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Development and Activity of the Symbiosis Between *Bradyrhizobium* Strains, *Glomus* Species and *Cicer arietinum*: Effect of Timing of Inoculation and Photon Irradiance

J.M. RUIZ-LOZANO and R. AZCON
Departamento de Microbiologia, Estación Experimental del Zaidin CSIC, Prof. Albareda 1, 18008-Granada, Spain
Tel. (958) 121011, Fax (958) 129600

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Abstract

The compatibility of dual inoculation of *Glomus* species and *Bradyrhizobium* strains was determined in relation to the time of inoculation and light intensity. The effects of simultaneous or sequential inoculation of two arbuscular mycorrhizal fungi, *Glomus mosseae* or *Glomus fasciculatum* and two *Bradyrhizobium* strains (Br 185 or Br 192) on the growth and nutrient uptake by *Cicer arietinum* were evaluated.

Plant development depended on the particular combination of *Bradyrhizobium* strain and *Glomus* species. Symbiotic efficiency was also influenced by the relative time of inoculation of the endophytes involved. Prior inoculation with the mycorrhizal fungus was usually the most effective.

Colonization by mycorrhizal fungi was negatively affected when fungi were applied to the seedlings after inoculation with *Bradyrhizobium*. Nodule number, however, was not increased by prior inoculation with bradyrhizobia. A crucial factor determining the lack of successful nodulation by Br 192 in plants colonized by *G. fasciculatum* (the most infective AM endophyte) was found to be a low level of photosynthetic activity. Symbiotic efficiency (plant growth and nutrition) also decreased when the photosynthetic capability of the plants was not sufficient to respond to the demand of the endophytes regarding *G. fasciculatum*, which was not the case with *G. mosseae* treatments.

Reduction of light intensity to 81.3 μE $s^{-1}m^{-2}$ (PAR), particularly affected plants colonized by *G. fasciculatum* as determined by the total sugar content of

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root extracts. Nearly stressed levels showed higher competitive ability of *Glomus* species than those of *Bradyrhizobium* strains, the most sensitive microsymbiont to plant colonization under these limited C resources. *Glomus* species were less affected and extensive AM colonization reduced the spread of nodulation. The fact that *G. mosseae* can maintain its activity under stressed light conditions is important in relation to the selection of AM endophytes to be used with leguminous plants.

Keywords: *Bradyrhizobium* strains, *Glomus fasciculatum*, *Glomus mosseae*, *Cicer arietinum*, inoculation efficiency, photon irradiance

1. Introduction

In a previous study (Ruiz-Lozano and Azcón, 1993) it was observed that symbiotic efficiency (as determined by plant growth and nutrition) was dependent on the use of specific combinations of isolates of *Glomus mosseae* and *Glomus fasciculatum* and strains of *Bradyrhizobium*. The interactions between these two types of microsymbionts were more or less efficient, depending on the strains and isolates of microorganisms involved. These results were more evident when *Cicer arietinum* was used as the host plant than with *Medicago sativa* (Azcón et al., 1991).

This incompatibility between endophytes was not expected since it is well established that legumes require both *Rhizobium* and arbuscular mycorrhizal (AM) fungi for efficient nodule formation and function (Asimi et al., 1980). The growth of nodulated plants requires a high level of phosphorus. In view of the role of P regulating CO_2 fixation (Sivak and Walker, 1986) and the relevance of AM fungi in P uptake, the relationship between photosynthesis and AM colonization is of particular importance for the success of tripartite symbiosis.

Rhizobia and AM fungi can interact either in the root-soil interface and/or in the root system of a common host plant. Nodular and mycorrhizal formation occurs at the same time and the two endophytes probably do not compete for infection site (Barea and Azcón-Aguilar, 1983). Neverthess, Bethlenfalvay et al. (1985) demonstrated a competitive interaction between an AM fungus and *Rhizobium*. However, AM fungal establishment actively alters the microbial populations in the rhizosphere (Ames et al., 1984; Meyer and Linderman, 1986). This in turn, affects the dynamic processes in the root-soil interface that are related to the distribution and development of the rhizobial population and the nodulating abilities of rhizobia.

The sink demand resulting from the growth and metabolic activity of these root endophytes has been associated with a compensatory increase in activity

by the carbon source of the association (Brown and Bethlenfalvay, 1988). A balanced physiological interaction between the microsymbionts and plant is thus required for their mutual compatibility so that carbohydrates from the root can be appropriately shared. Prior establishment of the fungus can inhibit subsequent nodule development (Bethlenfalvay et al., 1985). Possibly, the growth and activity of symbiotic partners was limited by the available carbon supply being unable to maintain a balanced system. Carbon allocation to the microbial symbionts can account for 28% of the total photosynthate (Harris et al., 1985).

Another aspect of the carbon balance in the tripartite association which has not been fully addressed is whether the plant is more advantaged from the dual symbiosis following delayed (sequential) inoculation. Carbon stress to the plant can be minimized by separate inoculation. A reduction in nodulation and mycorrhization appears to be a regulatory mechanism of the host plant to lower the carbon cost of the symbiosis.

In the present study, two different aspects related to endophyte interactions were studied. Firstly, the efficiency of tripartite symbiosis in *Cicer arietinum* was investigated using combinations of two *Glomus* species and two *Bradyrhizobium* strains in relation to the timing of their relative application. Secondly, the effect of two low light intensities on the development and effectivity of the tripartite symbiosis was determined and the relationship of the amount of available host carbohydrate to the specific interactions of the endophytes was examined.

