Symbiosis, 33 (2002) 23–38
Balaban, Philadelphia/Rehovot

Interactive Effect of Suspension or Encapsulated Inoculum of *Bacillus thuringiensis* Associated with Arbuscular Mycorrhizal Fungus on Plant Growth Responses and Mycorrhizal Inoculum Potential

A. MARULANDA, A. VIVAS, M. VASSILEVA, and R. AZCON*
Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain, Tel. +34-958-121011 (ext. 298), Fax. +34-958-129600, E-mail. rosario.azcon@eez.csic.es

Received April 7, 2002; Accepted July 2, 2002

Abstract

The inoculation of *Bacillus thuringiensis* applied as cells in suspension or encapsulated in combination or not with an arbuscular mycorrhizal (AM) fungus *Glomus occultum* was examined in a microcosm system on *Trifolium repens* plants. Succinate dehydrogenase (SDH) and alkaline phosphatase (ALP-ase) fungal activities, as index of active fungal metabolism, were determined in single and dual inoculated plants. The efficiency of *G. occultum* in increasing plant growth, nutrient content and nodulation was maximum when co-inoculated with the bacterium particularly in the encapsulated form. The percentage increases in growth and nutrition in AM plants co-inoculated with the encapsulated bacterium were 199 (shoot), 170 (N) and 199 (P) compared with plants inoculated with *G. occultum* only. Dual inoculation with these locally isolated microorganisms enhanced plant capacity for nutrient uptake. The mycorrhizal responses were correlated with the number of nodules and the amount of root cortex that became mycorrhizal. The bacterium, independent of the form of application, increased the extent of fungal mycelium showing ALP-ase activity. The impact of *B. thuringiensis* was greater on the ALP-ase activity in the mycelium than in the arbuscules.

*The author to whom correspondence should be sent.

0334-5114/2002/$05.50

This led to the hypothesis that the highest nodulation induced by the bacterium would interact with ALP-ase arbuscular activity. The increased metabolic mycelial ALP-ase activity as affected by *B. thuringiensis*, improved N and P content in shoots of co-inoculated plants. Furthermore, *B. thuringiensis* increased the inoculum potential of *G. occultum* from 129% (suspension) to 147% (encapsulated). Cell encapsulation was the treatment that particularly improved the efficiency of the bacterial inoculum when associated with the mycorrhizal fungus. Thus, encapsulation can be considered as a good form for bacterial inoculum application.

Keywords: Bacterial encapsulation, mycorrhizal fungus bacterium interaction, mycorrhizal succinate dehydrogenase (SDH) and alkaline phosphatase (ALP) activities

1. Introduction

Efforts have been made to increase the plant growth stimulating effect by combining inoculants from different microbial groups. It is generally assumed that the beneficial effect of AM fungi and bacterial co-inoculations is a result of interactions that increase nutrient uptake by the host plant. Root colonization and activity of AM fungi are affected by different factors including soil bacteria (Azcón-Aguilar et al., 1985; Azcón, 1987; Mayo, 1986; Grindler et al., 1995). The enhancement of mycorrhizal formation by selected microorganisms has been used to maximize growth and nutrition of plants (Gryndler et al., 2000).

In recent studies we showed that a locally isolated strain of *Bacillus thuringiensis*, able to produce 2.28 ppm of indol acetic acid (IAA), increased the physiological and metabolic mycorrhizal status of *Glomus mosseae* and *G. intraradices* colonized plants increasing plant nutrition in a soil-less growth medium. Thus, the next step was to verify if the bacterial effect would be maintained and expressed in the presence of different *Glomus* spp. using more realistic conditions (soil as growing medium) where microbial interactions normally result in increased host nutritional status. On the other hand, testing influence of bacterial inoculum formulations is an interesting study topic.

Until recently immobilized-cell technology for agricultural purposes has been limited to preparations of nitrogen-fixing bacteria (Elsas and Heijnen, 1990).

Although it has been shown that rhizosphere bacteria could positively interact with AM fungi in a wide range of media and host plants (Galleguillos et al., 2000; Grindler et al., 2000) little information has been presented to demonstrate the influence of different inoculum formulations. The preparation of microbial inoculants by alternative methods has shown considerable advantages to the traditional methodologies (Vassilev and Vassileva, 1992).

