$) and applied (1 mL containing 10^8 cells per pot) at transplanting time. Fifteen days after transplantation, pots inoculated with Rh II and transplanted with the three *Phaseolus* varieties (L-2, L-3 and L-4) received a solution of $(^{15}NH_4)_2SO_4$ with 10% N atom excess, which supplied 2 mg N Kg^{-1} soil, equivalent to 5 kg N ha^{-1}.

Plants were grown for 45 d under greenhouse conditions with temperature ranging from 19 to 25 °C, 16/8 light/dark photoperiod and a relative humidity of 50–70%. A photosynthetic photon flux density of 400–700 μmol m^{-1} s^{-1} was applied as supplementary light. All plants were grown under moderate drought conditions by keeping soil water capacity at 75%. Soil moisture was measured daily with an ML2 ThetaProbe (AT Delta-T Devices Ltd. Cambridge, UK), and the water needed to reach 75% soil water capacity was added (Marulanda et al., 2003).

Plant analyses

Once the plants were harvested, the dry weights of shoots, pods and roots were recorded after 2 d at 75 °C.

Leaf relative water content (RWC) was calculated as follows: (Fresh weight–Dry weight)/(Turgid weight–Dry weight) × 100 (Aroca et al., 2003). Stomatal conductance was measured in five plants from each treatment by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in one leaflet of every plant at midday on the day before harvest.

Nodules formed were visually determined. The percentage of mycorrhizal root length infected was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v) (Phillips and Hayman, 1970). Quantification was carried out using the grid-line intersect method (Giovannetti and Mosse, 1980).

After analyses of plant growth data, only shoot tissue of *P. vulgaris* vars. Romano Bush (L-2), Contender (L-3) and Borlotto (L-4) single inoculated with Rh II, and dually inoculated with Rh II plus AM fungi (I or M) or controls without any inoculation were analyzed for ^{15}N (Fiedler and Proksch, 1975) by mass spectrometry. It was assumed that, under equal exposure to ^{15}N-labeled fertilizer with the same ^{15}N enrichment, microbial treatments applied that improve N_2 fixation will reduce the percentage of ^{15}N excess in the plant tissue (Danso, 1988). In the same shoots, K was determined by flame photometry and P by the method described by Olsen and Dean (1965). Ca and N were determined by micro Kjeldahl assay, and Mg and S were also measured after wet digestion of the air-dried plant samples with HNO_3+H_2O_2 by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Takács et al., 2001).

Statistical analysis

Five replicates were made per treatment and data were subjected to analysis of variance. Differences between means were analyzed by Duncan's multiple range test ($P < 0.05$). For the percentage values, the data were arcsin square transformed before the statistical analysis was applied.

Results

Plant growth parameters

Total aerial dry weight of the four bean varieties was separated into shoot and pod dry weights (Table 1). Total aerial dry weight was increased by Rh I inoculation in all bean varieties except in L-3, where no effect was observed (Table 1). Rh II inoculation increased total aerial dry weight of L-2 and L-4 bean varieties, but caused no effect in L-1 and L-3 varieties (Table 1). Thus, the L-3 bean variety was not affected by any of the *Rhizobium* strains in terms of total aerial plant growth. The positive effects of Rh I on L-2 and L-4 aerial dry weight as well as that of Rh II on L-4 were due mainly to an increase in pod dry weight (Table 1). Therefore, pod dry weight was more greatly affected by *Rhizobium* inoculation than shoot dry weight.

Any of the sixteen possible combinations among the four bean varieties, the two *Rhizobium* strains and the two AM fungi increased total aerial plant growth with respect to single

Table 1
Shoot, pod and total aerial dry weights (g) of non-inoculated control (C) either single inoculated with one of two rhizobial strains (Rh I or Rh II) or in dual combination with *Glomus mosseae* (M) or *Glomus intraradices* (I) in bean vars. (L-1, L-2, L-3 and L-4).

