ELSEVIER

Applied Soil Ecology 22 (2003) 15–28

Applied Soil Ecology

www.elsevier.com/locate/apsoil

Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin)

A. Medina [a], A. Probanza [b], F.J. Gutierrez Mañero [b], R. Azcón [a,*]

[a] *Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*

[b] *Departamento de Ciencias Ambientales y Recursos Naturales, Facultad de Ciencias Experimentales y de la Salud Universidad San Pablo CEU, 28668 Boadilla del Monte, Madrid, Spain*

Received 28 January 2002; received in revised form 29 July 2002; accepted 30 July 2002

Abstract

The effects of two *Bacillus* strains (*Bacillus pumillus* and *B. licheniformis*) on *Medicago sativa* plants were determined in single or dual inoculation with three arbuscular-mycorrhizal (AM) fungi and compared to P-fertilization. Shoot and root plant biomass, values of thymidine and leucine incorporation as well as ergosterol and chitin in rhizosphere soil were evaluated to estimate metabolic activity and fungal biomass, respectively, according to inoculation treatments. For most of the plant parameters determined, the effectiveness of AM fungal species was influenced by the bacterial strain associated. Dual inoculation of *Bacillus* spp. and AM fungi did not always significantly increase shoot biomass compared to single AM-colonized plants. The most efficient treatment in terms of dry matter production was the dual *Glomus deserticola* plus *B. pumillus* inoculation, which produced similar shoot biomass and longer roots than P-fertilization and a 715% (shoot) and 190% (root length) increase over uninoculated control. The mycorrhizas were more important for N use-efficiency than for P use-efficiency, which suggests a direct mycorrhizal effect on N nutrition not mediated by P uptake. Both chemical and biological treatments affected thymidine and leucine incorporation in the rhizosphere soil differently. Thymidine was greater in inoculated than in control rhizospheres and *B. licheniformis* was more effective than *B. pumillus* in increasing thymidine. Non-inoculated rhizospheres showed the lowest thymidine and leucine values, which shows that indigenous rhizosphere bacteria increased with introduced inocula. The highest thymidine and leucine values found in P-fertilized soils indicate that AM plants are better adapted to compete with saprophytic soil bacteria for nutrients than P-amended plants. Chitin was only increased by coinoculation of *B. licheniformis* and *G. intraradices*. *B. pumillus* increased ergosterol (indicative of active saprophyte fungal populations) in the rhizosphere of AM plants and particularly when colonized by *G. mosseae*. The different AM fungi have different effects on bacterial and/or fungal saprophytic populations and for each AM fungus, this effect was specifically stimulated or reduced by the same bacterium. This is an indication of ecological compatibilities between microorganisms. Particular *Glomus*–bacterium interactions (in terms of effect on plant growth responses or rhizosphere population) do not seem to be related to the percentage of AM colonization. The effect on plant growth and stimulation

* Corresponding author. Tel.: +34-958-121-011; fax: +34-958-129-600.
E-mail address: razcon@eez.csic.es (R. Azcón).

PII: S0929-1393(02)00112-9

of rhizosphere populations, as a consequence of selected microbial groups, may be decisive for the plant establishment under limiting soil conditions.

Keywords: *Bacillus* spp. AM colonization; Thymidine and leucine incorporation; Rhizosphere metabolic activity; Fungal biomass (ergosterol and chitin)

1. Introduction

Arbuscular-mycorrhizal (AM) fungi can increase the capability of the root system to absorb and translocate phosphorus (P) and minor elements through an extensive network of mycelium (Mosse, 1973). AM fungi are commonly associated with legumes and can increase nutrient uptake of plants growing in high phosphate fixing soils, as are most of the Mediterranean soils. However, biological N fixation by rhizobia is weak, due to the limited availability of P and minor nutrients in these soils. Both *Sinorhizobium* and AM fungi together improve legume nutrition (Barea and Azcón-Aguilar, 1983; Requena et al., 2001).

A biotechnological goal is to use a combined inoculation of selected rhizosphere microorganisms to minimize fertilizer application and to maximize plant growth and nutrition (Barea et al., 1998; Barea and Jeffries, 1995; Linderman, 1986; Probanza et al., 2001). Selected combinations of microbial inocula enhanced the positive effect achieved by each microbial group, improving plant development in infertile soils (Toro et al., 1997, 1998). Free living bacteria producing certain stimulating metabolites enhance plant growth, particularly when associated with AM fungus and *Sinorhizobium* strains (Ahmad, 1995; Azcón, 1993). The wide genetic variation within microbial species explains the high potential of the microorganisms to adapt to different environments. Therefore, there is a need to develop specific host-strain combinations with higher effectiveness under a wide range of experimental conditions.

Variability in functional compatibilities among the microbial components is known to occur (Andrade et al., 1995, 1998; Azcón, 1989; Vázquez et al., 2001). In general, the functional compatibility of the rhizosphere system, which is the physiological ability of partners to contribute to the nutrition of the association (Smith et al., 1994), is poorly documented. However, plant responses depend on this compatibility of the microbial interaction at the physiological and biochemical level (Ruiz-Lozano et al., 1995a). AM fungi differ in their ability to enhance nutrient uptake and the different physiology of AM-colonized roots may alter conditions for rhizosphere microbial groups. Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria, colonizing plant roots or niches close by (Kloepper and Schroth, 1978). Several mechanisms have been proposed which account for the stimulating effect, including a range of metabolites (amino acids, vitamins, phytohormons), solubilization, or mineralization processes.

In this respect, it is necessary to consider that the establishment of inoculated or naturally occurring microorganisms in the rhizosphere, able to develop a range of activities when appropriate carbon sources are available, is very important for plant growth (Bowen and Rovira, 1999). The supply of photosynthates to the root-associated microbiota is a key issue in rhizosphere formation and functioning, but the biological balance in the rhizosphere is affected by AM colonization (Amora-Lazcano and Azcón, 1997; Amora-Lazcano et al., 1998; Schreiner et al., 1997). This may be the consequence of AM mycelium releasing energy rich organic compounds and thus positively influencing the persistence of inoculated microorganisms (Andrade et al., 1998). On the other hand, Christensen and Jacobsen (1993) reported that AM symbiosis decreased the amount of plant root-derived organic matter available for microbial growth. This contradictory insight is the result of AM colonization, which changes several aspects of plant physiology, according to conditions (Smith et al., 1994), and also introduces modifications in the surrounding root system. The effect of inoculants on bacterial and fungal populations in the rhizosphere is decisive for maximizing plant nutrient availability, since the soil microbial community in the rhizosphere plays a key role in plant nutrition. Microbial

inoculation could be favorable for the microbial community or not so.

To estimate bacterial populations, ^{3}H-thymidine incorporation into bacterial DNA and ^{14}C-leucine incorporation into bacterial protein have been used (Bååth, 1990, 1992, 1994). One of the advantages is that this provides an estimate of all living bacterial groups living in the soil without the need for cultivation. As fungal biomass in soil represents the main component in the microbial populations, fungus-specific compounds were determined. Ergosterol and chitin (the major cell wall component) have been considered as good fungal biomass indicators (Nylund and Wallander, 1992). Ergosterol can be considered a marker for living fungal biomass, since it decays rapidly in dead tissue. Chitin is indicative of both living and dead fungal biomass because of its more stable compounds. These marker substances for microorganisms were analyzed in the root free soil.

In previous studies, it was shown that the presence of *Bacillus pumillus* and *B. licheniformis* in the rhizosphere promoted the growth of alder (Probanza et al., 1996). The bacterial effect on plant growth was attributable to auxins (Gutiérrez-Mañero et al., 1996) and gibberellins (Gutiérrez-Mañero et al., 2001). Microorganisms producing phytohormones cohabit in the rhizosphere with AM fungi and could play a helper role in the plant–fungus interaction. But this possible interactive effect needs to be proved.