These authors demonstrated that the effectiveness of microbial processes in fermentation conditions could be increased by inoculating immobilized microbial cells (Vassilev et al., 1996, 1997b; Vassileva et al., 1998, 2000). In particular, encapsulation and gel-entrapment methods were successfully applied in preparation of inoculum formulations containing P-solubilizing microbial cultures (Vassilev et al., 1997a; b). Similarly, co-encapsulation of soil microorganisms with different rhizosphere functions such as N_2-fixing bacteria, P-solubilizing bacteria or fungi, mycorrhizal fungi or mycorrhiza helper microorganisms proved to be a highly efficient method of application for plant growth response when inoculated in soil-plant systems even after a long-term storage (Vassilev et al., 2001abc)

Encapsulated inocula possess better physical attributes such as being less weight and easy to transport (Bashan, 1998). The immobilization of viable microbial cells in natural, biodegradable polymers is an effective and safe technique (Cassidy et al., 1996; Vassilev et al., 2001d).

In this study we determined how different forms of application of a bacterial inoculum interacted with AM symbiosis at the level of plant growth responses, mycorrhizal development and activity. To compare the activity of mycorrhiza, both alkaline phosphatase (ALP-ase) and succinate dehydrogenase (SDH) have proved to be useful (Boddington and Dodd, 1998, 1999).

Changes in the metabolic characteristics of *G. occultum* colonization was tested using fungal SDH and ALP-ase activities. These both fungal values, determining the effectivity AM symbiosis, were proposed by Tisserant et al. (1993) and Guillemin et al. (1995) as indexes of fungal vitality and activity.

The aim of this study was to determine if the isolate of *Bacillus thuringiensis*, applied as suspension (BS) or as encapsuled inoculum (BE), enhanced the vitality and activity (in terms of SDH and ALP-ase activities) of the autochthonous mycorrhizal inoculum of *G. occultum* (M) and also to demonstrate the effectivity of the bacterial encapsulated inoculum on plant growth and nutrition in combination or not with the AM fungus *G. occultum*.

An additional objective was to determine the importance of the bacterium as a possible factor contributing to the inoculum potential of *G. occultum*. Changes in AM propagules left in soil at harvest time influence the next crop. Thus, bacterial effect on mycorrhizal propagules remaining in soil for the subsequent crop, their viability and infective capacity was also determined.

2. Materials and Methods

Experimental design

Plants were inoculated or not (control treatments) with one arbuscular

mycorrhizal fungus and/or one bacterium applied in two forms, as suspension or as encapsuled inoculum. Single or dual AM fungus-bacterium combinations were assayed. Five replicates per treatment were made given 30 pots placed in a randomized block design.

Soil characteristics

The soil was collected from the experimental field of the Estación Experimental del Zaidín, Granada, sieved (2 mm pore size), mixed with quartz sand (1/1, v/v) and autoclaved (100°C, 1 h on 3 consecutive days) and then re-inoculated with a soil filtrate containing the specific soil microbial populations except AM propagules. The characteristics of this agricultural soil were: pH of 7.8; 2.07% organic matter; 0.1% total N; 32 μg P/g ($NaHCO_3^-$ extractable P) (Olsen et al., 1954); 311.2 μg K/g (exchangeable); with 35.86% sand; 43.6% silt and 20.54% clay. Pots were filled with 500 g of sterilized soil/sand mixture.

Inoculation treatments

The mycorrhizal inoculum consisted of spores, hyphae and AM colonized root fragments (70% of colonization) of a local isolate of *Glomus occultum* (Walker) multiplicated and maintained as a stock culture of this AM fungus with *Lactuca sativa*. Inoculum (10 g/pot) was placed directly below the seeds in each pot.

The culture of *Bacillus thuringiensis* in this study was isolated from a local rhizosphere soil. Soil samples of 1 g (wet weight) were used to prepare a ten-fold dilution series in sterile water. Tubes containing 10 ml of soil dilutions were plated (0.1 ml/plate) from 10^4 to 10^8 dilutions following a standard procedure as described by Pochon and Tardieux (1962) and cultivated in our laboratory. It was identified using molecular techniques (see below). The bacterium was grown in a shaker at 150 rpm at 28°C for 30 h in 250 ml flasks containing 50 ml of a nutrient broth medium (Oxid) (8 g l^{-1}) pH 7.5 to a density of 10^8 cfu ml^{-1}. This culture was used to inoculate plants as free-suspension application (1 ml per plant) or to provide bacterial mass for immobilization (see below).

Encapsulated cells were prepared by mixing 100 ml of bacterial cell suspension with 100 ml of previously dissolved and sterilized sodium alginate (3%), supplemented with 3% skim milk powder. The mixture was homogenized for 3–4 min at low speed and then dropped into sterile 0.1 M $CaCl_2$ solution to form 2 mm beads. Encapsulated bacterial cells were separated from the liquid after hardening for 2 h and washed with sterile distilled water. The bacterial density applied per pot (10^8 cfu ml^{-1}) was the same as free-suspension or encapsulated inoculum.

The *Rhizobium* inoculum consisted of 1 ml per pot of *R. leguminosarum* biovar *trifoli* culture applied bellow the seeds. It was prepared following standard procedures (Azcón et al., 1991) and containing 10^9 cells ml^{-1}.