Bean vars.	C	Rh I	Rh I+I	Rh I+M	Rh II	Rh II+I	Rh II+M
Shoot dry weight							
L-1	2.75b	2.77b	3.19c	2.15a	2.46ab	2.32a	2.23a
L-2	2.20a	2.40a	2.36a	2.51a	2.71b	2.46a	2.47a
L-3	1.81a	2.22b	2.26b	1.70a	2.35b	1.76a	2.24b
L-4	2.66b	2.97b	2.77b	2.68b	2.72b	1.79a	2.81b
Pod dry weight							
L-1	1.27ab	1.49b	1.70c	1.44b	1.40b	0.99a	1.20ab
L-2	1.12a	1.84bc	1.57b	1.93c	1.64b	1.59ab	1.22a
L-3	1.15b	0.99ab	1.08b	0.88a	0.97ab	0.85a	0.99ab
L-4	0.38a	0.82b	1.41d	1.09c	0.86b	1.05bc	0.97bc
Total (shoot+pod) dry weight biomass							
L-1	4.02b	4.26c	4.89c	3.59ab	3.86b	3.31a	3.43a
L-2	3.32a	4.24b	3.93ab	4.44b	4.35b	4.05b	3.69a
L-3	2.96b	3.21b	3.34b	2.58a	3.32b	2.62a	3.23b
L-4	3.04a	3.78b	4.18b	3.77b	3.59b	2.84a	3.77b

The values presented are averages (n=5), and different letters within each row indicate significant differences within the specific bean variety ($P \leq 0.05$) after ANOVA and Duncan tests.

Table 2
Root dry weight of non-inoculated control (C) either single inoculated with one of two rhizobial strains (Rh I or Rh II) or in dual combination with *Glomus mosseae* (M) or *Glomus intraradices* (I) and AM-colonization (percentage) in four bean vars. (L-1, L-2, L-3 and L-4).

Bean vars.	Root dry weight (g)						
	C	Rh I	Rh I+I	Rh I+M	Rh II	Rh II+I	Rh II+M
L-1	0.80a	1.02ab	1.01ab	0.841a	1.11b	0.94a	1.07ab
L-2	1.05a	1.07a	1.06a	1.17a	1.16a	1.04a	1.12a
L-3	0.80a	1.43bc	1.37bc	1.72c	1.50bc	1.15b	1.47bc
L-4	1.00a	1.04a	0.96a	1.04a	1.10a	0.93a	1.11a
	AM (%)						
	Rh I+I	Rh I+M	Rh II+I	Rh II+M			
L-1	11a	27c	17b	14ab			
L-2	18b	6a	23b	20b			
L-3	1a	16b	3a	27c			
L-4	3a	7b	6b	11c			

The values presented are averages (n=5), and different letters within each row indicate significant differences within the specific bean variety ($P \leq 0.05$) after ANOVA and Duncan tests.

inoculation with Rh I or Rh II strains. However, nine combinations had no effect at all and seven had a negative effect on total aerial plant dry weight when compared to single inoculation with either Rh I or Rh II strains (Table 1). These negative combinations were Rh I+M plus L-1 and L-2, Rh II+I plus L-1, L-3 and L-4, and Rh II+M plus L-1 and L-2 bean varieties (Table 1). Thus, 44% of the possible triple combinations had negative effects on plant growth and 56% had no effect in comparison with single Rh I and Rh II inoculations. These negative effects were caused primarily by reductions in the shoot dry weight and not by reductions in pod dry weight (Table 1).

Root dry weight was less susceptible to microorganism inoculation than aerial dry weight. Thus, Rh I single inoculation only increased root dry weight in the L-3 bean variety and Rh II single inoculation increased root dry weight both in the L-1 and in L-3 bean varieties (Table 2). Surprisingly, L-3 was the only bean variety that did not respond to single *Rhizobium* inoculation in terms of aerial dry weight (Table 1). Among the sixteen possible triple combinations of bean lines, *Rhizobium* strains and AM fungi, only one combination (Rh II+I plus L-1) had an effect, although negative, on root dry weight in comparison with single Rh II (Table 2).