2. Materials and Methods

Experiment 1: Experimental design

The experiment consisted of twelve treatments receiving dual inoculation with *Bradyrhizobium* strain 185 or strain 192 plus the AM fungus *G. mosseae* or *G. fasciculatum*. Five replicate treatments were made to give a total of 60 pots. One-third or these pots (20) was simultaneously inoculated with both microbial endophytes at planting. A further twenty pots were inoculated with the *Bradyrhizobium* strains at sowing and then were inoculated with the AM fungus 15 days later. The remaining twenty pots were inoculated at sowing with the AM fungus and then were inoculated with *Bradyrhizobium* 15 days later.

Soil and biological materials

Soil was collected from Granada city (Field around the Zaidin Experimental Station), sieved (2 mm pore size), diluted with quartz (1/1 v/v) and autoclaved

(100° C, 1 hr 3 consecutive days). The characteristics of the agricultural soil used were: pH 8.09; 1.81% organic matter; 2.5 mg N g^{-1}; 6.24 mg P g^{-1} ($NaHCO_3$-extractable P); 132 mg K g^{-1}; 35.8% sand; 46.3% loam and 20.5% clay.

Pots were filled with 1000 g of sterilized soil/sand mixture. Mycorrhizal inoculation consisted of a stock culture of *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe from Dijon (INRA) (28 spores g^{-1} and 76% AM infection) or *Glomus fasciculatum* (Taxter sensu Gerd.) Gerd. and Trappe from Rothamsted Experimental Station (35 spores g^{-1} and 79% AM infection) comprising soil, spores, mycelium and infected root fragments from an *Allium cepa* open pot culture. Five grams of inoculum was added to each pot at sowing just below the surface of sterilized seeds of *Cicer arietinum* or was added 15 days after sowing. *Bradyrhizobium* strains, obtained from Icarda (Syria), were grown in rotary shake culture at 28° C for 7 days in sterilized 250 ml flasks containing 75 ml of Allen 79 (Allen 1957) medium. Inoculum consisted of 2 ml of medium containing 10^9 cells/ml^{-1} and was added to the seeds at sowing or 15 days after sowing.

Growth conditions

Plants were cultivated in a greenhouse with a 16/8 hr day/night cycle maintained at 80% relative humidity. Photosynthetic photon flux density (PPFD) was 314 $\mu E\ m^{-2}s^{-1}$ as measured with a lightmeter (LICOR, model LI-188B). Day and night temperatures were variable, but did not exceed 35° C or fall below 21° C during the experiment. Water was supplied daily to maintain soil moisture close to 80% field capacity during the period of plant growth. At sowing time, and two weeks after sowing, water was added to reach an optimum level for seed germination. Each week plants received 10 ml of Hewitt's nutrient solution (Hewitt, 1952) lacking N and P.

Determinations

At harvest (8 weeks after planting), the root system was separated from the shoot, and dry weights were determined after drying for 36 hr at 70° C. The number of nodules on the main and lateral fresh roots were counted by direct observation using a magnifying glass. Visual observation of mycorrhizal infection was made by clearing the washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v) according to Phillips and Hayman (1970). Quantification was carried out using the method of Giovannetti and Mosse (1980).

Total N content of dried, ground plant material was determined by micro-Kjeldahl assay, phosphorus content was measured by the ammonium molybdate method (Olsen and Dean, 1965) and K, Ca and Mg contents were quantified by atomic absorption spectrophotometry (Lachica et al., 1973). Nutrient concentrations were also evaluated. For determination of chlorophyll content, 1 g of fresh leaf tissue was homogenized in 10 ml of Tris-ClH 50 mM, pH 7.5 and filtered. Aliquots of the filtrate (0.4 ml) plus water (0.6 ml) and 4 ml of acetone were shaken 30 s in a Heidolph reax 2000 shaker. After 10 min of centrifugation at 6000 g, the chlorophyll concentration of the supernatant was determined colorimetrically at 625 nm. Results were expressed as mg per g fresh shoot weight. The results were statistically evaluated by analysis of variance and Duncan's multiple range test.

Experiment 2: Experimental design

Plants were inoculated at sowing simultaneously with either *G. mosseae* or *G. fasciculatum* and *Bradyrhizobium* (strain 185 or 192). Plants were grown under two low light regimes. Thus the experiment consisted of eight treatments, of five replicates each, giving a total of 40 pots.

Soil and biological materials were as described for Experiment 1.

Growth conditions

Plants were cultivated in a growth chamber with day/night regimes of 16/8 hr, 27/21° C. Each set of twenty pots were placed in one or two lighting compartments. Compartment A: With a photosynthetic photon flux density of 125.5 μE $s^{-1}m^{-2}$ (PAR). Compartment B: With a photosynthetic photon flux density of 81.3 μE $s^{-1}m^{-2}$ (PAR). Water was supplied daily to maintain soil moisture content close to 80% field capacity during the period of plant growth. At sowing time and two weeks after, water was added to reach an optimum level for seed germination. Plants received 10 ml/week of Hewitt's nutrient solution (Hewitt, 1952) lacking N and P.

Determinations of shoot and root dry weights, number of nodules, mycorrhizal infection, nutrients content and concentration, and chlorophyll content were as described in experiment 1.

Total sugar content was evaluated in the root extracts of plants grown under the lowest light intensity (81.3 μE $s^{-1}m^{-2}$) and determined by the anthrone method after extraction in 80% ethanol (Loewus, 1952). All the results were evaluated statistically by ANOVA and Duncan's multiple range test for multiple comparisons.

3. Results

The time at which *Bradyrhizobium* strains and AM fungus isolates were inoculated was important in relation to the growth and nutrition of *Cicer arietinum*. Specific interactions between the endophytes (*Glomus* species and the strains of *Bradyrhizobium*) were affected by the relative inoculation time (Fig. 1).