Identification of the bacterial isolate

Total DNA from the bacterial isolate used in this experiment was obtained as described by Giovannetti et al. (1990) and characterized by sequence analysis of the small ribosomal subunit (16S ribosomal DNA). PCR amplification was carried out with the eubacterial primers 27f and 1495r (Lane, 1991), located respectively, at the extreme 5' and 3' of the ribosomal rDNA sequence, which enable the amplification of nearly the entire gene. The amplification reactions were performed in a 20-µl volume containing 0.5 µM concentrations of each primer, 100 µM dNTPs, 1X PCR buffer (Sigma, St. Louis, MO, USA), 2.5 mM $MgCl_2$, 10 ng of genomic DNA and 0.25 U Taq DNA polymerase (Sigma). A PerkingElmer/Cetus DNA Thermal Cycler was employed with the following parameters: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, elongation at 72°C for 1 min and a final elongation at 72°C for 5 min. The amplified DNA was purified following electrophoresis through a 1.2% agarose gel with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pGME plasmid (Promega) for sequencing. Database searches for 16S rDNA sequence similarity using FASTA and BLAST algorithms unambiguously identified the selected bacterial isolate as a member of the genus *Bacillus*. The 16S rDNA sequence showed its highest similarity (more than 98%) with *Bacillus thuringiensis*.

Host plant and growth conditions

Trifolium repens L. was used as the test plant. Five surface sterilized seeds were sown and thinned to four seedling per pot after shoot emergence in pots containing 500 g soil/sand mixture (see above).

Plants were grown in a controlled environmental chamber under conditions of 50% relative humidity, day and night temperatures of 27°C and 18°C, respectively, and a photoperiod of 14 h. Photosynthetic photon flux density (PPFD) was 503 $\mu mol/m^2/s$ as measured with a lightmeter (LICOR, model LI-188B). Water was supplied by daily weighing to maintain the required water level (100% of water holding capacity) of the test soil/sand mixture through the experiment.

Plants from each treatment were harvested after 60 days of growth. At harvest, the root system was separated from the shoot, and dry weights were determined of the shoot and root tissues.

Measurements

Plant shoots were weighed and dried in a forced-draught oven at 70°C for 1 day, weighed and ground in a Wiley Mill to pass a 0.5 mm mesh. Concentrations of N and P were measured colorimetrically on the Autoanalyzer according to the manufacturer's instruction (Technicon, 1974).

Roots were carefully washed and divided into three batches: one was stained by the classical non-vital trypan blue (TB) staining all fungal tissues (Phillips and Hayman, 1970) and the others were used for histochemical vital staining (SDH or ALP-ase activities) of the mycorrhizal roots to measure total (TB), living (SDH) or functional (ALP-ase) mycorrhizal fungal development.

Succinate dehydrogenase (SDH) activity was revealed according to the procedure described by Smith and Gianinazzi-Pearson (1990). The roots were immersed in a freshly made solution containing 0.2 M Tris-ClH, pH 7.0, 2.5 M sodium-succinate 6-hydrate, 4 mg/ml nitroblue tetrazolium, 5 mM $MgCl_2$. Root fragments were stained overnight at room temperature and then cleared for 15–20 min in a 3% active chlorine solution of sodium hypochlorite.

Alkaline phosphatase (ALP-ase) was determined according to the procedure described by Tisserant et al. (1993), which reported the specificity of staining methods for ALP-ase. The roots were immersed in a freshly made solution containing 50 mM Tris-citric acid, pH 9.2, 1 mg/ml alfa-naphthyl acid phosphate (monosodium salt), 0.05% $MgCl_2$ anhydrous, 0.05% $MnCl_2$ tetrahydrate and 1 mg/ml Fast Blue RR salt. Root fragments were stained overnight at room temperature and after that were cleared for 15–20 min in a 1% active chlorine solution of sodium hypochlorite

The percentage of root length colonized by mycorrhizal fungus was estimated by visual observation (×40 magnification) of mycorrhizal infection after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips and Hayman (1970). Parameters of mycorrhizal colonization were determined according to Trouvelot et al. (1986). The colonization frequency (F %) is a ratio between colonized root fragments and total number of root fragments observed. It gives an estimation of the length of root which is colonized by the fungus. The colonization intensity in the root fraction of 1 cm (%m) or total length of the root system (M %) is an estimation of the amount of root cortex which became mycorrhizal. Finally, the parameters of arbuscule abundance a% and A% give an estimation of the arbuscule abundance in the mycorrhizal root fraction of 1 cm and total root system, respectively. Nodule number per plant root was estimated by direct observation and counting using a binocular microscope.