Symbiosis development

Root AM fungus development was quantified by the grid-line intersect method after trypan blue staining (Giovannetti and Mosse, 1980). No AM infection was observed in non-inoculated or in single *Rhizobium* inoculated roots. In the L-1 line, the dual inoculation of Rh I+M presented the broadest AM root infection and the Rh I+I showed the lowest (Table 2). In the L-3 and L-4 lines, Rh I+I also showed the lowest AM root infection, and the Rh II+M treatment showed the broadest in both bean lines (Table 2). Finally, in the L-2 line, the broadest AM root infection was found in the Rh II+I treatment and the lowest in the Rh I+M treatment (Table 2). Therefore, it seems that *G. intraradices* in combination with Rh I had the lowest root infectivity. *G. mosseae* appeared to be more infective than *G. intraradices*.

Rhizobium development was estimated by counting total nodule number per root system and by measuring total nodule fresh weight per root system (Table 3). No nodules were found in non-inoculated roots. In the L-1 and L-2 bean lines, Rh I and Rh II had the same effectiveness in terms of nodule number and weight. In the L-3 line, both *Rhizobium* strains developed the same number of nodules per root system, but the nodules of Rh II were heavier. However, in the L-4 line, the Rh I strain was more effective in terms of nodule number and weight than the Rh II strain.

The infectivity of the Rh II strain was reduced drastically by co-inoculation with either AM fungus, independently of the bean line involved (Table 3). However, when the Rh I strain was involved, a negative effect of AM co-inoculation in nodule number was found only in the L-2 line independently of the AM fungus involved. In fact, when Rh I was co-inoculated with *G. intraradices*, an increase in the total nodule fresh weight per root system was found regardless of the bean variety (Table 3).

Water relations parameters

Leaf water status was estimated by measuring leaf relative water content (RWC). Single inoculation with Rh I or Rh II only altered RWC in the L-4 line, by decreasing it (Table 4). In bean variety L-4, treatments Rh I+I, Rh II+I and Rh II+M increased RWC values in comparison with single Rh I or Rh II inoculated plants

Table 3
Total nodule fresh weight and nodule number per plant of single inoculated with one of two rhizobial strains (Rh I or Rh II) or in dual combination with *Glomus mosseae* (M) or *Glomus intraradices* (I) in four bean vars. (L-1, L-2, L-3 and L-4).

Bean vars.	Rh I	Rh I+I	Rh I+M	Rh II	Rh II+I	Rh II+M
Total nodule fresh weight (mg)						
L-1	204c	538d	305c	292c	2a	85b
L-2	508c	479bc	586c	502c	315b	52a
L-3	240b	810d	640c	550c	10a	5a
L-4	466b	648c	893d	268b	11a	22a
Number of nodule formed per plant						
L-1	27.2c	36.0d	22.8c	45.8cd	0.2a	3.6b
L-2	65.2d	34.4c	32.2c	63.8d	14.4b	3.2a
L-3	43.0b	98.2d	80.4c	57.0b	21.8a	12.2a
L-4	58.4c	57.8c	57.8c	23.6b	2.6a	1.4a

The values presented are averages (n=5), and different letters within each row indicate significant differences within the specific bean variety ($P \leq 0.05$) after ANOVA and Duncan tests.

(Table 4). Negative effects of dual inoculation in RWC values were observed with Rh I+I treatment in the L-1 variety. No other changes in RWC by dual inoculation were observed (Table 4).

The L-1 and L-2 bean varieties did not change their leaf stomatal conductance as a result of the single inoculation with any of the *Rhizobium* strains (Table 4). However, Rh I inoculation increased stomatal conductance in L-3 and L-4, while Rh II inoculation decreased it in these bean varieties (Table 4). The L-1 and L-2 bean varieties did not change their stomatal conductance when they were co-inoculated with Rh I strain and either of the two AM fungi (Table 4). On the contrary, in L-3 and L-4, stomatal conductance decreased when Rh I was involved in the dual inoculation, except for the Rh I+M combination (Table 4). When Rh II was implicated in the dual inoculation, stomatal conductance increased in the L-2 bean variety when interacting with *G. intraradices*, and in the L-4 bean variety when interacting with *G. mosseae* (Table 4). No other changes in stomatal conductance with the dual inoculation were observed in comparison with single *Rhizobium* inoculation.