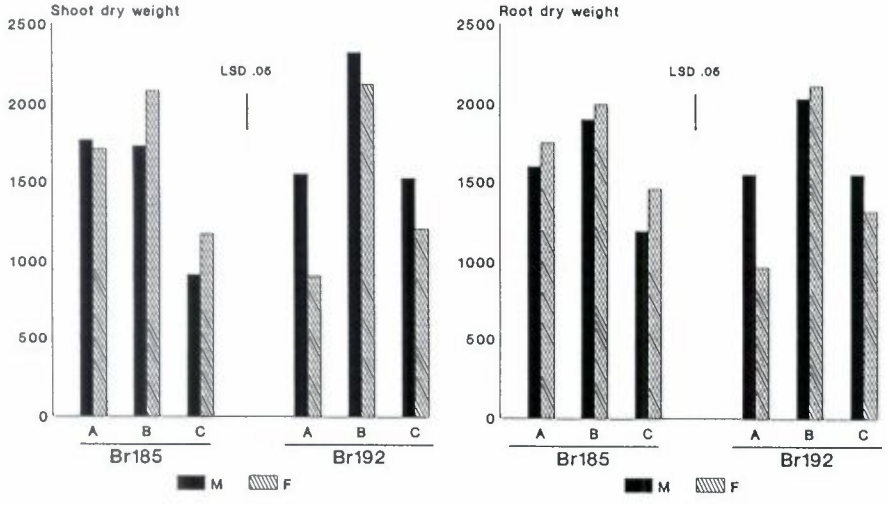

Figure 1. Shoot and root dry weights (mg plant^{-1}) of *Cicer arietinum* plants dually inoculated with *Bradyrhizobium* (185 or 192 strains) and AM endophytes (*G. mosseae*, M or *G. fasciculatum*, F) in simultaneous application (A) or 15 days delayed: AM fungus at sowing (B), *Bradyrhizobium* strain at sowing (C)

The effect of *G. mosseae* on plant growth varied depending on the associated *Bradyrhizobium* strain and the relative time of inoculation of the endophytes. Simultaneous or prior inoculation with the AM fungus relative to strain Br 185 was more effective than the application of *G. mosseae* 15 days after Br 185 inoculation (Fig. 1). Inoculation of the plants with *G. mosseae* and strain Br 192 increased plant growth if the AM endophyte was inoculated prior to Br 192. The simultaneous or earlier inoculation with Br 192 resulted in less effective growth. Simultaneous inoculation of *G. mosseae* and each of the *Bradyrhizobium* strains affected *Cicer* root and shoot growth (Fig. 1) similarly.

Plant N content was not affected in plants colonized by *G. mosseae* in any of the treatments. P, K, Ca and Mg plant contents, however, were affected by the relative time of inoculation. The most effective treatments were by

prior inoculation with the AM fungus and the least effective being by prior inoculation with *Bradyrhizobium* (particularly strain 185) (Figs. 3 and 4).

Chlorophyll content increased by the simultaneous inoculation with Br 185 and *Glomus*. Mycorrhizal root colonization was low in all the treatments and the nodule numbers were lower when plants were inoculated first with the AM fungus (Fig. 2).

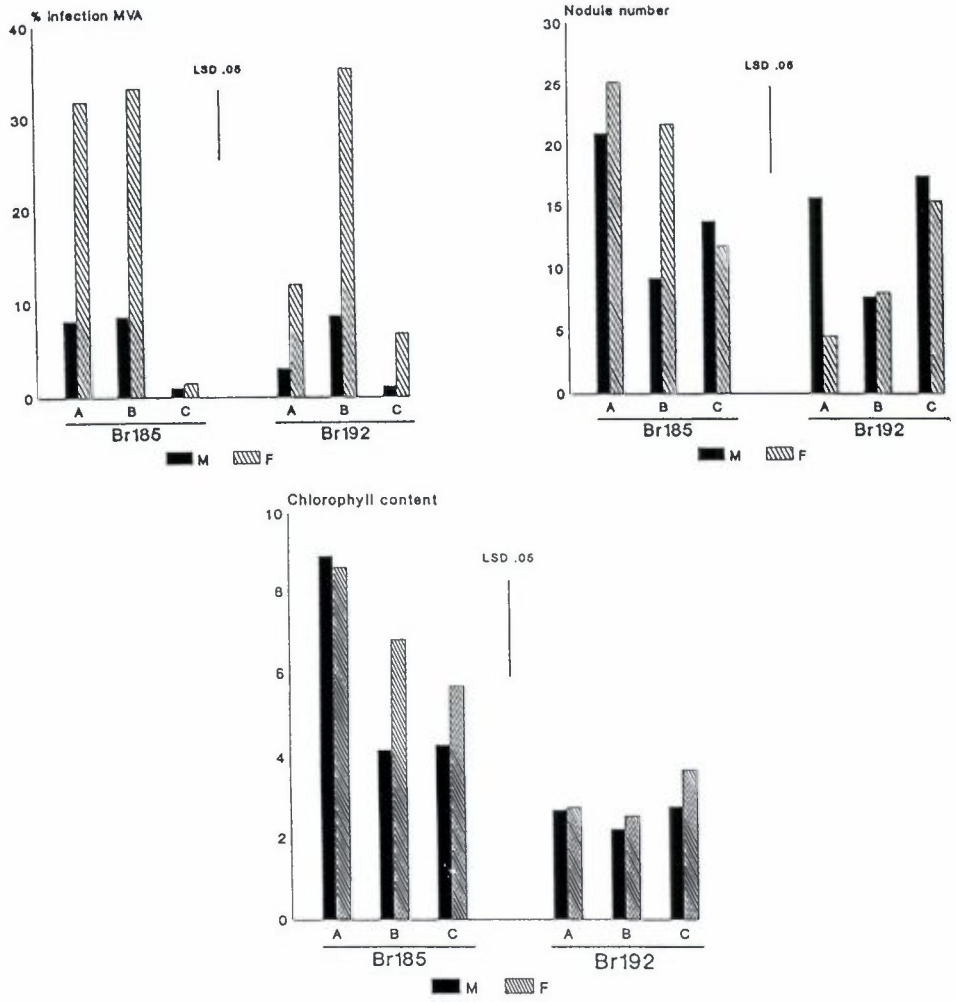

Figure 2. Chlorophyll content (mg g^{-1}f.w), AM colonization and nodule number of *Cicer arietinum* plants dually inoculated with *Bradyrhizobium* (185 or 192 strains) and AM endophytes (*G. mosseae*, M or *G. fasciculatum*, F) in simultaneous application (A) or 15 days delayed: AM fungus at sowing (B), *Bradyrhizobium* strain at sowing (C).

