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Effect of encapsulated cells of *Enterobacter* sp on plant growth and phosphate uptake

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Abstract

Cells of *Enterobacter* sp., a phosphate-solubilizing bacterium, were encapsulated in calcium-alginate beads supplemented with skim milk. Free-cell and encapsulated-cell formulations were introduced into a soil, amended with rock phosphate, to compare their effect on non-mycorrhizal and mycorrhizal *Lactuca sativa* plants. Bacterial inoculation affected plant growth and P uptake but encapsulated-cell formulation caused the better plant response. Combined inoculation of encapsulated cells and *Glomus deserticola* provided the best microbial inoculum and enhanced plant growth by 96% as compared to the uninoculated control. The higher number of bacterial cells (log 4.4 CFU/g soil) detected in soil inoculated with encapsulated cells stimulated the plant mycorrhization. The continuous flow of bacterial cells from the skim-milk-enriched carrier beads appeared to be the main reason for the enhanced plant growth and P uptake.

Keywords: Encapsulated cells; *Enterobacter* sp.; *Glomus deserticola*; P uptake; Plant growth

1. Introduction

Soil microorganisms are the key factor in the maintenance of soil fertility and plant establishment thus playing an important role in the sustainable agricultural concept. It is now well established that introduction of plant-beneficial microorganisms can substitute, at least partly, for the widespread, often costly, input of chemical agro-materials. For this reason, there is considerable interest in preparation of microbial inoculants by methods alternative to the traditional peat-based technology. Encapsulation technology offers a solution to many of the problems of liquid and peat-based microbial inoculant-formulations (Cassidy et al., 1996). However, the application of encapsulated soil microorganisms has been limited mostly to preparations of nitrogen-fixing bacteria (Van Elsas and Heijnen, 1990).

Several experiments have shown that some soil microorganisms, mainly filamentous fungi and bacteria, are able to solubilize inorganic phosphate-bearing compounds in fermentation and soil conditions by excreting organic acids. However, only recently has it been demonstrated that the effectiveness of this process could be increased by applying immobilized microbial cells (Vassilev et al., 1996, 1997a–c), although the advantages of immobilized systems are well known (Vassilev and Vassileva, 1992).

The aim of this study was to develop efficient microbial plant-growth promotion by introducing encapsulated cells of *Enterobacter* sp. and an arbuscular-mycorrhizal fungus into soil.

2. Methods

2.1. Microorganism and culture medium

The microorganism used was *Enterobacter* sp. [a soil isolate from Granada province (Spain), naturally resistant to 150 μg streptomycin/ml], maintained on agar RC medium at 4°C (Ramos and Callao, 1967) by subculturing monthly. The growth medium (RC liquid medium) was used to obtain bacterial mass for immobilization and contained (g/l of soil extract-filtrate of 1:10, soil:water): commercial (Panreac, Spain) nutrient broth (8), glucose (20), yeast extract (2), pH 7.5.

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2.2. Encapsulation

The free-cell cultivation for obtaining cell material for encapsulation was performed in 250 ml Erlenmeyer flasks with 100 ml RC liquid medium under agitation at 220 rpm, and at 30°C, for 30 h. The encapsulated cells were prepared by mixing 100 ml of *Enterobacter* sp. cell suspension (6×10^8 CFU/ml), obtained after a 30-h fermentation, 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 0.1M $CaCl_2$ 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.

2.3. Soil–plant experiment

The soil used was the top 0–20 cm of a Granada field soil. The characteristics of the test soil, a Cambisol, were as follows: pH (H_2O), 6.8; available ($NaHCO_3$ extractable) P, 15 mg/l; total N, 2.6 mg/l; organic C, 0.8%; and a texture of sand (58.7%), silt (26,4%), and clay (14.9%). The soil was sieved (2 mm), mixed with quartz sand (<1 mm) (1:1, soil:sand, v/v) and rock phosphate (1.4 g/kg soil) and sterilized by steaming (100°C for 1 h on three consecutive days). The rock phosphate source was finely ground (100-mesh) Morocco sedimentary flourapatite with 12.8% total P. Pots were filled with 500 g of the sterilized soil/sand/phosphate mixture. Four seeds of *Lactuca sativa* L. cv. Romana were planted in each pot inoculated or not with the arbuscular mycorrhizal (AM) fungus *Glomus deserticola* and free or encapsulated *Enterobacter* sp. A 5 g sample of the AM inoculum (spores, mycelium and mycorrhizal root fragments) was applied to each of the required pots in the bottom of a 5-cm deep hole. Two mililiters of free-cell bacterial suspension (log 9.1 CFU) were added drop-wise to the soil surface or ten beads (log 8 CFU) of alginate-encapsulated *Enterobacter* sp. cells were mixed into the soil (5-cm subsurface layer) using a sterile metal spatula. After emergence, plants were thinned to one seedling per pot. Plants were grown in a controlled environmental chamber with a day/light/temperature cycle of 16/8 h, 21/15°C, at 50% relative humidity. Water loss was compensated by watering every day after weighing the pots.

2.4. Analytical methods

The number of colony-forming units in the different types of bacterial culture was determined by spreading samples of free-cell culture, encapsulated cells (physically disrupted), and soil–water extract (1:10) at low dilutions on to RC-agar plates containing 150 μg streptomycin/ml.

The two-month-old shoot dry weight was determined after drying at 70°C. Shoot P content was determined by the molybdo-vanado method described by Lachica et al. (1973). Roots were treated for determination of mycorrhizal colonization according to Giovannetti and Mosse (1980) and Phillips and Hayman (1970).