Shoot nutrient contents

Nutrient contents were analyzed just in shoots of control, Rh II, Rh II+I and Rh II+M treatments in the L-2, L-3 and L-4 bean varieties, in which three dual inoculations had a negative effect and the other three had no effect in terms of aerial growth.

In L-2 bean variety, Rh II single inoculation increased shoot C and N contents without altering any other nutrient analyzed (Tables 5 and 6). Rh II single inoculation increased only N shoot content in the L-4 variety, with the other nutrient contents unaltered (Tables 5 and 6). On the contrary, in the L-3 variety, Rh II single inoculation increased the content of all the nutrients tested except that of Ca (Tables 5 and 6).

Shoot N content decreased with dual inoculation in comparison with single Rh II inoculated plants in all bean varieties except in the L-2 bean variety when it was co-inoculated with *G. intraradices*. In that case, no significant changes were observed (Table 5). On the other hand, shoot C content decreased in the three lines analyzed in comparison to single inoculated Rh II plants when *G. intraradices* was co-inoculated. The results were unaltered when *G. mosseae* was the fungus involved (Table 5).

Shoot S content was unaltered in the L-3 bean variety by dual inoculation in comparison with single Rh II inoculated plants, regardless of the fungus used (Table 5). Rh II+I treatment caused a reduction in shoot S content only in the L-4 variety, while Rh II+M treatment caused an increase in shoot S content in the L-2 and L-4 varieties (Table 5). Shoot K and Mg contents responded similarly to dual inoculation (Table 6). The contents of both minerals decreased in Rh II+I treated L-3 and L-4 varieties and increased in Rh II+M treated L-2 bean varieties. No other changes in the contents of these two minerals were observed with dual inoculation in comparison with single Rh II inoculated plants. Shoot Ca content responded as K and Mg responded. Rh II+M treatment also increased shoot Ca content in the L-3 variety and decreased it in the L-4 variety (Table 6). Finally, shoot P content was increased in the L-2 variety by dual inoculation with any the fungus species and decreased in the L-3 variety when *G.*

Table 4
Leaf relative water content (%) and stomatal conductance of non-inoculated control (C) either single inoculated with one of two rhizobial strains (Rh I or Rh II) or in dual combination with *Glomus mosseae* (M) or *Glomus intraradices* (I) in four bean vars. (L-1, L-2, L-3 and L-4).

Bean vars.	C	Rh I	Rh I+I	Rh I+M	Rh II	Rh II+I	Rh II+M
Leaf relative water content							
L-1	90.1bc	90.3bc	81.0a	88.4b	91.9bc	93.8c	89.6b
L-2	91.8a	90.1a	89.8a	88.0a	87.8a	88.4a	91.9a
L-3	92.5b	92.5b	91.4a	93.2b	93.4bc	90.5a	95.7c
L-4	87.4b	82.9a	82.3a	96.5c	83.9a	88.5b	90.3b
Stomatal conductance (mmol H_2O m^{-2} s^{-1})							
L-1	56.3b	49.6ab	32.7a	47.6b	55.9b	55.3b	48.2b
L-2	26.4ab	22.5ab	29.9b	18.6a	17.3a	29.6b	23.1b
L-3	47.7c	65.4d	30.8b	16.4a	30.3ab	23.6ab	29.5ab
L-4	41.5b	69.2c	33.8a	74.7d	34.7a	35.9a	40.8b

The values presented are averages (n=5), and different letters within each row indicate significant differences within the specific bean variety ($P \leq 0.05$) after ANOVA and Duncan tests.

Table 6
P, K, Ca and Mg shoot content (mg) of non-inoculated control (C) either single inoculated with rhizobial strains Rh II or in dual combination with mycorrhizal fungi *Glomus mosseae* (M) or *Glomus intraradices* (I) in bean vars. (L-2, L-3 and L-4).