The viability and infectivity of the mycorrhizal inoculum as affected by the bacterial inoculation was determined as described Ruiz-Lozano and Azcón (1996). Inoculum (soil containing spores, hyphae AM colonized root fragments)

of *G. occultum* was stored for two weeks in soil soil/sand (1:1, v/v) sterile mixture inoculated or not with *B. thuringiensis* (a density of 10^8 cfu ml^{-1}) as free-suspension on as encapsulated form as previously described. This was kept in black plastic bags and stored under greenhouse conditions. After two weeks period the inocula were diluted with sterilized soil/sand mixture (1/4, 1/16 or 1/64) following the most probable number [MPN method, (Porter, 1979) as described by Sieverding (1991)] to assay for AM fungal colonization in small pots of 100 cc of capacity. Five replicates per dilution were done.

The data were subjected to ANOVA. When the main effect was significant, differences among means were evaluated for significance ($P<0.05$) by Duncan's multiple range test. For the percentage values, an Arc Sin transformation was made before the statistical analysis.

3. Results

The growth of mycorrhizal plants was best when associated with *B. thuringiensis*. The beneficial effect of this bacterium on mycorrhizal plants was highest when applied in an encapsulated form. Increases in shoot weight by the most effective treatments compared to the non-inoculated control were 310% (mycorrhizae plus encapsulated bacterium) and 246% (mycorrhizae plus free-suspension bacterium) and from 181% and 160% regarding root responses. The greatest shoot growth corresponded with the highest number of nodules developed in the root system (Table 1). The number of nodules formed was increased by mycorrhizal colonization as well as by the bacterium applied as suspension. These beneficial effects of single AM fungus or bacterium inoculation were not significantly different compared with the uninoculated control plants. Encapsulated *B. thuringiensis* in non-mycorrhizal plants did not increase the number of nodules, which was similar to the controls. However, in mycorrhizal plants, encapsulated bacterium increased nodulation to a greater extent (non-significant difference) than as suspension inoculum. Encapsulated *B. thuringiensis* increased number of nodules by 258% when associated with *G. occultum* (Table 1).

Root/shoot ratios decreased as a result of AM colonization, particularly (non-significantly) when encapsulated bacterium was involved (Table 1).

Shoot N, P and K content displayed similar trends as those described for plant growth. The content of these nutrients were increased to a greater extent in plants inoculated with *B. thuringiensis* in association with the AM fungus. The encapsulated bacterium resulted particularly efficient in increasing N and K by 170% (N) and 192% (K) compared to the mycorrhizal plants alone. The mycorrhizal effect on P plant acquisition was also enhanced by the co-inoculation with the bacterium by 199% regardless of which form of the

Table 1. Effect of *B. thuringiensis* (B) applied as suspension (S) or encapsulated (E) inoculum on shoot, root and root/shoot ratio fresh weight (mg per plant) and nodule formation (number of nodules per plant root) of non-mycorrhizal and mycorrhizal (M) *Trifolium repens*

Treatments	Shoot	Root	No. of nodules	R/S
Control	1,482 c	3,426 a	7.6 c	2.31 a
BS	2,336 bc	6,096 a	57.2 abc	2.60 a
BE	1,723 c	3,952 a	10.0 c	2.30 a
M	2,367 bc	4,260 a	47.2 bc	1.80 b
M+BS	3,649 a	5,486 a	96.2 ab	1.50 b
M+BE	4,592 a	6,230 a	121.8 a	1.35 b

Table 2. Effect of *B. thuringiensis* (B) applied as suspension (S) or encapsulated (E) inoculum on N, P and K content (mg per plant) in non-mycorrhizal and mycorrhizal (M) *Trifolium repens*

Treatments	N	P	K
Control	13.77 b	1.12 b	9.88 b
BS	16.12 b	1.48 b	11.83 b
BE	12.81 b	1.08 b	9.46 b
M	19.64 b	1.41 b	12.31 b
M+BS	29.74 a	2.81 a	22.67 a
M+BE	33.46 a	2.81 a	23.64 a

bacterial inoculum was used (Table 2). Differences in N, P and K nutrition in single inoculated plants versus non-inoculated plants were non-significant.

Differences were found between lengths of colonized roots, as percentage (TB staining) in single mycorrhizal-only and dual inoculated plants (Table 3). The effect of the bacterium was evident regarding the amount of root cortex which became mycorrhizal (%F, %m, %M). In contrast, the arbuscule abundance in the root system fraction (%a) was not increased by the inoculation with *B. thuringiensis*. However, the proportion of mycelial colonization showing ALP-ase activity was greatly increased, by 348%, in dual inoculated plants. But on the contrary, the arbuscules formed in dual inoculated plants showed lower ALP-ase activity than those formed in single *G. occultum* mycorrhizal plants without bacteria.