3. Results and discussion

3.1. Establishment of microbial inoculants in the plant–soil system

The development of *Enterobacter* sp. in soil microcosmos was investigated to evaluate the effects of free- and alginate-encapsulated-cell soil inoculation (Table 1). Cells of *Enterobacter* sp. were found in all inoculated treatments regardless of the inoculum form. It should be noted, however, that the number of bacterial cells was higher in soil inoculated with alginate-encapsulated cells than in soil inoculated with free cells. This observation was much more pronounced in the treatments with mycorrhizal plants where a higher number of bacterial cells than amongst the other treatments inoculated with *Enterobacter* sp. was detected.

Microscopic observations of *Lactuca sativa* root samples showed that only AM-inoculated plants were root-colonized. A higher percentage of mycorrhization was found in the treatment inoculated with encapsulated bacterial cells (Table 1).

3.2. Plant growth and P uptake

In general, bacterial inoculants affected plant growth and P uptake (Table 2). Significantly higher plant growth was obtained in treatments inoculated with

Table 1
Bacterial and mycorrhizal establishment in soil–plant system after 60 days of cultivation

Treatment	log CFU (g^{-1})	Mycorrhization (%)
Non-mycorrhizal		
Control (C)	ND	ND
C+free cells	3.40^a	ND
C+encapsulated cells	4.17^b	ND
Mycorrhizal		
Control (C)	ND	49^a
C+free cells	3.50^a	52^b
C+encapsulated cells	4.40^b	58^c

ND: not determined
For response variables, values (means of five replicates) not sharing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test).

Table 2
Shoot dry weight and phosphorus uptake of non-mycorrhizal or mycorrhizal lettuce plants grown in the presence or absence of free and encapsulated PGPR *Enterobacter* sp.

Treatment	Shoot dry weight (g/plant)	P content in shoot (mg/plant)
Nonmycorrhizal		
Control (C)	0.53[a]	1.01[a]
C+free cells	0.59[a]	1.24[a]
C+encapsulated cells	0.79[b]	1.90[b]
Mycorrhizal		
Control (C)	0.68[a,b]	1.50[a,b]
C+free cells	0.78[b]	1.83[b]
C+encapsulated cells	1.04[c]	2.57[c]

For response variables, values (means of five replicates) not sharing a letter in common differ significantly from each other (Duncan's multirange test).

encapsulated bacterial cells. It was higher by 49 and 47% than the respective non-mycorrhizal and mycorrhizal controls. Free-cell amended treatments also stimulated the growth of *Lactuca sativa*, but in this case in the range of 11–15% depending on the presence of AM fungus. Dual inoculation with *G. deserticola* and alginate-encapsulated *Enterobacter* sp. resulted in the most effective microbial inoculant system which led to a two-fold increase of *Lactuca sativa* growth as compared to that of the non-inoculated control. Plant P uptake in all treatments inoculated with bacterial cells was higher than in the control treatments. Mycorrhizal plants enhanced this effect particularly when encapsulated cells were applied as a microbial inoculum. The total shoot P content in mycorrhizal plants that received encapsulated cells was 150% higher than that in the control without microbial inoculants.

3.3. General discussion

There has been a growing interest in the application of microbial inoculants during recent years as an alternative strategy for reducing the use of agro-chemicals and as a part of the sustainable, environmentally sound, agriculture. Introduction of specific beneficial microorganisms into soil for different purposes is a well known practice. The main problem, which is the subject of many investigations, is connected with the fate and activity of introduced microorganisms (Van Veen et al., 1997). Normally, microbial cells in soil, where they are subjected to a set of adverse conditions, often exist in a form characterized by non-growth and/or low activity (Van Elsas and van Overbeek, 1993).

The present study demonstrated the advantageous effect of encapsulated phosphate-solubilizing *Enterobacter* sp. on plant growth, in combination or not with the AM fungus. Overall, the plant dry matter production was stimulated by inoculation with bacterial cells. Recently, we have shown that the agar-encapsulated form of *Enterobacter* sp. added to non-mycorrhizal plants was equally effective as the free-cell inoculated control (Vassilev et al., 1997c). In the present study, alginate-encapsulated, skim milk amended, inoculum preparation provoked a different, significantly higher, growth response and P uptake of non-mycorrhizal plants than did the free-cell inoculum. The presence of the AM fungus, in combination with the phosphate-solubilizing bacterium, obviously improved the efficiency of these processes in combination with the phosphate-solubilizing bacterium, particularly with encapsulated cells. The most simple explanation is the higher number of cells obtained in the case of encapsulated-cell inoculated soil than in the case of free-cell treatment. The addition of skim milk during the preparation of the encapsulated inoculant probably provided increased survival of the bacterial cells in the soil (Van Elsas et al., 1992). On the other hand, by applying a ^{32}P isotopic technique, we have demonstrated the ability of the same bacterial culture to solubilize the added rock phosphate more easily when it was co-inoculated with AM fungi (Toro et al., 1997). The enhanced, although slightly, level of root-mycorrhization in the bacteria-supplemented soil–plant system was not surprising bearing in mind that *Enterobacter* sp. falls into the category of the mycorrhization helper bacteria (Azcon and Barea, 1992). Again, this effect was more pronounced in the alginate-encapsulated-cell treatment.

The advantage of using encapsulated microbial inoculants is not in doubt. Such formulations provide a microenvironment that protects cells from adverse biotic and abiotic factors. On the other hand, the continuous release of cells into the surrounding environment ensures a prolonged effect in the frame of plant–soil systems, thus inhibiting microbial dispersal to adjacent sites (Trevors et al., 1992). Particularly in the case of phosphate-solubilizing microorganisms, the presence of AM fungi, with their structural and functional properties, plays an additional positive role, increasing the beneficial effect of encapsulated inoculant.

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