Bean vars.	P				K			
	C	Rh II	Rh II+I	Rh II+M	C	Rh II	Rh II+I	Rh II+M
L-2	4.19a	4.01a	5.01b	5.65c	42.4a	41.3a	38.5a	46.0b
L-3	4.46b	5.30c	3.85a	5.54c	38.1a	49.6b	39.9a	49.2b
L-4	4.61a	4.70a	4.65a	4.77a	62.3b	65.5b	51.0a	64.6b
	Ca				Mg			
L-2	46.6b	39.5ab	33.9a	51.1b	5.33a	5.86a	5.08a	7.19b
L-3	37.2b	37.3b	24.5a	46.0c	5.02b	6.50c	4.2a	6.47c
L-4	53.6c	55.0c	31.1a	45.5b	6.77b	6.76b	4.47a	7.30b

The values presented are averages (n=5), and different letters within each row indicate significant differences within the specific bean variety ($P \leq 0.05$) after ANOVA and Duncan tests.

Table 5
S, C, N and N^{15} content in shoot (mg) of non-inoculated control (C) either single inoculated with the rhizobial strains Rh II or in dual combination with mycorrhizal fungi *Glomus mosseae* (M) or *Glomus intraradices* (I) in bean vars. (L-2, L-3 and L-4).

Bean vars.	S				C			
	C	Rh II	Rh II+I	Rh II+M	C	Rh II	Rh II+I	Rh II+M
L-2	2.33a	2.29a	2.66b	3.08c	920a	1029b	970a	1002b
L-3	1.67a	2.65b	2.62b	2.54b	743a	967b	708a	929b
L-4	4.60bc	4.11b	3.37a	4.78c	1082b	1188b	698a	1126b
	N				N^{15}%			
L-2	24.9a	38.8b	34.3b	25.5a	0.539c	0.486a	0.501b	0.550c
L-3	27.2a	40.3c	28.1a	32.6b	0.453ab	0.437a	0.478b	0.450ab
L-4	27.3b	45.9c	22.0a	27.9b	0.531c	0.488a	0.553c	0.506b

The values presented are averages (n=5), and different letters within each row indicate significant differences within the specific bean variety ($P \leq 0.05$) after ANOVA and Duncan tests.

intraradices was co-inoculated in comparison with single Rh II inoculated plants (Table 6).

Estimation of N_2 fixation efficiency

Fixation efficiency of atmospheric N_2 was estimated by the ^{15}N dilution method (Fried and Broeshart, 1975), by which roots able to fix N_2 reduce the shoot ^{15}N percentage. Single Rh II inoculation reduced shoot ^{15}N percentage in bean varieties L-2 and L-4, but no changes were detected in the L-3 bean variety (Table 5). When *G. intraradices* was inoculated together with the Rh II *Rhizobium* strain, an increase in shoot ^{15}N percentage was recorded in all three bean varieties tested (Table 5). Co-inoculation of *G. mosseae* with the Rh II *Rhizobium* strain also increased shoot ^{15}N percentage in L-2 and L-4 and did not alter this parameter in bean variety L-3 (Table 5).

Discussion

There are several reports in which a positive effect of dual inoculation with different *Rhizobium* strains and AM fungi in legume growth, including *P. vulgaris*, has been found (Aryal et al., 2003; Mortimer et al., 2008). Here, we examined whether this positive effect also occurred under drought conditions. Surprisingly, nine out of sixteen combinations involving four different bean varieties, two different *Rhizobium* strains and two different AM fungi showed no effect on bean total aerial growth in comparison with plants single inoculated with only one *Rhizobium* strain. However, more surprising was that seven of these combinations decreased bean total aerial growth in comparison with single *Rhizobium* inoculated plants. We attempted to elucidate the causes of this negative effect through several analyses.

The two *Rhizobium* strains were effective in improving aerial plant growth of all the bean lines tested except for L-3, in which no effects were detected. A positive effect of Rh I inoculation on bean shoot dry weight under drought conditions was found previously (Figueiredo et al., 2008). To our knowledge, no data regarding the Rh II *Rhizobium* strain are available.