Table 3. Effect of *B. thuringiensis* (B) applied as suspension (S) or encapsulated (E) inoculum on AM colonization (M) observed as trypan blue (TB), succinate dehydrogenase (SDH) or alkaline phosphatase (ALP) staining in *Trifolium repens* roots

Treatments	%F	%m	%M	%a	%A
TB staining*					
M	98 a	39.8 bc	39.1 b	40.2 a	15.72 b
M+BS	100 a	60.4 a	60.6 a	37.1 ab	22.48 a
M+BE	100 a	54.2 a	54.1 a	39.4 a	21.30 a
SDH staining					
M	93 b	35.1 c	32.7 c	35.8 b	11.70 b
M+BS	100 a	44.6 b	44.6 b	30.9 bc	13.80 b
M+BE	100 a	40.0 bc	30.0 c	26.5 c	7.90 c
ALP staining					
M	90 b	12.7 e	11.5 d	32.1 bc	3.70 c
M+BS	100 a	42.9 bc	42.4 b	13.9 d	5.90 c
M+BE	100 a	31.9 d	31.9 c	19.3 d	6.20 c

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P<0.05$). *%F, percentage of root fragments with fungal colonization. %M, percentage of the fractional colonization extent. %A, percentage or root cortex with arbuscules. %m, percentage of colonization in the root fraction of 1 cm. %a, percentage of arbuscule abundance in the root fraction of 1 cm.

Table 4. Effect of *B. thuringiensis* (B) applied as suspension (S) or encapsulated (E) on *G. occultum* (M) potential as AM inoculum as indicated most probably number method (MPN)

Treatments	Propagules per g of soil
M	1.7 b
M+BS	2.2 a
M+BE	2.5 a

Means (five replicates) sharing a letter are not significantly different according to Duncan's test ($P<0.05$).

While the bacterium increased the ALP-ase activity in the mycelium by 216% and by 277% as free-suspension or encapsulated, respectively, it reduced

the ALP-ase activity (by 57% and 40%) in the arbuscules formed in the root system fraction.

This negative bacterial effect on the arbuscular ALP-ase activity could be related to the greater number of nodules formed in dual-inoculated plants (Tables 1 and 3).

In general, intraradical *G. occultum* development increased by *B. thuringiensis* inoculation. Regarding living fungal tissue (SDH) such stimulation was not determined in the arbuscules formed. In any way, the stimulating effect of the bacterium on the mycorrhizal colonization was correlated with an increase of the nutritional status in the host plant. Encapsulation did not improve the effect of the bacterial suspension culture on these values (Table 3).

An additional bacterial effect was observed on the viability and infectivity potential of *G. occultum* inoculum (Table 4). No significant differences were found between such values when suspensions or encapsulated bacterial inoculum was applied. The increase of the mycorrhizal inoculum potential as affected by the bacterium ranged from 129% (BS) to 147% (BE) (Table 4).

4. Discussion

Improvements in growth and in N and P plant acquisition as well as in AM colonization due to the interaction between AM fungus and *B. thuringiensis* were found in this study, irrespective of the form of bacterial inoculum applied. The positive effect of dual inoculations of *Glomus* sp. plus bacterium (applied as free-suspension) is a well documented effect in previous studies (Azcón-Aguilar and Barea, 1985). Mechanisms such as production of phytohormons (Azcón et al., 1976), vitamins or amino acid can be involved in the bacterial effect (Bowen and Rovira, 1999). The IAA production in the culture of this bacterium may be a main factor involved in the effect found.

Nevertheless, the shoot increases by these microbial associations has been often attributed to changes in characteristics and growth of the roots (Galleguillos et al., 2000). However, in this study, non significant differences in root growth were observed between treatments. Thus, decreases in R/S ratios in AM plants indicated that mycorrhizal roots were more efficient in increasing shoot biomass and nutrient uptake, particularly when associated with the bacterium.

The results clearly demonstrated that the encapsulated *B. thuringiensis* retained its PGPR and MHB capabilities.

The effect of the microbial interaction on dry matter yield (shoots) was more related to the intraradical mycelium development and activity (% m, % M) than to the ALP-ase activity in the arbuscules (% a). This is a new and

interesting observation since in recent studies when this bacterium was co-inoculated with *G. mosseae* or *G. intraradices* (Marulanda et al., 2002) enhanced all the parameters of AM colonization formed by both these fungi in lettuce, a non-legume plant. Moreover, in this previous study, the mycorrhizal vitality and activity (values from SDH and ALP-ase staining) were increased in co-inoculated treatments to a greater extent than did colonization values determined by TB staining. These results also indicated more efficient mycorrhizal functioning in the presence of the bacterium. In the present study, using a legume host plant, the totality of these bacterial effects on *G. occultum* colonization were not reproduced.