The aim of this study, was to determine how different AM fungi (*Glomus mosseae*, *G. intraradices* and *G. deserticola*) and two PGPR bacteria (*B. pumillus* and *B. licheniformis*) interacted with *Sinorhizobium meliloti* on *Medicago sativa* and to identify when P-fertilization substitutes for the microbial effect. The compatibility of the microbial plant system was evaluated for plant growth, N and P use-efficiency and symbiotic parameters. As marker substances for rhizosphere microorganisms, changes in thymidine and leucine incorporation as well as ergosterol and chitin content in rhizosphere soil were also determined to provide a better insight into changes in rhizosphere activity and fungal biomass, according to microbial treatments applied. Information of the metabolic activity in rhizosphere soil is required to obtain an understanding of the role of inoculated species in the diverse microbial communities existing in the rhizosphere zone.

2. Material and methods

2.1. Experimental design

The experiment consisted of three treatments for each *Glomus* species. The *Glomus* species (*G. mosseae*, *G. intraradices* and *G. deserticola*) were assayed inoculated singly or in coinoculation with *B. pumillus* or *B. licheniformis*. Non-mycorrhizal controls inoculated with each bacterium, supplemented with P, or non-treated, were also used. All treatments were replicated five times with a total of 78 pots and placed in a random complete block design.

2.2. Soil and microorganisms

The soil used was collected from the Granada Province (Spain) and had 73.11% sand, 6.6% clay and 20.29% loam, containing 1.17% organic matter, 1.2 mg P kg^{-1}, 0.071% (N) and 20 mg K kg^{-1} with a pH of 8.23. The soil was sieved (<2 mm) and mixed with sand (soil:sand 1:1 v/v).

The soil/sand mixture was autoclaved at 100 °C for 1 h on three consecutive days and distributed in 500 ml pots. A soil extract (10 ml per pot) of natural soil/water at equal v/v, filtered through Whatman no. 1 paper, was added to reintroduce the native microbial population, except for propagules of AM fungi.

To produce mycorrhizal plants, pots from each *Glomus* species were inoculated either with *G. mosseae* Taxter sensu Gerd. & Trappe, *G. intraradices* Schenk & Smith or *G. deserticola* Trappe, Bloss and Menge. The mycorrhizal inoculum consisted of spores, mycelia and mycorrhizal root fragments from a stock culture of each fungus with *Lactuca sativa*. Twenty grams of inoculum per pot, having similar characteristics (an average of 30 spores per g and roots with 75% of AM colonization), was placed directly below the seedlings in the planting hole. Non-mycorrhizal treatments received the same amounts of autoclaved inoculum.

The rhizobacteria assayed were the *B. pumillus* strain CECT 5105 and the *B. licheniformis* strain CECT 510, selected on the basis of their ability to produce indol-acetic-acid (IAA) and gibberellins (Probanza et al., 1996; Gutiérrez-Mañero et al., 1996, 2001). Both bacteria were grown in nutrient broth (8 g l^{-1}) in shake culture in 250 ml flasks containing

50 ml of medium. One milliliter of bacterial culture (10^7 cfu ml^{-1}) per pot was added to the corresponding treatments at sowing.

S. meliloti strain (GR4) was grown in Ty medium (Beringer, 1974) and applied at a rate of 1 ml per pot (10^8 cfu ml^{-1}).

2.3. Plant growing conditions, inoculation and treatments

The *M. sativa* plants were grown in a controlled greenhouse under a 16 h light (21 °C) and 8 h dark (15 °C) cycle, with 50% relative humidity and a photosynthetic photon flux density of 700 μmol m^{-2} s^{-1} for the compensating photophase. During the assay the plants were fertilized at the rate of 10 ml per week per pot, with a macronutrient-free micronutrient solution (Hewitt, 1952).

Some uninoculated non-mycorrhizal plants were fertilized with a KH_2PO_4 solution at a rate of 10 ml per week per pot, giving a total P supplement of 70 mg kg^{-1}. The rate of P supply was selected to match the effect on growth and nutrition of the AM fungi (on the basis of previous studies), thus providing an appropriate control for the mycorrhizal plants. Pots were weighed daily throughout the experiment, and water loss replaced daily by top watering to maintain soil moisture close to 100% field capacity during the period of plant growth.

At harvest (12 weeks after planting) the root system was separated from the shoots and the dry weight of the shoots was recorded after drying at 70 °C. Image analysis methods were applied to study the effect of biological treatments on root development. Mycorrhizal colonization was microscopically assessed using the gridline intersect method (Giovannetti and Mosse, 1980), after staining (Phillips and Hayman, 1970).

2.4. [^3H]-Thymidine (TdR) and L-[^{14}C] leucine (Leu) incorporation

These values were determined in rhizosphere root free soil. Bacterial activity was simultaneously determined by the incorporation of TdR and Leu into cold TCA-insoluble material, following the procedure described by Bååth (1994). The samples (1 g of rhizosphere soil) were homogenized in 20 ml of distilled water, centrifuged at 1000 × *g* for 10 min and then the supernatant was filtered though glass wool. Two milliliters of the bacterial suspension obtained was incubated with 100 nM methyl-[^{3}H]-TdR (925 Gbq $mmol^{-1}$, Amersham, UK) and 387 nM L-[^{14}C]-Leu (11.9 Gbq $mmol^{-1}$, Amersham, UK) at 22 °C for 2 h. The incorporation was then stopped by adding 1 ml of 5% formalin. Zero time blanks, where formalin was added together with the labelled substrate, were always included. For each bacterial suspension duplicate measurements were made. The labelled bacteria were filtered into glass fiber filters (Whatman GF/F) and washed with ice-cold 80% ethanol and 5% TCA. Solubilization of macromolecules (in 0.1 M NaOH at 90 °C for 1 h) and scintillation counting were carried out following Bååth (1992).

2.5. Concentration of N and P in plant tissues

The concentrations of N and P were determined in plant tissues (Lachica et al., 1973). Nitrogen and phosphorus utilization efficiency, defined as the amount of biomass produced per unit of nitrogen or phosphorus in plant tissues, was calculated.

2.6. Chitin and ergosterol content

In rhizosphere soil samples both fungal components were determined. Chitin was measured according to the method described by Ehbald et al. (1998), with modifications. Samples (1 g) were crushed in liquid N_2 to obtain a fine powder. The extract was resuspended in 3.0 ml of methanol and centrifuged at 4500 rpm for 20 min at 4 °C. This procedure was twice repeated. Supernatants were assigned to analyze the ergosterol content, whereas the pellets were subjected to chitin analysis. Each washed and freeze-dried pellet was treated with 0.2N NaOH to remove proteins and amino acids, which could interfere with glucosamine determination. An acid hydrolysis (6N HCl, v/v) was performed at 80 °C for 6 h in order to release glucosamine residues and followed by neutralization with 3 M sodium acetate. Glucosamine residues were evaluated colorimetrically at 653 nm.

Ergosterol was measured according to Salmanovicz and Nylund (1988) and Nylund and Wallander (1992), with modifications. Free ergosterol, as well as that bound forming sterol esters, contained in

the supernatant, were measured together. The sample processing, in brief, consisted of an evaporation of the methanolic fraction, saponification (KOH 4% in ethanol, 80 °C for 30 min) and a final partition with cyclohexane (4 ml). The alkaline ethanolysis was stopped with 2 ml of the mixture Na_2HPO_4 and KH_2PO_4 (0.1 g ml). The organic phase was evaporated under a stream of N_2 and the final dried residue was stored at −20 °C and dissolved in 200 μl of methanol. Ergosterol was separated by HPLC with a C18 reversed phase column (150 mm × 4.5 mm, 5 μm i.d.) and detected with an UV detector at 282 nm. The mobile phase was 100% methanol (HPLC grade) with a gradient flow rate, which began with 1.5 ml min^{-1} for 3 min, then decreased to 1.0 ml min^{-1} for 5 min. The chromatographic run was ended after 12 min.