There was similarity in the growth of *Cicer* if inoculated simultaneously or inoculated with *G. fasciculatum* before Br 185. Inoculation with the fungus *G. fasciculatum* after 15 days of sowing was less stimulatory to plant growth. When *G. fasciculatum* was inoculated in association with Br 192, the relative time of inoculation affected plant growth in a different magnitude than when Br 185 was the bacterial partner. In this case, prior inoculation of *G. fasciculatum* resulted in better plant growth than when it was inoculated simultaneously or 15 days after sowing. Simultaneous application of *G. fasciculatum* was more effective in association with Br 185 than with Br 192 (Fig. 1). The root and shoot dry weight results showed a similar trend (Fig. 1).

The highest plant N content was recorded when strain Br 185 was applied with *G. fasciculatum*. When strain Br 192 was used, no significant differences in levels of this nutrient were observed for any of the endophyte inoculations. Plant P, K, Ca and Mg content was the highest when the mycorrhizal fungus *G. fasciculatum* was inoculated in advance of Br 185 or Br 192. When *G. fasciculatum* and Br 185 or Br 192 were inoculated simultaneously, however, different results were obtained: with Br 185, the nutrient contents reached high levels, but with Br 192, the nutrient levels decreased (Figs. 3 and 4).

The chlorophyll content was affected by the strain of *Bradyrhizobium* used.

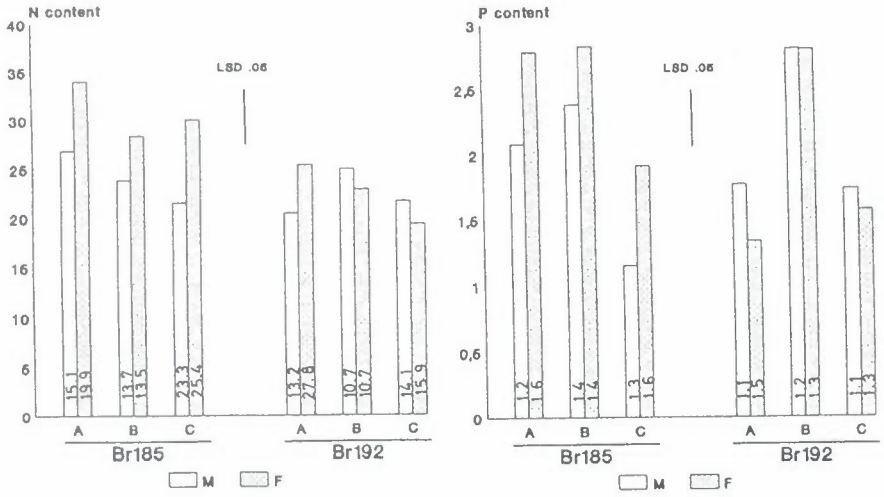

Figure 3. N and P contents (mg plant^{-1}) of *Cicer arietinum* plants dually inoculated with *Bradyrhizobium* (185 or 192 strains) and AM endophytes (*G. mosseae*, M or *G. fasciculatum*, F) in simultaneous application (A) or 15 days delayed: AM fungus at sowing (B), *Bradyrhizobium* strain at sowing (C). The nutrient concentration as mg g^{-1} is given inside the bars.