B. thuringiensis was able to increase the intraradical mycelium developed by *G. occultum*, but it did not affect the arbuscular development and even decreased its ALP-ase activity in the root system fraction (%a). This negative effect could be interpreted as certain incompatibility between the *Rhizobium* and *G. occultum*, at the intracellular level, due to the increased nodulation produced in co-inoculated bacterium-AM fungus plants. In dual symbiotic relationship, host root carbohydrates are required by both microsymbionts as nutrition and energy sources. Here, C was not limiting at the infective level of *Rhizobium* (see nodule formation) and/or AM fungus (see AM colonization) since the bacterium enhanced the establishment and development of both symbiotic structures.

Nevertheless, the effect of the bacterium on arbuscular ALP-ase activity (% a) did not follow this tendency. The observed reduction of arbuscular ALP-ase activity (% a) in highly nodulated plants could be explained by the increased C requirements of greatly developed symbionts (nodules number and AM infection) stimulated by the bacterium. Results suggest that high amount of soluble sugars in the root were required for the arbuscular activity. On the basis of these results, we speculate that the arbuscular ALP-ase activity and function seem to be sensitive to the carbohydrate availability in roots and a high C demand for symbiotic structures formation reduced the arbuscular activity. Ezawa et al. (1999) suggested links between ALP-ase and C/P transfer in AM fungi.

Our results, in agreement with Tisserant et al. (1993), suggest that ALP-ase fungal activity is induced by the host plant.

But the arbuscule abundance and activities (SDH, ALP-ase) in the total root system (% A) were not decreased by the bacterium.

The inoculation with *B. thuringiensis* resulted in an enhancement of ALP-ase staining of AM mycelium that indicated a higher activity as shown by the greatest nutrient content in these plants. The highest infectivity resulted in increased plant N and P contents

In this study, in agreement with Smith et al. (1994b), AM fungal efficiency was not closely correlated with the extent of arbuscular developments but it

was with differences in rates of P transfer per unit area across the symbiotic interface. In fact, P plant acquisition was stimulated, more than root development, in the presence of the bacterium. Comparatively, no different activity on P uptake was demonstrated by encapsulated and free-suspension of *B. thuringiensis*. The average amount of P uptake increased by 199% in mycorrhizal plants co-inoculated with the bacterium (as free suspension or encapsulated) and root weight by 129% (BS) and by 146% (BE) over singlely inoculated mycorrhizal plants. The results of this study do not allow the generalization that fungal enzyme activities were correlated with the symbiotic efficiency of AM infection.

G. occultum propagules quantity and/or vitality, determined in terms of infectivity, were increased by the association with *B. thuringiensis*. The results indicate the importance of this bacterium on the viability of the mycorrhizal inoculum. Under natural conditions, mycorrhizal fungal propagules must remain viable from one period of root growth to the next. Thus, increased viability may be critical in the infective potential of the inoculum. Here, *B. thuringiensis* (encapsulated) was able to increase by a 147% the most probable number of infective propagules of *G. occultum*. But each form of bacterial inoculum application was able to enhance positively with the mycorrhizal inoculum. The germination of extramatrical chlamydospores and mycelial growth is known to be affected not only by physical (Sylvia and Schenck, 1983) but also by biological (Azcón, 1987; Azcón-Aguilar et al., 1985, 1986a,b) factors.

The beneficial bacterial effect on plant growth and nutrition was more pronounced when the encapsulated bacterial cells were co-inoculated with *G. occultum*. The explanation is the higher number of viable cells maintained in the case of encapsulated-cell-inoculated soil than in the case of free-cell treatments. Thus, encapsulation could be considered as the ideal form of bacterial inoculum application under stress conditions. Encapsulated cell formulations provide a microenvironment that protects microbial cells from stress conditions such as drought, salinity and heavy metals. Such adverse detrimental factors can affect cells viability. The encapsulated system probably increased survival of the bacterial cells in the soil.

Moreover, the encapsulated system allows the continuous release of cells ensuring a prolonged effect in the surrounding environment of the rhizosphere. Into the technological approaches to increase inocula viability in stressed areas the inoculum management has practical importance (Elsas et al., 1992; Trevors et al., 1992; Vassilev et al., 2001). Additionally, the importance of *B. thuringiensis* as a factor contributing to the *G. occultum* potential as inoculum was also manifested.

Acknowledgements

Adriana Marulanda and Astrid Vivas are grateful to ICI (Spanish CICYT) and Fundación Gran Mariscal de Ayacucho (Venezuela) for the grants. Financial support for this study was obtained from FEDER-CICYT (REN 2000-1724-C03-02). Thanks to Edson Souchie for correcting the English text.