2.7. Statistics

The results were statistically evaluated by factorial analysis of variance with bacterial treatment, mycorrhizal treatment and bacterial treatment–mycorrhizal treatment interaction as sources of variation. Percentage values were arcsine-transformed before statistical analysis.

3. Results

The results of factorial ANOVA are given in Table 1. While leucine incorporation and ergosterol were significantly highly increased by all microbial treatments, only mycorrhiza increased plant parameters.

Single bacterial treatments did not affect any determined growth parameter (Figs. 1 and 2). Two of the *Glomus* spp. caused increases in shoot weight. *G. mosseae* did not significantly increase the alfalfa yield, but the association of this AM fungus with each of the bacteria enhanced plant yield. On the contrary, *G. intraradices* did not change the effectiveness in coinoculation with the bacterium and *G. deserticola* associated with *B. pumillus* matched the growth of plants fertilized with P. In fact, the alfalfa biomass was much increased when the root system was infected with *G. deserticola*, associated with this particular *Bacillus* strain (Fig. 1). Plant growth responses did not arise from a direct bacterial effect on the percentage of AM colonization determined.

Although, the effectiveness of mycorrhizal inoculation in improving plant growth varied with the fungal isolates and the bacterium associated, the level of infectivity of each AM fungus was not modified by the bacterial strains. Thus, the enhancement of plant growth was not closely correlated with the level of mycorrhizal colonization (Fig. 1). Nevertheless *G. mosseae* was less infective and the least effective fungus increasing shoot and root growth parameters. *G. mosseae*-colonized plants also showed the lowest amount of total mycorrhizal roots.

The number of nodules formed was greatly stimulated by the fungal symbiont and P-fertilization.

Table 1
Significance of the main treatment effects and their interactions based on factorial ANOVA

	F-values		
	AM treatment	Bacterial treatment	Mycorrhiza* bacteria
Shoot growth	141.8***	2.8 NS	1.9 NS
Number of nodules	70.1***	2.7 NS	5.0***
AM colonization	340.7***	1.3 NS	1.6 NS
Root length	14.8***	1.0 NS	6.4***
Root surface	4.0*	1.7 NS	4.4**
Root dry weight	54.8***	0.4 NS	0.4 NS
Thymidine incorporation	3.3 NS	15.1***	17.1***
Leucine incorporation	18.7***	13.4***	38.8***
Ergosterol	19.9***	10.1***	6.7***
Chitin	6.8**	4.9*	9.1***

NS: not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

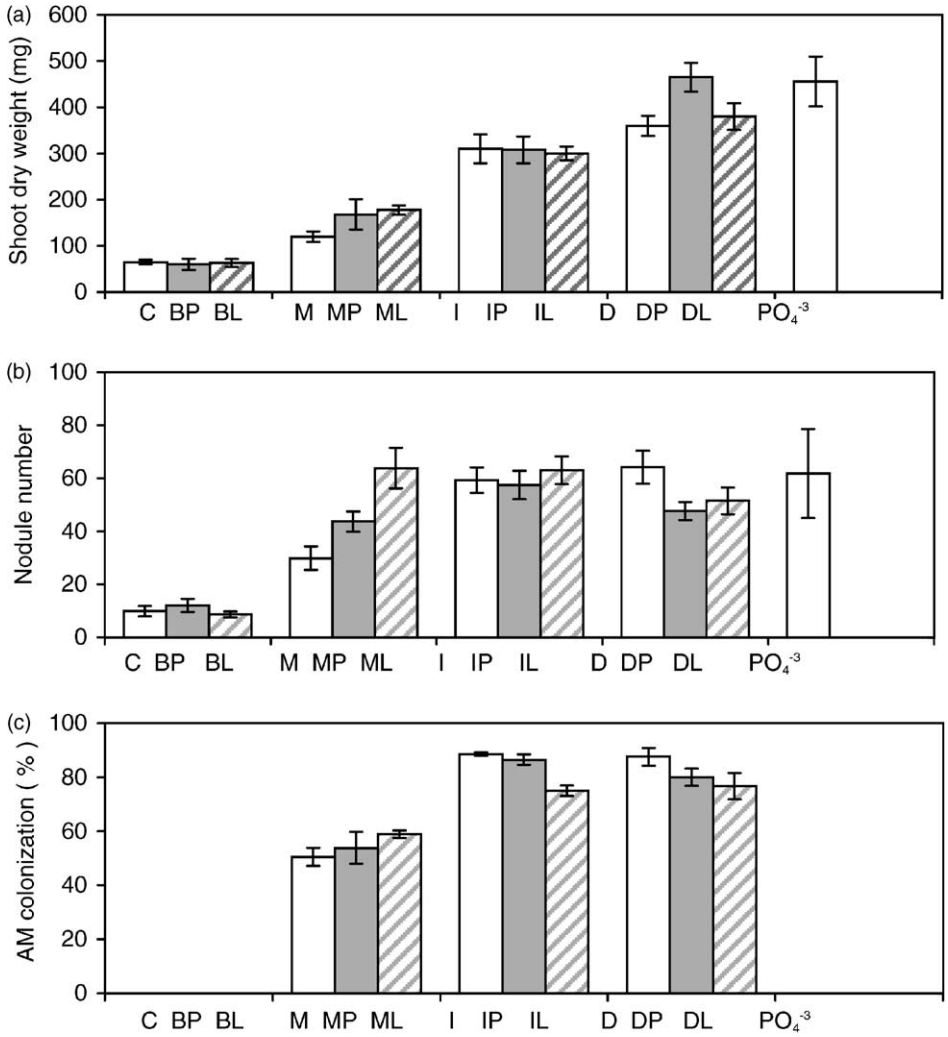

Fig. 1. Shoot dry weight (a), number of nodules (b) and AM colonization (c) of alfalfa plants. Treatments are designed as C, non-fertilized uninoculated control; PO_4^{3-}, P-fertilized uninoculated control; BP, *Bacillus pumillus*; BL, *Bacillus licheniformis*; M, *Glomus mosseae*; MP, *G. mosseae* + *B. pumillus*; ML, *G. mosseae* + *B. licheniformis*; I, *G. intraradices*; IP, *G. intraradices* + *B. pumillus*; IL, *G. intraradices* + *B. licheniformis*; D, *G. deserticola*; DP, *G. deserticola* + *B. pumillus*; DL, *G. deserticola* + *Bacillus licheniformis*. Vertical bars represent standard errors.

Non-mycorrhizal control plants show a restricted nodulation but in most AM plants, the number of nodules formed were similar to those in P supplied plants (Fig. 1).

AM colonization also increased root biomass. The total root length (axis plus lateral roots) and root surface were more increased by some biological treatments than by P-fertilization. Coinoculation of *G. deserticola* plus *B. pumillus* maximized all these values (Fig. 2).