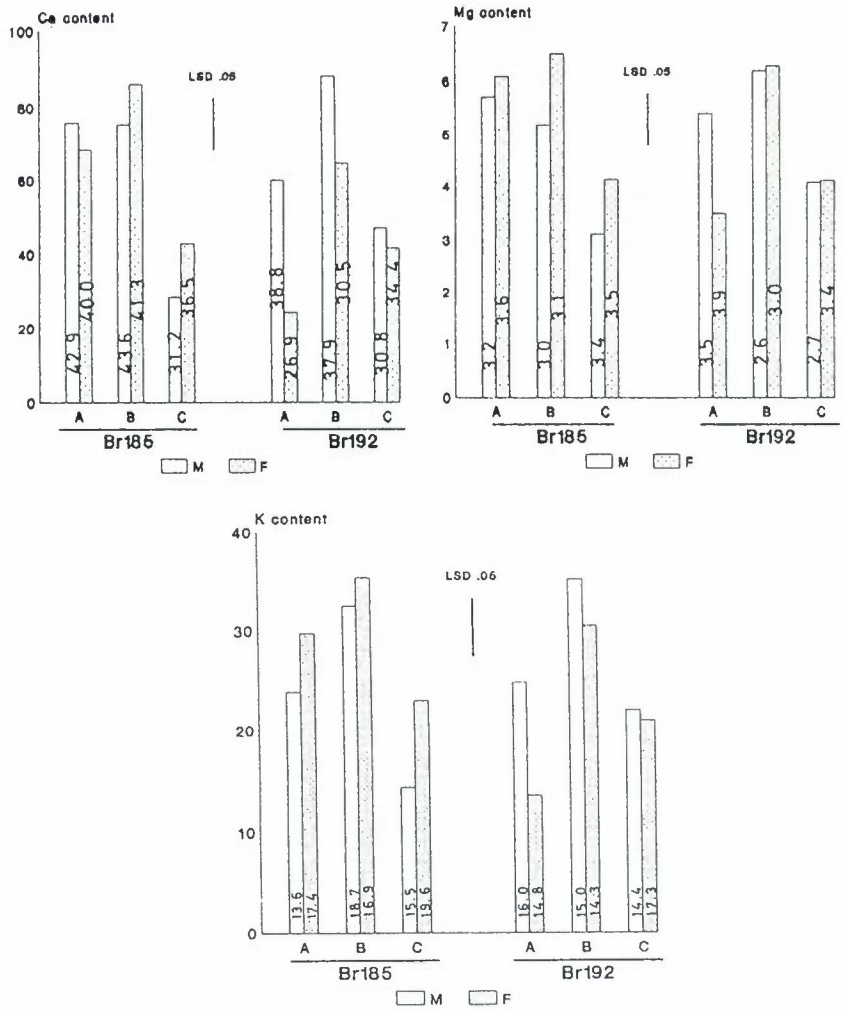

Figure 4. K, C and Mg contents (mg plant^{-1}) of *Cicer arietinum* plants dually inoculated with *Bradyrhizobium* (185 or 192 strains) and AM endophytes (*G. mosseae*, M or *G. fasciculatum*, F) in simultaneous application (A) or 15 days delayed: AM fungus at sowing (B), *Bradyrhizobium* strain at sowing (C). The nutrient concentration as mg g^{-1} is given inside the bars.

Br 185 was the most effective strain in improving this parameter. The time of endophyte inoculation also affected chlorophyll content in the case of Br 185 (Fig. 2).

AM colonization was maximal in *G. fasciculatum*-inoculated plants under simultaneous inoculation with Br 185 and in the case of prior inoculation with

both *Bradyrhizobium* strains. AM colonization decreased when *G. fasciculatum* was inoculated on seedlings 15 days after bradyrhizobia inoculation and also when it was added to the seed simultaneously with Br 192 (Fig. 2). The nodule number was also different for the two *Bradyrhizobium* strains in plants colonized by *G. fasciculatum*, except when it was applied on seedlings 15 days after bradyrhizobia (Fig. 2).

The results of experiment 2 (Tables 1, 2 and 3) show that the reduction of light intensity affected the tripartite symbiosis in different ways. Each simultaneous application of endophytes affected plant growth and nutrition (except N content) in a similar way when the light intensity was 125.4 μE $s^{-1}m^{-2}$, (PAR). An increase in mycorrhizal colonization was accompanied by a reduction in nodule numbers in all of the dual associations under this light regime (Table 1).

When the light intensity was reduced to 81.3 μE $s^{-1}m^{-2}$ PAR, the differences were more pronounced (Table 2). Under such conditions, the maximum plant growth, K and Ca nutrition was reached in plants colonized by *G. mosseae*, particularly when the plants were inoculated with Br 192. In plants with *G. fasciculatum* and Br 192, the mycorrhizal colonization level of the fungus was greatly reduced. From Tables 1 and 2, it can be seen that light limitation

Table 1. Shoot dry weight (mg $plant^{-1}$), N-P-K-Ca-Mg shoot contents (mg $plant^{-1}$) and symbiotic structures formation of *Cicer arietinum* plants dually inoculated with *Glomus* sp. and *Bradyrhizobium* strains under light intensity of μE $s^{-1}m^{-2}$12.5

Microbial treatments	Shoot dry weight	Nutrient contents					Nodule number	% Mycorrhizal infection
		N	P	K	Ca	Mg		
C. mossea + Br 185	1018a	16.6b	2.1a	13.7a	33.2a	3.4a	2.9a	61a
G. fasciculatum + Br 185	1228a	29.6a	2.3a	14.9a	35.0a	3.9a	1.4a	60a
G. mosseae + Br 192	1294a	26.5ab	2.1a	16.8a	37.8a	4.2a	.0b	68a
G. fasciculatum + Br 192	1424a	32.2a	2.6a	17.3	40.7a	4.4a	1.8ab	65a

Means followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple range test, n = 5.

Table 2. Shoot dry weight (mg $plant^{-1}$), N-P-K-Ca-Mg shoot contents (mg $plant^{-1}$) and symbiotic structures formation of *Cicer arietinum* plants dually inoculated with *Glomus* sp. and *Bradyrhizobium* strains under light intensity of μE $s^{-1}m^{-2}$81.3

Microbial treatments	Shoot dry weight	Nutrient contents					Nodule number	% Mycorrhizal infection
		N	P	K	Ca	Mg		
C. mossea + Br 185	1520b	41.1a	3.3a	29.1b	35.6b	5.6a	4.4a	62.1a
G. fasciculatum + Br 185	960c	26.4b	2.3b	19.6a	24.2c	3.5b	2.6a	61.1a
G. mosseae + Br 192	1942a	41.7a	3.7a	34.0a	43.1a	6.4a	4.4a	65.8a
G. fasciculatum + Br 192	976c	27.6b	2.2b	20.5	24.5c	3.5b	0.4b	53.5b

Means followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple range test, n = 5.

Table 3. Nutrient concentration (mg g^{-1}) of *Cicer arietinum* plants dually inoculated with *Glomus* sp. and *Bradyrhizobium* strains under two light intensities

Microbial treatments	125.5 μE $s^{-1}m^{-2}$				
	N	P	K	Ca	Mg
G. mosseae + Br 185	16.3c	2.1a	13.5a	32.6a	3.3a
G. fasciculatum + Br 185	24.1a	1.9ab	12.1a	28.5a	3.2a
G. mosseae + Br 192	20.5b	1.6b	13.0a	29.2a	3.2a
G. fasciculatum + Br 192	22.6ab	1.8ab	12.1a	28.6a	3.1a
Microbial treatments	81.3 μE $s^{-1}m^{-2}$				
	N	P	K	Ca	Mg
G. mosseae + Br 185	27.0a	2.2ab	19.1a	23.4a	3.7a
G. fasciculatum + Br 185	27.5a	2.4a	20.4a	25.2a	3.6a
G. mosseae + Br 192	21.5b	1.9b	17.5a	22.2a	3.3a
G. fasciculatum + Br 192	28.3a	2.3a	21.0a	25.1a	3.6a

Means followed by the same letter are not significantly different (P = 0.05) by Duncan's multiple range test, n = 5.