REFERENCES

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

Azcón, R., Barea, J.M., and Hayman, D.S. 1976. Utilization of rock phosphate in alkaline soils by plants inoculated with mycorrhizal fungi and phosphate solubilizing bacteria. *Soil Biology and Biochemistry* **8**: 135–138.

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 Phytologist* **117**: 399–404.

Azcón-Aguilar, C. and Barea, J.M. 1985. Effect of soil microorganisms on formation of VA mycorrhizas. *Transaction of British Mycology Society* **84**: 536–539.

Azcón-Aguilar, C., Barea, J.M., Azcón, R., and Díaz-Rodríguez, R.M. 1985. Assessment of field situations for the feasibility of VA mycorrhizal inosulation using a forage legume as test plant. *Agriculture, Ecosystems & Environment* **15**: 241–252.

Azcón-Aguilar, C., Díaz-Rodríguez, R.M., and Barea, J.M. 1986a. Effect of free-living fungi on the germination of *Glomus mosseae* on soil extract. In: *Physiological and Genetical Aspects of Mycorrhizae*. V. Gianinazzi-Pearson and S. Gianinazzi, eds. INRA, Paris, pp. 515–521.

Azcón-Aguilar, C., Díaz-Rodríguez, R.M., and Barea, J.M. 1986b. Effect of soil microorganisms on spore germination and growth of the VA mycorrhizal fungus *Glomus mosseae*. *Transaction of British Mycology Society* **91**: 337–340.

Bashan, Y. 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnology Advances* **16**: 729–770.

Boddington, C.L. and Dodd, J.C. 1998. A comparison of the development and metabolic activity of mycorrhizas formed by arbuscular mycorrhizal fungi from different genera on two tropical forage legumes. *Mycorrhiza* **8**: 149–157.

Boddington, C.L. and Dodd, J.C. 1999. Evidence that differences in phosphate metabolism in mycorrhizas formed by species of *Glomus* and *Gigaspora* might be related to their life-cycle strategies. *New Phytologist* **142**: 531–538.

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

Cassidy, M.B., Lee, H., and Trevors, J.T. 1996. Environmental applications of immobilized cells. *Journal of Industrial Microbiology* **16**: 79–101.

Elsas, J.D. Van and Heijnen, C.E. 1990. Methods for the introduction of bacteria into soil. *Biology and Fertility of Soils* **10**: 127–133.

Elsas, J.D. Van, Trevors, J.T., Jain, D., Wolters, A.C., Heijnen, C.E., and Overbeek, L.S. Van. 1992. Survival of, and root colonization by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soil. *Biology and Fertility of Soils* **14**: 14–22.

Ezawa, T., Kuwahara, S., Sakamoto, K., Yoshida, T., and Saito, M. 1999. Specific inhibitor and substrate specificity of alkaline phosphatase expressed in the symbiotic phase of the arbuscular mycorrhizal fungus, *Glomus etunicatum*. *Mycologia* **91**: 636–641.

Galleguillos, C., Aguirre, C., Barea, J.M., and 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 Science* **159**: 57–63.

Giovannetti, L., Ventura, S., Bazzicalupo, M., Fani, R., and Materassi, R. 1990. DNA restriction fingerprint analysis of the soil bacterium *Azospirillum*. *Journal of General Microbiology* **136**: 1161–1166.

Gryndler, M., Hrselova, H., and Striteska, D. 2000. Effect of soil bacteria on hyphal growth of the arbuscular mycorrhizal fungus *Glomus claroideum*. *Folia Microbiologica* **45**: 545–551.

Guillemin, J.P., Orozco, M.O., Gianinazzi-Pearson, V., and Gianinazzi, S. 1995. Influence of phosphate fertilization on fungal alkaline phosphatase and succinate dehydrogenase activities in arbuscular mycorrhiza of soybean and pineapple. *Agriculture Ecosystems & Environment* **53**: 63–69.

Lane, D.J. 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E. and Goodfellow, M., eds. *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, New York, pp. 115–147.

Mayo, K., Davis, R.W., and Motta, J. 1986. Stimulation of germination of spores of *Glomus versiforme* by spore associated bacteria. *Mycologia* **78**: 426–431.

Olsen, S.R., Cole, C.V., Watanabe, G.S., and Dean, L.A. 1954. Estimation of available phosphorus in soil by extraction with sodium bicarbonate. Circular No. 939 US Department of Agriculture, Washington, DC. 19 p.

Ponchon, J. and Tardieux, Y. 1962. Techniques d'analyse en microbiologie du sol. De la Tourelle, Paris.

Porter, W.M. 1979. Most probable number method for enumeraing infective propagules of vesicular arbuscular mycorrhizal fungi in soil. *Australian Journal of Soil* **17**: 515–519.