First, the percentage of root length colonized by both AM fungi ranged from 1% to 27%. Lambais et al. (2003) found an activation of several antioxidant enzyme activities in *P. vulgaris* roots even with low values of mycorrhizal colonization similar to those found here. Moreover, Fay et al. (1996) found a photosynthetic sink effect in barley plants, even with very low mycorrhizal infection. Thus, the low percentage of mycorrhizal infection found here could explain the negative effects caused by AM inoculation. However, no correlation between percentage of mycorrhizal root length and growth effect of the dual inoculation was found here. On the other hand, Sampedro et al. (2007) found a positive growth effect in bean plants when they were inoculated separately by the Rh I *Rhizobium* strain or by the *G. mosseae* fungus isolate used here under unstressed conditions. However, the same authors did not find any further increase in bean growth when plants were co-inoculated with both symbionts. Thus, we do not know exactly if the negative effects observed here are particular to the bean lines used here or if it could represent a broad effect. Unfortunately, Sampedro et al. (2007) did not specify which bean variety was employed. This question will be analyzed in future research.

Contrary to other reports (Aryal et al., 2006; Mortimer et al., 2008), a general negative effect of AM inoculation on nodule development was observed here. This general negative effect on nodule development matched up with less shoot N content and less N_2 fixation efficiency. Again, five out of six dual inoculation treatments analyzed for N content and N_2 fixation efficiency decreased these parameters, but only three also caused a diminution in plant growth. Therefore, this reduction on the capacity to assimilate N was not the only cause in inhibiting bean plant growth, although it could be the cause of the lack of positive response to dual inoculation. In fact, both parameters (N content and N_2 fixation efficiency) have been found to increase in dual inoculated bean plants (Ibijbijen et al., 1996; Mortimer et al., 2008), but not under drought conditions. Both microbial symbionts (*Rhizobium* and AM fungi) act as C sinks for the host plant (Kaschuk et al., 2009). In the present research, bean plants were grown under drought conditions, and thus the photosynthetic rate was reduced (Lawlor and Tezara, 2009). It is therefore possible that AM fungi inoculated had a higher "C-sink capacity" than co-inoculated *Rhizobium* strains. In fact, Mortimer et al. (2008) showed that AM symbionts can dominate upon rhizobial symbionts in terms of "C-sink capacity".

Neither P, S nor C shoot contents showed a clear correlation with the negative effect of dual inoculation on bean total aerial growth. However, K, Ca and Mg shoot contents decreased in two out of three negative dual interactions, but increased in the other one. Monzón and Azcón (1996) also found a reduction in the content of these three elements in four *Medicago* species inoculated with several AM fungi, although a negative effect on plant growth was observed only in one of them (*M. rigidula*). Again, neither increase nor decrease of these three elements correlated with the negative effect of dual inoculation on bean total aerial growth. Similarly, a lack of correlation was found between diminution of bean total aerial growth with dual inoculation and leaf RWC or stomatal conductance. The same lack of correlation between RWC and growth was found by Martínez et al. (2007) for several *P. vulgaris* varieties growing under drought conditions. Thus, it seems that RWC is not strongly related to growth capacity under moderate water stress conditions in *P. vulgaris*.

In summary, dual inoculation (*Rhizobium* plus AM fungi) in comparison with single inoculation with only one *Rhizobium* strain in *P. vulgaris* plants under moderate drought conditions ultimately had no effect or a negative effect in plant growth, depending on the specific composition of the triple combination in the symbiosis. Although none of the nutritional or physiological parameters analyzed can totally explain the absence of effect or the negative effect, the reduction of nodule development and of atmospheric N_2 fixation efficiency could be a major cause for such effects. In fact, Mortimer et al. (2008) found that, at initial developmental stages of AM symbiosis, nodule development was restricted in *P. vulgaris* roots. Here, the AM root colonization values were low and could be inhibiting nodule development and functionality. More precise experiments on this topic are needed to confirm or rule out this hypothesis.

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