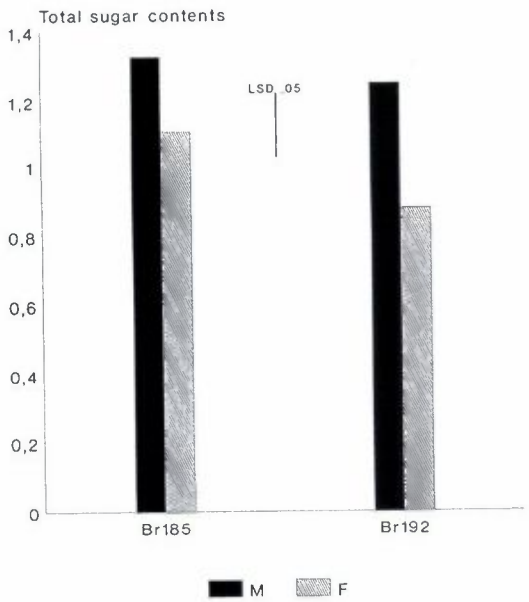

Figure 5. Total sugar contents in root extracts (mg g^{-1}) of *Cicer arietinum* plants dually inoculated with *Bradyrhizobium* (185 or 192 strains) and AM endophytes (*G. mosseae*, M or *G. fasciculatum*, F) under light intensity of 81.3 μE $s^{-1}m^{-2}$.

had different effects on plants colonized by *G. mosseae* or *G. fasciculatum*. The total sugar content in roots of *Cicer* plants colonized by both microorganisms was determined at the light level of 81.3 μE $s^{-1}m^{-2}$. The results show (Fig. 5) that the lowest sugar content occurred in *G. fasciculatum*-colonized

plants. Differences between *Glomus* species colonized plants ranged from 17% in association with Br 185 and up to 30% in dual inoculation with Br 192.

4. Discussion

As the results have shown, the compatibility of the microsymbionts in *Cicer arietinum* was affected by altering the timing of dual inoculation. Maximum growth and nutritional benefit was obtained when the AM fungus was inoculated before the *Bradyrhizobium*, particularly in the case of plants inoculated with *G. fasciculatum* and Br 192. Mycorrhizal plants nodulated with Br 192 had the lowest chlorophyll content and *G. fasciculatum* was the most infective AM endophyte. This plant response according to simultaneous or to delayed inoculation of the endophytes can be explained by competition between the symbionts for carbon supply when this energy source was limited. Kucey and Paul (1982) have suggested that *G. fasciculatum* has a higher C requirement or confers a lower photosynthetic compensation than *G. mosseae*. The combination of *G. fasciculatum* plus Br 192 inoculated together, resulted in a considerable reduction in nodulation. In these experiments, the physiological status of the host was such that simultaneous application of *G. fasciculatum* Br 192 was less effective than sequential inoculation. The establishment of *Glomus* species in advance was the most effective strategy.

In the case of plants colonized by *G. mosseae* and nodulated by Br 192, the allocation of a proportion of the host photosynthate to the root and to the maintenance of the symbiosis may result in a more energy-efficient system than in plants colonized by *G. fasciculatum* plus Br 192. Delayed inoculation of one endophyte with respect to the other symbiont only benefited the plant in certain situations. This may reflect competition between endophytes and the autotroph-host for a limited supply of photosynthates, but other mechanisms can also be involved.

Plants with a higher chlorophyll content (Br 185 colonized plants) had a higher number of nodules than plants containing less chlorophyll (Br 192 colonized plants). The presence of AM fungi can increase the photosynthetic output and plant growth (Bethlenfalvay et al., 1983). This fact cannot be observed here because nonmycorrhizal treatments were not available.

When mycorrhizal formation occurred prior to nodulation, the presence of the AM fungus within roots did not usually alter the subsequent pattern of nodulation, in the case of *G. fasciculatum*. These results confirm that in some microbial associations the endophytes probably do not compete for infection sites, i.e. there is no competitive interaction between microbial symbionts for infection. This result does not completely agree with those from Bethlenfalvay

et al. (1985) who reported that prior establishment of the fungus inhibited subsequent development of nodules and indeed, when *G. mosseae* was the endophyte involved, a decrease in nodulation values was observed.

The low level of mycorrhizal colonization found when the AM fungus was inoculated on 15-day old seedlings is probably due to a reduction of root susceptibility at this developmental stage (Hepper, 1985) rather than being due to the prior establishment of *Bradyrhizobium*, since nodule formation was not inversely correlated with AM colonization in this study.

One important aspect discussed previously (Ames and Bethlenfalvay, 1987) was the demonstration that *Glomus* has a localized, non-systemic effect on nodule activity, suggesting that there is a much more complex and localized interaction. In the preinfective stages, the effect between each component of the system may be relevant (Gonzalez, 1988; Azcón-Aguilar et al., 1980). Interactions cannot be attributed to plant physiology and nutrition alone. Regulation of the symbiosis involves a combination of mechanisms that either limit or promote infection by specific endophytes, depending upon conditions. Symbiotic associations between plants and microorganisms depend for their formation and function on interactions between their constituents. A reduction in fungal and/or rhizobial activity could be the result of competition between symbionts for their energy supply from the plant.