Thymidine and leucine incorporation were increased by P-application and, to a lesser extent, by microbial inoculations. Both values were lower in non-inoculated control. The highest levels of thymidine and leucine incorporation in the inoculated treatments were reached with single *G. mosseae* or single

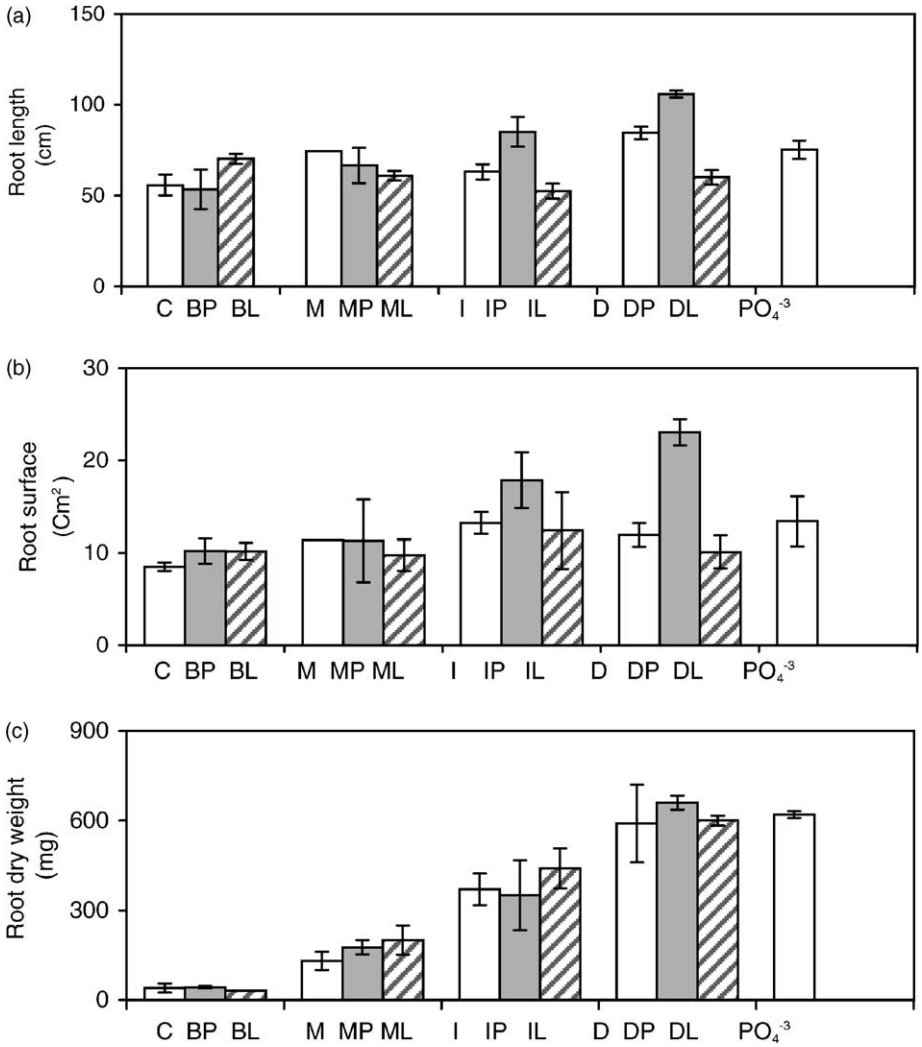

Fig. 2. Root length (a), root surface (b) and root dry weight (c) of alfalfa plants. Treatments are designed as C, non-fertilized uninoculated control; PO_4^{3-}, P-fertilizer uninoculated control; BP, *Bacillus pumillus*; BL, *Bacillus licheniformis*; M, *Glomus mosseae*; MP, *G.mosseae*+*B. pumillus*; ML, *G. mosseae* + *B. licheniformis*; I, *G. intraradices*; IP, *G. intraradices* + *B. pumillus*; IL, *G. intraradices* + *B. licheniformis*; D, *G. deserticola*; DP, *G. deserticola* + *Bacillus pumillus*; DL, *G. deserticola* + *B. licheniformis*. Vertical bars represent standard errors.

B. licheniformis inoculation, respectively, *B. licheniformis* associated with *G. intraradices* enhanced both values. Bacterial treatments had different effects according to the associated *Glomus* species (Fig. 3). The thymidine/leucine ratio was lowest in P fertilized treatments and highest in control or *G. intraradices* inoculated rhizosphere (Fig. 3).

The maximum ergosterol and chitin content was found when dual *B. pumillus* plus *G. mosseae* (ergosterol) or plus *G. intraradices* (chitin) were inoculated (Fig. 4). Differences between control and P-fertilized or inoculated treatments were higher for ergosterol than for chitin (Fig. 4). Ergosterol more than chitin was increased by mycorrhizal colonization and the bacterial effect on this value was more relevant in the presence of *G. mosseae*.

Nitrogen and P nutrient use-efficiency values were different for each nutrient according to the

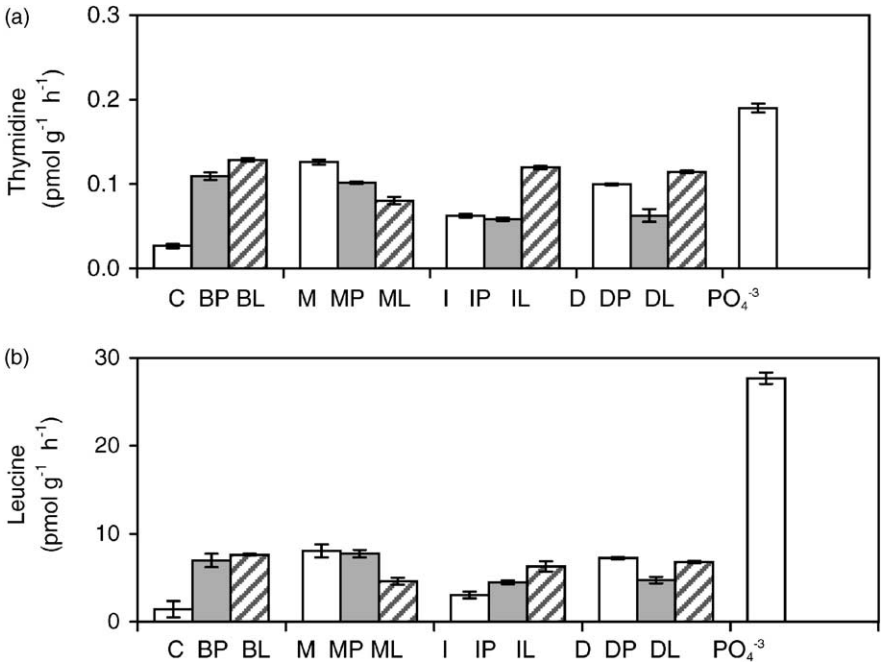

Fig. 3. Thymidine (a) and leucine (b) incorporation (pmol g^{-1} h^{-1}, *x*-axis) of alfalfa plants. Treatments are designed as C, non-fertilized uninoculated control; PO_4^{3-}, P-fertilizer uninoculated control; BP, *Bacillus pumillus*; BL, *Bacillus licheniformis*; M, *Glomus mosseae*; MP, *G. mosseae* + *B. pumillus*; ML, *G. mosseae* + *B. licheniformis*; I, *G. intraradices*; IP, *G. intraradices* + *B. pumillus*; IL, *G. intraradices* + *B. licheniformis*; D, *G. deserticola*; DP, *G. deserticola* + *B. pumillus*; DL, *G. deserticola* + *Bacillus licheniformis*. Vertical bars represent standard errors.

microbial treatments. The mycorrhizal treatment was more important for N use-efficiency (particularly by *G. mosseae*) than for P use-efficiency. Considering N and P use-efficiency, the mycorrhizal effect was higher than the P-fertilized effect. However, the bacterial inoculation was not relevant in increasing N and P use-efficiency in mycorrhizal colonized plants (Table 2). Differences in the N/P ratio between P-fertilized and mycorrhizal plants were also found (Table 3). These values indicate the different effectiveness of AM fungi on the uptake of those nutrients and the ability of *B. pumillus* to increase this ratio in mycorrhizal plants.