Phillips, J.M. and Hayman, D.S. 1970. Improved procedures for clearing and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of the infection. *Transaction of British Mycologia Society* **55**: 158-161.

Ruiz-Lozano, J.M. and Azcón, R. 1996. Viability and infectivity of mycorrhizal spores after long term storage in soils with different water potentials. *Applied Soil Ecology* **3**: 183–186.

Sieverding, E. 1991. Vesicular-arbuscular mycorrhiza management in tropical agrosystems. pp. 371.

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

Smith, S.E., Dickson, S., Morris, C., and Smith, F.A. 1994. 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 Phytologist* **127**: 93–99.

Smith, S.E. and Gianinazzi-Pearson, V. 1990. Phosphate uptake and arbuscular activity in mycorrhizal *Allium cepa* L. Effects of photon irradiance and phosphate nutrition. *Australian Journal of Plant Physiology* **17**: 177–188.

Sylvia, D.M. and Schenck, N.C. 1983. Application of super phosphate to mycorrhizal plants stimulates sporulation of phosphorus tolerant vesicular arbuscular mycorrhizal fungi. *New Phytologist* **95**: 655–661.

Technicon. 1974. Autoanalyzer II. Continuous-flow analytical instrument. Technical publication UA, 010A00, Technicon, New York.

Tisserant, B., Gianinazzi-Pearson, V., Gianinazzi, S., and Gollotte, A. 1993. In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. *Mycological Resources* **97**: 245–250.

Trevors, J.T., Elsas, J.D. Van, Lee, J., and Overbeek, L.S. Van. 1992. Use of alginate and other carriers for encapsulation of microbial cells for use in soil. *Microbiology Releases* **1**: 61–69.

Trouvelot, A., Fardeau, J.C., Plenchett, C., and Gianinazzi, S. 1986. Nutritional balance and symbiotic expression in mycorrhizal wheat. *Physiologia Vegetal* **24**: 300.

Vassilev, N. and Vassileva, M. 1992. Production of organic acids by immobilized filamentous fungi. *Mycological Research* **96**: 563–570.

Vassilev, N., Fenice, M., and Federici, F. 1996. Rock phosphate solubilization will gluconic acid produced by immobilized *Penicillium variable* P16. *Biotechnology Techniques* **10**: 585–588.

Vassilev, N., Toro, M., Vassileva, M., Azcón, R., and Barea, J.M. 1997a. Rock phosphate solubilization by immobilized cells of *Enterobacter* sp. in fermentation and soil conditions. *Bioresource Technology* **61**: 29–32.

Vassilev, N., Vassileva, M., and Azcón, R. 1997b. Solubilization of rock phosphate by immobilized *Aspergillus niger*. *Bioresource Technology* **59**: 1–4.

Vassilev, N., Vassileva, M., Azcon, R., and Medina, A. 2001a. Interactions of an arbuscular mycorrhizal fungus with free or co-encapsulated cells of *Rhizobium trifoli* and *Yarowia lipolytica* inoculated into a soil-plant system. *Biotechnology Letters* **23**: 149–151.

Vassilev, N., Vassileva, M., Azcon, R., and Medina, A. 2001b. Preparation of gel-entrapped mycorrhizal inoculum in the presence or absence of *Yarowia lipolytica*. *Biotechnology Letters* **23**: 907–909.

Vassilev, N., Vassileva, M., Azcon, R., and Medina, A. 2001c. Application of free and Ca-alginate-entrapped *Glomus deserticola* and *Yarrowia lipolytica* in a soil-plant system. *Journal of Biotechnology* **91**: 237–242.

Vassilev, N., Vassileva, M., Fenice, M., and Federici, F. 2001d. Immobilized cell technology applied in solubilization of insoluble inorganic (rock) phosphates and plant P acquisition. *Bioresource Technology* **79**: 263–271.

Vassileva, M., Azcón, R., Barea, J.M., and Vassilev, N. 1998. Application of an encapsulated filamentous fungus in solubilization of inorganic phosphate. *Journal of Biotechnology* **63**: 67–72.

Vassileva, M., Azcón, R., Barea, J.M., and Vassilev, N. 1999. Effect of encapsulated cells of *Enterobacter* sp on plant growth and phosphate uptake. *Bioresource Technology* **67**: 229–232.

Vassileva, M., Azcón, R., Barea, J.M., and Vassilev, N. 2000. Rock phosphate solubilization by free and encapsulated cells of *Yarrowia lipolytica*. *Process Biochemistry* **35**: 693–697.

Wolf, B. 1982. A comprehensive system of leaf analysis and its use for diagnosing crop nutrient status. *Communications in Soil Science and Plant Analysis* **13**: 1035–1059.