The second objective of this study was to evaluate how different levels of irradiance can affect the interaction of the endophytes. The results showed that growth differences of plants inoculated with different AM endophytes were also evidenced at the lower light level (Table 2), but did not show similar trends to those described in the first experiments. No differences were found in colonization levels between *Glomus* species, but the efficiency of *G. fasciculatum* was lower, compared to that of *G. mosseae* at the lower light level. Son and Smith (1988) related reductions in C inflow at low irradiance with a reduced activity of the fungus, rather than to a reduced amount of infection. This is similar to the situation for plants, in this experiment, colonized by *G. fasciculatum*. The differential effect of environmental conditions (in this case irradiance) on the growth response of specific endophytes could be explained by considering that one fungus has shown a lower requirement for C than the other endophytes in spite of possessing a similar amount of intraradical growth (% AM infection). It is not easy to explain the result in the complex system as several causes and effect relationships may be operating (Fig. 5, Table 2) (Azcón and Ocampo, 1984; Ocampo and Azcón, 1985). The results may indicate that *G. fasciculatum* has a different demand for C compounds than *G. mosseae*, particularly in association with Br 192, or that it induces a different photosynthetic compensation. The former explanation (Kucey and Paul, 1982) is more likely when

the results of chlorophyll content also are considered (Fig. 2). *G. fasciculatum* appears to be less efficient than *G. mosseae* (irrespective of the Br strains) when photosynthesis is limited. Recent results have shown that fungal colonization and nodulation are differently affected by the carbohydrate content of the host. Not much information is available on the efficiency performed of *Bradyrhizobium* strains and mycorrhizal species under stress conditions (Azcón et al., 1988). An explanation of the mechanisms related to the particular efficiency of each part of the tripartite symbiosis is important for understanding the behavior of the complex associations under limiting conditions. Energetic balance in plants is a major component of an adaptation strategy as a stress reaction (Gale and Zerony, 1985). A decrease in irradiance levels negatively affected *Bradyrhizobium* colonization (number of nodules). The effect of "light stress" depressed formation of the N_2-fixing symbiosis, yet increased mycorrhizal formation. These results are similar to those of Bayne et al. (1984) who also showed that AM colonization and nodulation are affected differently by plant carbohydrate status. The reduction in nodulation as a consequence of light limitation, which did not suppress fungal colonization in roots, is evidence for the ability of arbuscular endophytes to survive under light-reduced conditions. The high activity of *G. mosseae* under limited light conditions is an unusual result which needs further investigation. The knowledge of differences in the tolerance of endophytes to stress is important for the utilization of efficient isolates which may provide ecological competitive advantages to the host plant. The selection of organisms that are the most appropriate for a specific situation is an important goal. The effects of inoculation may not always be beneficial. Paulitz and Linderman (1989) determined that some *Glomus* species colonized plants more rapidly than others. At an early stage of plant growth (e.g. 1–3 week old seedlings) the fungus may exert a significant C drain on the plants when their photosynthetic capacity is low. The cost-benefit relationship is complicated in interactions between symbionts and the environment. Under conditions of low irradiance, a reduced growth response results from the drain of carbohydrates to the endophytes, which is not compensated by a more efficient N and P uptake. Thus, successful nodulation (N_2 fixation) and AM colonization requires a system in which the C demand by the endophytes is balanced by enhanced uptake of limited mineral nutrients.

As a result of our experiments presented here, we can state that the behavior of the endophyte-used *Glomus* species and *Bradyrhizobium* strains differed considerably, affecting physiological plant parameters (carbohydrate status) in the common host *Cicer arietinum*. Indeed, the relative time of inoculation and endophytes involved affected the functional compatibility of the association.

The limited light situation negatively affected the symbiosis formed by the

most infective fungus (*G. fasciculatum*) and the *Bradyrhizobium* strain with the lowest chlorophyll content in the host (Br 192). The incompatibility of such microsymbionts in simultaneous inoculation seems to be associated with a limited carbohydrate status in root tissue of plants colonized by both symbionts.

Using AM fungi as biological fertilizers is of great interest in agrobiology (Jeffries, 1987). *Cicer arietinum* is one of the important grain legumes in the semiarid and arid tropics. Experiments on the chickpea response to fertilizer application, especially to nitrogen and phosphorus have been reported (Hirata, 1982) and conflicting results have been obtained. Regarding the present results, the optimization of benefits to the growth and N-P nutrition of plants with the dual symbiosis requires a goal-oriented selection of organisms that are most appropriate to the specific situation (Abbott and Robson, 1982; Bethlenfalvay et al., 1988).

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REFERENCES

Abbot, L.K. and Robson, A.D. 1982. The role of vesicular-arbuscular mycorrhizal fungi in agriculture and the selection of fungi for inoculation. *Aust. J. Agric. Res.* **33:** 389–408.

Ames, R.N. and Bethlenfalvay, G.J. 1987. Mycorrhizal fungi and the integration of plant and soil nutrient dynamics. *J. Plant. Nutri.* **10:** 1313–1321.

Ames, R.N., Reid, C.P.P., and Ingham, E.R. 1984. Rhizosphere bacterial population responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. *New Phytol.* **96:** 555–563.

Asimi, S., Gianinazzi-Pearson, V., and Gianinazzi, S. 1980. Influence of increasing soil phosphorus levels on interactions between vesicular-arbuscular mycorrhizae and *Rhizobium* in soybeans. *Can. J. Bot.* **58:** 2200–2205.

Azcón, R., El-Atrach, F., and Barea, J.M. 1988. Influence of mycorrhiza vs. soluble phosphate on growth, nodulation, and N_2 fixation (^{15}N) in alfalfa under different levels of water potential. *Biol. Fertil. Soils* **7:** 28–31.