Table 2
Nutrient (N or P) use-efficiency (mg biomass/mg N or P recovered) of non-mycorrhizal P fertilized (PO_4^{3-}) or mycorrhizal plants with *Glomus mosseae*, *G. intraradices* or *G. deserticola* either non-inoculated or inoculated with bacteria [*Bacillus pumillus* (BP) or *Bacillus licheniformis* (BL)]

	N use-efficiency			P use-efficiency		
	–	BP	BL	–	BP	BL
PO_4^{3-}	181 ± 6.7	–	–	804 ± 8.0	–	–
Control	271 ± 4.9	428 ± 14.8	485 ± 27.8	1083 ± 4.3	2000 ± 25.2	2100 ± 50.0
G. mosseae	500 ± 28.9	259 ± 3.8	342 ± 5.7	1191 ± 58.4	1200 ± 57.7	1271 ± 73.1
G. intraradices	239 ± 5.5	208 ± 9.9	244 ± 4.5	1069 ± 91.4	1141 ± 17.3	1000 ± 62.9
G. deserticola	321 ± 5.9	256 ± 3.1	309 ± 8.6	1091 ± 67.7	1107 ± 42.9	1058 ± 103.2

Standard errors of the means are given.

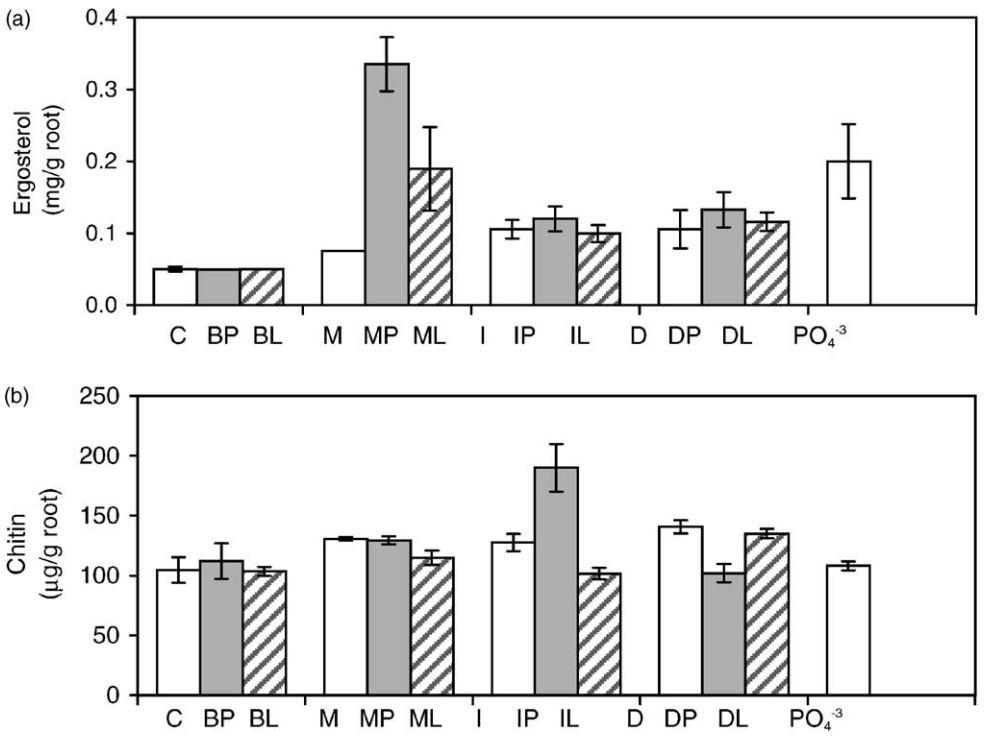

Fig. 4. Ergosterol (a), chitin (b) of alfalfa plants. Treatments are designed as C, non-fertilized uninoculated control; PO_4^{3-}, P-fertilizer uninoculated control; BP, *Bacillus pumillus*; BL, *Bacillus licheniformis*; M, *Glomus mosseae*; MP, *G. mosseae* + *B. pumillus*; ML, *G. mossea* + *B. licheniformis*; I, *G. intraradices*; IP, *G. intraradices* + *B. pumillus*; IL, *G. intraradices* + *B. licheniformis*; D, *G. deserticola*; DP, *G. deserticola* + *B. pumillus*; DL, *G. deserticola* + *Bacillus licheniformis*. Vertical bars represent standard errors.

Table 3
Shoot N/P ratio of non-mycorrhizal (control or P fertilized PO_4^{3-}) or mycorrhizal plants with *Glomus mosseae*, *G. intraradices* or *G. deserticola* either non-inoculated or inoculated with bacteria (*Bacillus pumillus* (BP) or *Bacillus licheniformis* (BL))

	–	BP	BL
Control	4.0 ± 0.5	4.7 ± 0.2	4.3 ± 0.2
PO_4^{3-}	4.4 ± 0.2	–	–
G. mosseae	2.2 ± 0.3	4.6 ± 0.3	3.7 ± 0.1
G. intraradices	4.5 ± 0.3	5.5 ± 0.2	4.1 ± 0.3
G. deserticola	3.4 ± 0.2	4.3 ± 0.1	3.4 ± 0.2

Standard errors of the means are given.

4. Discussion

It was apparent from the results that the various treatments examined have a different inherent potential for improving plant growth and nutrition and for altering rhizosphere bacterial and fungal populations. The changes observed, regarding growth values by mycorrhizal and/or bacterial inoculation were sometimes comparable to those of P-application.

Results show that the effect of the *Glomus* species on shoot and root growth is related to the associated bacterial strain. Certain specific effects in the *Glomus*–bacterium interaction have previously been reported (Azcón, 1989). Nevertheless, in the selective effect previously found, the bacterial groups involved belonged to different taxonomic families (*Enterobacteriaceae* and *Azotobacteriaceae*), selected on the basis of their abilities to produce amino acid and vitamins or to solubilize rock-phosphate. But in the present study, differences in compatibilities were found in *Glomus* sp. *Bacillus* species selected as IAA and gibberellin producers (Gutiérrez-Mañero et al., 1996, 2001). In agreement with this, differences in *Rhizobium* strains, according to the mycorrhizas *Glomus* associated, which influence the mutual

development and activity of each other, have also been reported (Azcón et al., 1991; Vázquez et al., 2001).

Here, the most effective microbial combinations were not the result of an increased percentage of mycorrhizal colonization. In general, the level of AM root infection was not closely related to the ability of the coinoculated treatments to improve plant growth. Functional differences in the characteristics of extraradical mycelium developed by each AM fungus (not determined here) could be expected (Jakobsen et al., 1992). The *Glomus* species may have different abilities to form external mycelium with respect to the length of root infected (Abbott and Robson, 1985) and this can be changed as a result of the bacterial inoculation.

In the case of the most efficient treatment, the greatest amount of mycorrhizal roots was determined. Moreover, if the development of *G. deserticola* colonization was affected by *B. pumillus* at any time during the experiment, it was not detected here. This possible effect was not evident at harvest time where nearly the whole root system was AM colonized. Bacterial activity can cause a stimulation of early infection (Barea and Azcón-Aguilar, 1983) and this effect correlates with the extent of growth enhancement (Abbott and Robson, 1985). A greater ability of dual inoculated roots to form external hyphae could also explain the bacterial effectivity (Azcón, 1987), since it affects mycorrhizal nutrient uptake.

The direct effect of *B. pumillus* on root growth (biomass, length and surface) as phytohormone producers may be the main cause of the growth response found in *G. deserticola* colonized plants, as reported by Piccini and Azcón (1987). The ability of this bacterium for auxin production, which regulates adventitious root formation (Nordström et al., 1991; Alvarez et al., 1989), suggests the involvement of these mechanisms. Nevertheless, this activity was not generalized and only *G. deserticola*-colonized plants take significant advantage of this bacterial ability (Azcón, 1989).