Azcón, R. and Ocampo, J.A. 1984. Effect of root exudation on VA mycorrhizal infection at early stages of plant growth. *Plant Soil* **82:** 133–138.

Azcón, R., Rubio, R., and Barea, J.M. 1991. Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2 fixation (^{15}N) and nutrition of *Medicago sativa* L. *New Phytol.* **117:** 399–404.

Azcón-Aguilar, C., Barea, J.M., and Olivares, J. 1980. Effects of *Rhizobium* polysaccharides on VA mycorrhiza formation. *Second International Symposium on Microbial Ecology*, University of Warwick, Coventry, UK, Abstr. No. 187.

Barea, J.M. and Azcón-Aguilar, C. 1983. Mycorrhizas and their significance in nodulating nitrogen-fixing plants. *Adv. Agron.* **36:** 1–54.

Bayne, H.G., Brown, M.S., and Bethlenfalvay, G.J. 1984. Defoliation effects on mycorrhizal colonization, nitrogen fixation and photosynthesis in the *Glycine-Glomus-Rhizobium* symbiosis. *Physiol. Plant.* **62:** 576–580.

Bethlenfalvay, G.J., Brown, M.S., Ames, R.N., and Thomas, R.S. 1988. Effects of drought on host and endophyte development in mycorrhizal soybeans in relation to water-use and phosphate-uptake. *Physiol. Plant.* **72:** 565–571.

Bethlenfalvay, G.J., Bayne, H.G., and Pacovsky, R.S. 1983. Parasitic and mutualistic associations between a mycorrhizal fungus and soybean: The effect of phosphorus on host-endophyte interactions. *Physiol. Plant.* **57:** 543–548.

Bethlenfalvay, G.J., Brown, M.S., and Stafford, A.E. 1985. The *Glycine-Glomus-Rhizobium* symbiosis. II. Antagonistic effects between mycorrhizal colonization and nodulation. *Plant Physiol.* **79:** 1054–1058.

Brown, M.S. and Bethlenfalvay, G.J. 1988. The *Glycine-Glomus-Rhizobium* symbiosis. VII. Photosynthetic nutrient-use efficiency in nodulated, mycorrhizal soybeans. *Plant Physiol.* **86:** 1292–1297.

Gale, J. and Zerony, M. 1985. The cost to plants of different strategies of adaptation to stress and the alleviation of stress by increasing assimilation. *Plant Soil* **89:** 57–67.

Giovannetti, M. and Mosse, B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytol.* **84:** 489–500.

Gonzalez, S.B. 1988. *Ecología y Biotecnología de Micorrizas en Leguminosas (Soja y Alfalfa).* Ph.D. Thesis, University of Granada.

Harris, D., Pacovsky, R.S., and Paul, E.A. 1985. Carbon economy of soybean-*Rhizobium-Glomus* associations. *New Phytol.* **101:** 427–440.

Hepper, C.M. 1985. Influence of age of roots on the pattern of vesicular arbuscular mycorrhizal infection in leek and clover. *New Phytol.* **101:** 685–693.

Hewitt, E.J. 1952. Sand and water culture methods used in the study of plant nutrition. *Tech. Commun. 22, Farnhan Royal, Commonwealth Agricultural Bureau, Bucks.*

Hirata, H. 1982. Responses of pulses to spot placements of P and N on deep vertisol in semi-arid tropics some experiences at ICRISAT. *Fertil. Sci.* **5:** 113–135.

Jeffries, P. 1987. Use of mycorrhizae in agriculture. *CRC Crit. Rev. Biotechnol.* **5:** 319–358.

Kucey, R.M.N. and Paul, E.A. 1982. Carbon flow, photosynthesis, and N_2 fixation in mycorrhizal and nodulated faba feans (*Vicia faba* L.). *Soil Biol. Biochem.* **14:** 407–412.

Lachica, M., Aguilar, A., and Yañez, J. 1973. Análisis foliar, métodos utilizados en la Estación Experimental del Zaidín. *Anal. Edafol. Agrobiol.* **32:** 1033–1047.

Loewus, F.A. 1952. Improvement in the anthrone method for determination of carbohydrates. *Anal. Chem.* **24:** 219.

Meyer, J.R. and Linderman, R.G. 1986. Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. *Soil Biol. Biochem.* **18:** 191–196.

Ocampo, J.A. and Azcón, R. 1985. Relationship between the concentration of sugars in the roots and VA mycorrhizal infection. *Plant Soil* **86:** 95–100.

Olsen, S.R. and Dean, L.A. 1965. Phosphorus. In: *Methods of Soil Analysis.* Madison, American Society of Agronomy, pp. 1035–1049.

Paulitz, T.C. and Linderman, R.G. 1989. Interactions between fluorescent pseudomonas and VA mycorrhizal fungi. *New Phytol.* **113:** 37–45.

Phillips, J.M. and Hayman, D.S. 1970. Improved procedures for cleaning roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Brit. Mycol. Soci.* **55:** 158–161.

Ruiz-Lozano, J.M. and Azcón, R. 1993. Specificity and functional compatibility of VA mycorrhizal endophytes in association with *Bradyrhizobium* strains in *Cicer arietinum*. *Symbiosis* **15:** 217–226.

Smith, S.E., Nicholas, D.J.D., and Smith, F.A. 1979. Effect of early mycorrhizal infection on nodulation and nitrogen fixation in *Trifolium subterraneum* L. *Aust. J. Plant Physiol.* **6:** 305–311.

Sivak, M.N. and Walker, D.A. 1986. Photosynthesis *in vivo* can be limited by phosphate supply. *New Phytol.* **102:** 499–512.

Son, C.L. and Smith, S.E. 1988. Mycorrhizal growth-response: Interactions between photon irradiance and phosphorus nutrition. *New Phytol.* **108:** 305–314.