Mycorrhizal plants maximized the efficient use of N and P, but the bacterial effect was not relevant. Mechanisms such as the highest rates of nutrient translocation or root growth and activity may be involved. In a physiological sense, the utilization of a nutrient is the unit of dry matter produced per unit of nutrient (Marschner, 1995) and AM colonization promoted N and P utilization. Morphological and physiological root characteristics and the acquisition of nutrients by roots represent the most important role in nutrient use-efficiency (Gutschick, 1993). AM colonization and rhizosphere microorganisms have been shown to alter root morphology (Kothary et al., 1990; Azcón-Aguilar et al., 1996; Galleguillos et al., 2000) but here, a non-consistent relationship was found between root length and surface and N and P use-efficiency.

The N/P ratio increased as a consequence of *B. pumillus* inoculation, which indicates that the bacterium increased N uptake more efficiently than P uptake in *Medicago* plants. The different effect of *Glomus* sp. on this N/P ratio means that the uptake of N (in relation to P uptake) was differently affected by each AM fungus.

The two *Bacillus* species had different effects on thymidine and leucine values according to the associated *Glomus* species. It was expected that these values decrease with time. Nevertheless, the activities detected at the end of the experiment were higher in inoculated than in control treatments. The *Bacillus* spp. used here did not survive in inoculated soil for longer than 1 month (personal communication). Soil commonly reacts as a “biological buffer”, hence any change in its microbial population is only temporary (Bashan, 1999). In most soils, the inoculated bacterial population declined rapidly within the first 2 weeks. The P fertilizer effect, increasing leucine and thymidine incorporation was expected since nutrients amendment increased the bacterial population (Germida and Walley, 1996; Smith et al., 1984). Data reported by Bashan (1999) show that the viability of *A. brasilense* declined within 35 days of inoculation.

The inoculated *Bacillus* strain was effective, increasing thymidine and leucine values, above all, when singly inoculated. These results are probably due to good adaptation and the stimulating effect on root surface of these bacteria on the experimental soil/plant conditions used.

Phosphorus and AM fungi also increased these values over control. We can confirm that metabolic activity of the indigenous rhizosphere bacteria increased with the inocula introduced. But the stimulation generated for dual inoculation was lower in some cases than that generated by individual inocula. In general, these values remained higher in the inoculated than in non-inoculated control rhizosphere. Changes in the

physicochemical properties of the soil may have a much greater effect on the survival of the inoculated bacterial (Stotzky, 1997).

Meyer and Linderman (1986) show that AM colonization changed the population of specific groups of bacteria and Amora-Lazcano and Azcón, 1997; Amora-Lazcano et al., 1998) reported the specific effect of AM fungi on sulfur and nitrogen cycling microorganisms, respectively. This mycorrhizal activity on rhizosphere communities may be a consequence of changes in physical and/or chemical characteristics in the environment close to mycorrhizal roots. Mycorrhizal mycelium might act as a typical rhizosphere organism and show synergism or microbial competence with other rhizosphere populations. Moreover, the different physiology of AM roots may alter root exudation as well as rooting patterns, influencing the microbial growth conditions (Christensen and Jacobsen, 1993; Andrade et al., 1995; Germida and Walley, 1996). Mycorrhizal fungi do not consume organic C from the soil as a growing medium due to their symbiotic condition, but their effect on the host's physiological processes affects the hydrocarbonate status of the rhizosphere environment (Ruiz-Lozano et al., 1995a,b; Paulitz and Linderman, 1989). In the case of legume plants, where dual endophytes may compete for carbon compounds in the host (Ruiz-Lozano and Azcón, 1993) it is difficult to predict the outcome of interaction between AM fungi-*Sinorhizobium* and the bacterium in relation to the microbial activity and plant growth.

In the particular interactions observed, the symbiont, *S. meliloti*, may be involved in spite of being uniformly applied to mycorrhizal and non-mycorrhizal plants However, the bacterial biomass seems to be considerably reduced by microbial inoculation, as compared to P-fertilization, since the inoculated plants have similar or higher root development (length and surface).

Considering these results, AM mycelium did not serve as a carbon source for microbial groups, as Bethlenfalvay and Schuepp (1994) suggested. Authors such as Paulitz and Linderman (1989) and Christensen and Jacobsen (1993), counting the inoculated bacteria in the rhizosphere soil, found a depression in the mycorrhizal rhizosphere zone. Recently, Olsson et al. (1996) reported that soil bacteria respond to the presence of roots but not to the mycelium of AM fungus.

According to Christensen and Jacobsen (1993), the AM colonization probably decreased the amount of plant root-derived organic matter available for bacterial growth. Thus, AM-colonized plants seem to be better adapted to compete with the saprophytic soil microorganisms for major nutrients (N, P) and micronutrients than non-mycorrhizal or P-amended plants. Regarding results, the bacterial biomass in *Bacillus* inoculated treatments increased to the highest extent in non-mycorrhizal rhizosphere soil. In mycorrhizal-colonized plants, the increased thymidine incorporation over control indicates a higher growth rate of bacterial population. Reports of interaction between bacteria and AM in the rhizosphere includes negative (Olsson et al., 1996) as well as positive (Andrade et al., 1995; Azcón-Aguilar and Barea, 1992) effects of bacteria because of the presence of mycorrhiza.

The chitin level, as a general measure of fungal biomass including saprophytes, AM fungi and even dead fungal mycelium, was particularly increased by *B. pumillus* in *G. intraradices* colonized rhizosphere soil. Ergosterol values would be indicative of active mycelium measurement (Frey et al., 1994) but the highest bacterial stimulation on ergosterol was determined only in *G. mosseae* colonized rhizosphere soil affected by *B. pumillus*. However, no consistent relationship between these fungal values and mycorrhizal effect on plant growth parameters was tested. These results provide evidence that in general, ergosterol increased in AM treatments coinoculated with *B. pumillus*.

Frey et al. (1994) found a correlation between ergosterol and chitin content and AM extraradical mycelium. Nevertheless, Frey et al. (1992) reported that ergosterol could be proposed as an indication of AM fungal biomass only in the early stages of AM colonization. Likewise, Schmitz et al. (1991) considered the chitin content of limited value since it apparently does not correlate well with the different AM structures. These authors also reported that ergosterol content cannot be taken as a useful index for AM colonization measurements. Beilby (1980) was unable to detect ergosterol in spores of *A. leavis* and Nordby et al. (1981) reported that $<0.1\%$ of the total sterol content in spores of *G. mosseae* accounted for the ergosterol found. Thus, the similar amount of both metabolites found in mycorrhizal

and P-fertilized plants is an indication of the reduced fungal populations (non-mycorrhizal ones) in mycorrhizal treatments, having longer root length.

Exceptionally, *B. pumillus* increases chitin content in *G. intraradices* colonized soil but in contrast, it decreases chitin when associated with *G. deserticola*. These results are in concordance with those reported by Andrade et al. (1998) about the preferences (ecological specificities) between the host plant and its associated AM fungi in the mycorrhizosphere and they may also extend to the soil associated microorganisms (McGonigle and Fitter, 1990). Results suggest that *B. pumillus* has a preference for *G. intraradices* as a fungal host, but it did not affect mycorrhizal response on plant growth. Andrade et al. (1998) pointed out that preference of the bacterial isolate for a specific AM fungal host may not be mutually advantageous, since AM hyphal length declines in the presence of the bacterium. The relationships between the mycorrhiza and the associated bacteria may affect other members of the mycorrhizosphere community. Different AM fungi have different effects on bacterial and fungal populations and this can be attributed to specific competition for growth substrates (Marschner and Crowley, 1996). Curiously, *B. licheniformis* increased thymidine and leucine incorporation and reduced chitin concentration in the rhizosphere of *G. intraradices* colonized plants. The understanding of the mechanisms through which rhizosphere microbial populations (bacteria and/or fungi) interact is very important for the management of sustainable systems (Hamel, 1996), but requires further study.

It is difficult to predict the outcome of interactions between bacteria and AM fungi in relation to microbial activity, as well as the meaning of this activity regarding the interaction of specific groups of microorganisms on plant growth. The infectivity of AM fungi did not correlate with the activity of rhizosphere bacteria or fungi determined. The soil microbial community appears to increase with both PO_4^{3-} fertilization and microbial inoculations, but the community structure changed consistently according to the chemical or biological treatments applied. Comparing PO_4^{3-} fertilization and *G. deserticola–B. pumillus* inoculated plants, which have similar effects on shoot and root growth, differences in leucine and chitin values indicated that in AM-colonized rhizosphere, fungi dominated bacteria and in P fertilized rhizosphere, bacteria dominated fungi.

The present results provide strong evidence about AM colonization in addition to the associated bacterial groups specifically change ecological soil conditions which affect the microbial community (bacteria and fungi). The role and contribution of AM symbiosis to plant growth and efficient use of N and P may be increased in association with selected rhizosphere bacteria. The microbial communities associated to the root system are key factors in the contex of sustainable systems since they regulate the availability of plant nutrient.

Acknowledgements

Financial support for this study was obtained from Comision Interministerial de Ciencia y Tecnología (CICYT) PN REN 2000-1506 GLO. One of us (A. Medina) is indebted to Junta de Andalucia for scholarship grants. The author thank very much Linda Hammalaimen for her linguistic revision of manuscript.

References

Abbott, L.K., Robson, A.D., 1985. Formation of external hyphae in soil by four species of vesicular arbuscular mycorrhizal fungi. New Phytol. 99, 257–265.

Ahmad, M.H., 1995. Compatibility and coselection of vesicular-arbuscular mycorrhizal fungi and rhizobia for tropical legumes. Crit. Rev. Biotech. 15, 229–239.

Alvarez, R., Nissen, S.J., Sutter, E.G., 1989. Relationship between indole-3-acetic acid levels in apple (*Malus pumila* Mill) rootstocks cultured in vitro and adventitious root formation in the presence of indole-3-butyric acid. Plant Physiol. 89, 439–443.

Amora-Lazcano, E., Azcón, R., 1997. Response of sulfur cycling microorganisms to arbuscular mycorrhizal fungi in the rhizosphere of maize. Appl. Soil Ecol. 6, 217–222.

Amora-Lazcano, E., Vázquez, M.M., Azcón, R., 1998. Response of nitrogen transforming microorganisms to arbuscular mycorrhizal fungi. Biol. Fert. Soil 27, 65–70.

Andrade, G., Azcón, R., Bethlenfalvay, G.J., 1995. A rhizobacterium modifies plant and soil responses to the mycorrhizal fungus *Glomus mosseae*. Appl. Soil Ecol. 2, 195–202.

Andrade, G., Linderman, R.G., Bethlenfalvay, G.J., 1998. Bacterial associations with the mycorrhizosphere and hyphosphere of the arbuscular mycorrhizal fungus *Glomus mosseae*. Plant Soil 202, 79–87.

Azcón, R., 1987. Germination and hyphal growth of *Glomus mosseae* in vitro: effect of rhizosphere bacteria and cell-free culture media. Soil Biol. Biochem. 19, 417–419.

Azcón, R., 1989. Selective interaction between free-living rhizosphere bacteria and vesicular-arbuscular mycorrhizal fungi. Soil Biol. Biochem. 21, 639–644.

Azcón, R., 1993. Growth and nutrition of nodulated mycorrhizal and non-mycorrhizal *Hedysarum coronarium* as a result of treatments with fractions from a plant growth-promoting rhizobacteria. Soil Biol. Biochem. 25, 1037–1042.

Azcón, R., Rubio, R., 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., Padilla, I.G., Encina, C.L., Azcón, R., Barea, J.M., 1996. Arbuscular mycorrhizal inoculation enhances plant growth and changes root system morphology in micropropagated *Annona cherimola* Mill. Agronomie 16, 647–652.

Azcón-Aguilar, C., Barea, J.M., 1992. Interactions between mycorrhizal fungi and other rhizosphere microorganisms. In: Allen, M.J. (Ed.), Mycorrhizal Functioning: An Integrative Plant-Fungal Process. Chapman & Hall, New York, pp. 163–198.

Bååth, E., 1990. Thymidine incorporation into soil bacteria. Soil Biol. Biochem. 22, 803–810.

Bååth, E., 1992. Thyamidine incorporation into macromolecules of bacteria extracted from soil by homogenisation-centrifugation. Soil Biol. Biochem. 24, 1157–1165.

Bååth, E., 1994. Measurement of protein synthesis by soil bacterial assemblages with the leucine incorporation technique. Biol. Fert. Soils 17, 147–153.

Barea, J.M., Azcón-Aguilar, C., 1983. Mycorrhizas and their significance in nodulating nitrogen-fixing plants. In: Brady, N.C. (Ed.), Advances in Agronomy, vol. 36. Academic Press, New York, pp. 1–54.

Barea, J.M., Jeffries, P., 1995. Arbuscular mycorrhizas in sustainable soil plant systems. In: Varma, A., Hock, B. (Eds.), Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology, Springer, Heidelberg, pp. 521–559.

Barea, J.M., Andrade, G., Bianciotto, V., Dowling, D., Lohrke, S., Bonfante, P., O'Gara, F., Azcón-Aguilar, C., 1998. Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for the biocontrol of soil-borne plant fungal pathogens. Appl. Environm. Microbiol. 64, 2304–2307.

Bashan, Y., 1999. Interactions of *Azospirillum* spp. in soils: a review. Biol. Fertil. Soils 29, 246–256.

Beilby, J.P., 1980. Fatty-acid and sterol composition of ungerminated spores of the vesicular-arbuscular mycorrhiza fungus *Acaulospora laevis*. Lipids 15, 949–952.

Beringer, J.E., 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84, 188–198.

Bethlenfalvay, G.J., Schuepp, H., 1994. Arbuscular mycorrhizas and agrosystem stability. In: Gianinazzi, S., Schüepp, H. (Eds.), Impact of Arbuscular-Mycorrhizas on Sustainable Agriculture and Natural Ecosystems. Birkhäuser, Basel, pp. 117–131.

Bowen, G.D., Rovira, A.D., 1999. The rhizosphere and its management to improve plant growth. Adv. Agronomy 66, 1–102.

Christensen, H., Jacobsen, I., 1993. Reduction of bacterial growth by a vesicular-arbuscular mycorrhizal fungus in the rhizosphere of cucumber (*Cucumis sativus* L.). Biol. Fertil. Soils 15, 253–258.

Ehbald, A., Wallander, H., Näsholm, T., 1998. Chitin and ergosterol combined to measure total living fungal biomass in ectomycorrhizas. New Phytol. 138, 143–149.

Frey, B., Buser, H.R., Schüepp, H., 1992. Identification of ergosterol in vesicular-arbuscular mycorrhizae. Biol. Fertil Soils 13, 229–234.

Frey, B., Vilariño, A., Schuepp, H., Arines, J., 1994. Chitin and ergosterol content of extraradical and intraradical mycelium of the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices*. Soil Biol. Biochem. 26, 711–717.

Galleguillos, C., Aguirre, C., Barea, J.M., Azcón, R., 2000. Growth promoting effect of two *Sinorhizobium meliloti* strains (a wild type and its genetically modified derivative) on a non-legume plant species in specific interaction with two arbuscular mycorrhizal fungi. Plant Sci. 159, 57–63.

Germida, J.J., Walley, F.L., 1996. Plant growth-promoting rhizobacteria alter rooting patterns and arbuscular mycorrhizal fungi colonization of field-grown spring wheat. Biol. Fert. Soils 23, 113–120.

Giovannetti, M., Mosse, B., 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84, 489–500.

Gutiérrez-Mañero, F.J., Acero, N., Lucas, J.A., Probanza, A., 1996. The influence of native rhizobacteria on European alder growth. Characterizacion and biological assay of metabolites produced by growth promoting and growth inhibiting bacterial. Plant Soil 182, 67–74.

Gutierrez Mañero, F.J., Ramos, B., Probanza, A., Mehouachi, J., Tadeo, F.R., Talón, M., 2001. The plant-growth promoting rhizobacteria *Bacillus pumillus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiol. Plantarum. 111, 206–211.

Gutschick, V.P., 1993. Nutrient-limited growth rates: roles of nutrient-use-efficiency and of adaptation to increase uptake rates. J. Exp. Bot. 44, 41–52.

Hamel, C., 1996. Prospects and problems pertaining to the management of arbuscular mycorrhizae in agriculture. Agric. Ecosyst. Environ. 60, 197–210.

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.

Jakobsen, I., Abbott, L.K., Robson, A.D., 1992. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. Part I. spread of hyphae and phosphorus inflow into roots. New Phytol. 120, 371–380.

Kloepper, J.W., Schroth, M.N., 1978. Plant growth-promoting rhizobacteria on radishes. In: Gibert-Clarey, T. (Ed.), Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, Station de Pathologie Végétale et Phytobactériologie, vol. 2. INRA, Angers, France, pp. 879–882.

Kothary, S.K., Marschner, H., Romheld, V., 1990. Direct and indirect effects of VA mycorrhizal fungi and rhizosphere microorganisms on acquisition of mineral nutrients by maize (*Zea mays* L.) in a calcareous soil. New Phytol. 116, 637–645.

Lachica, M., Aguilar, A., Yánez, J., 1973. Analisis foliar: métodos utilizados en la estación experimental del Zaidín. Anal. Edafol. Agrobiol. 32, 1033–1947.

Linderman, R.G., 1986. Managing rhizosphere microorganisms in the production of horticultural crops. Hortscience 21, 1299–1302.

Marschner, H., 1995. Mineral Nutrition of Higher Plants, 2nd ed. Academic Press, London.

Marschner, P., Crowley, D.E., 1996. Physiological activity of a bioluminescent *Pseudomonas fluorescens* (strain 2–79) in the rhizosphere of mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L.). Soil Biol. Biochem. 28, 869–876.

McGonigle, T.P., Fitter, A.H., 1990. Ecological specificity of vesicular arbuscular mycorrhizal associations. Mycol. Res. 94, 120–122.

Meyer, J.R., Linderman, T.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.

Mosse, B., 1973. Advances in the study of vesicular arbuscular mycorrhiza. Ann. Rev. Phytopathol. 11, 171–195.

Nordby, H.E., Nemec, S., Nagy, S., 1981. Fatty acids and sterols associated with citrus root mycorrhizae. J. Agric. Food Chem. 29, 396–401.

Nordström, A.C., Albarado, F., Eliasson, L., 1991. Effect of exogenous indole-3-acetic acid and indole-3-butyric acid on internal levels of the respective auxins and their conjugation with aspartic acid during adventitious root formation in pea cutting. Plant Physiol. 96, 856–861.

Nylund, J.E., Wallander, H., 1992. Ergosterol analysis as a means of quantifying mycorrhizal biomass. Methods Microbiol. 24, 77–88.

Olsson, P.A., Bååth, E., Jakobsen, I., Söderström, B., 1996. Soil bacteria respond to presence of roots but not to mycelium of arbuscular mycorrhizal fungi Soil. Biol. Biochem. 28, 463–470.

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

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Brit. Mycol. Soc. 55, 159–161.

Piccini, D.F., Azcón, R., 1987. Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizal fungi on the utilization de Bayovar rock phosphate by alfalfa plants using a sand-vermiculite medium. Plant Soil 101, 45–50.

Probanza, A., Lucas, J.A., Acero, N., Gutierrez Mañero, F.J., 1996. The influence of native rhizobacteria on european alder (*Alnus glutinosa* (L.) Gaertn.) growth. Part I. Characterization of growth promoting and inhibiting bacterial strains. Plant Soil 182, 59–66.

Probanza, A., Mateos, J.L., Lucas, J.A., Ramos, B., de Felipe, M.R., Gutierrez mañero, F.J., 2001. Effects of inoculation with PGPR *Bacillus* and *Pisolithus tinctorius* on *Pinus pinea* L. growth, bacterial rhizosphere colonization and mycorrhizal infection. Microb. Ecol. 41, 140–148.

Requena, N., Pérez-Solís, E., Azcón-Aguilar, C., Jeffries, P., Barea, J.M., 2001. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl. Environ. Microbiol. 67, 495–498.

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

Ruiz-Lozano, J.M., Azcón, R., Gómez, M., 1995a. Effects of arbuscular-mycorrhizal *Glomus* species on drought tolerance: physiological and nutritional plant responses. Appl. Environ. Microbiol. 61, 456–460.

Ruiz-Lozano, J.M., Gómez, M., Azcón, R., 1995b. Influence of different *Glomus* species on the time-course of physiological plant responses of lettuce to progressive drought stress periods. Plant Sci. 110, 37–44.

Salmanovicz, B., Nylund, J.E., 1988. High preformance liquid chromatography determination of ergosterol as a measure of ectomycorrhiza infection in scots pine. Eur. J. Forest Pathol. 18, 291–298.

Schreiner, R.P., Mihara, K.L., McDaniel, H., Bethlenfalvay, G.J., 1997. Mycorrhizal fungi influence plant and soil functions and interactions. Plant Soil 188, 199–209.

Schmitz, O., Danneberg, G., Hundeshagen, B., Klingner, A., Bothe, H., 1991. Quantification of vesicular-arbuscular mycorrhiza by biochemical parameters. J. Plant Physiol. 139, 106–114.

Smith, S.E., Dickson, S., Morris, C., Smith, F.A., 1984. Transfer of phosphate from fungus to plant in VA mycorrhizas: Calculation of the area of symbiotic interface and of fluxes of P from two different fungi to *Allium porrum* L. New Phytol. 127, 93–99.

Smith, S.E., Gianinazzi-Pearson, V., Koide, R., Cairney, J.W.G., 1994. Nutrient Transport in mycorrhizas: structure, physiology and consequences for efficiency of the symbiosis. Plant Soil 159, 103–113.

Stotzky, G., 1997. Soil as environment for microbial life. In: van Elsas, J.D., Trevors, J.T., Wellington, E.M.H. (Eds.), Modern Soil Microbiology. Marcel Dekker, New York, pp. 1–20.

Toro, M., Azcón, R., Barea, J.M., 1997. Improvement of arbuscular mycorrhizal development by inoculation with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. Appl. Environ. Microbiol. 63, 4408–4412.

Toro, M., Azcón, R., Barea, J.M., 1998. The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate-solublizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*. New Phytol. 138, 265–273.

Vázquez, M.M., Azcón, R., Barea, J.M., 2001. Compatibility of a wild type and its genetically modified *Sinorhizobium* strain with two mycorrhizal fungi on *Medicago* species as affected by drought stress. Plant Sci. 161, 347–358.