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Application of free and Ca-alginate-entrapped *Glomus deserticola* and *Yarowia lipolytica* in a soil–plant system

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Abstract

This study was performed to investigate the applicability of microbial inoculants entrapped in alginate gel. *Glomus deserticola* (AM) was inoculated into soil microcosms, enriched with rock phosphate, as either free form or entrapped in calcium alginate alone or in combination with a P-solubilizing yeast culture (*Yarowia lipolytica*). Plant dry weight, soluble P acquisition, and mycorrhizal index were equal in treatments inoculated with free and alginate-entrapped AM. Dual inoculation with entrapped *G. deserticola* and free cells of *Y. lipolytica* significantly increased all analyzed variables. Highest rates of the latter were obtained when both fungal microorganisms were applied co-entrapped in the carrier. The yeast culture behaved as a 'mycorrhiza helper microorganism' enhancing mycorrhization of tomato roots. These results indicate that dual inoculation with an AM fungus and a P-solubilizing microorganism co-entrapped in alginate can be an efficient technique for plant establishment and growth in nutrient deficient soils.
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Keywords: *Glomus deserticola*; *Yarowia lipolytica*; Encapsulation; Plant growth and P uptake; Interactive microbial effect

1. Introduction

The increasing interest in applying microorganisms beneficial to plants in the context of the so-called 'sustainable agriculture' and the efforts to avoid environmentally deleterious agro-chemicals explain the increasing number of studies on management of the soil–plant–microorganism systems (Bowen and Rovira, 1999). The widespread use of conventional fertilizers has meant that many soils have received amounts of nutrients in excess of crop needs. A typical example is the oversaturation of upper soil layers with phosphorus that easily passes to ground water thus causing its eutrophication (Del Campillo et al., 1999; Leinweber et al., 1999). An attractive alternative is the use of rock phosphate (RP) in combination with P-solubilizing microorganisms and arbuscular mycorrhizal fungi (AM) which can lead to remarkable yield increases as reported by

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several authors (Kim et al., 1998; Omar, 1998; Singh and Kapoor, 1999; Vassileva et al., 1998, 1999).

Rock phosphates, the cheapest P sources, are abundantly found and easily mined but their direct application is not always effective without chemical treatment, particularly for non-acidic soils. Considerable research has been conducted in recent years on developing novel, environmentally sound methods for its solubilization, different from the traditional processing with mineral acids. Solubilization of phosphate-bearing inorganic materials by microorganisms excreting organic acids seems to be an attractive approach that has been studied actively during the last decade (Whitelaw, 2000). Microbially mediated solubilization was examined with bacteria and filamentous fungi in fermentation and soil conditions (Kucey et al., 1989). Integrated approaches involving mycorrhizosphere interactions have been proposed to improve P bioavailability in soil within the context of the biogeochemical cycling of plant nutrients (Jeffries and Barea, 1994; Barea et al., 1997). In any case, all of the reported studies on the application of microorganisms able to solubilize rock phosphate involve free living cells in either fermentation or soil conditions. Only recently, the effectiveness of immobilized P-solubilizing microorganisms (mainly bacteria and filamentous fungi) has been proved successfully (Vassilev et al., 1996, 1997a,b; Vassileva et al., 1999, 2000). Immobilization methods have been also used in preparation of AM-bearing inoculant formulations (Calvet et al., 1996; Declerck et al., 1996). In fact, it is discussed that these fungi, upon biotrophic root colonization, develop an external mycelium, which is a bridge connecting the root with the surrounding microhabitats (Smith and Read, 1997).

The aim of this work was to verify the potential application of free and alginate-entrapped arbuscular mycorrhizal fungus (*Glomus deserticola*). *Yarowia lipolytica* was also introduced into the soil in the form of free culture or co-entrapped in the carrier in order to evaluate its effect on the rate of mycorrhization, plant growth and P acquisition.

2. Materials and methods

2.1. Microorganisms

All microorganisms were used from the Department of Microbiology Culture Collection, Estacion Experimental del Zaidin, Granada.

The AM fungus used for the experiment was *G. deserticola*. Mycorrhizal roots of stock pot cultures of *Lactuca sativa* L. colonized to between 80 and 90% (determined by the methods of Phillips and Hayman, 1970 and Giovanetti and Mosse, 1980) by the mycorrhizal *G. deserticola* were used as a source of the fungus. The yeast culture, applied as a solubilizer of insoluble phosphates, was *Y. lipolytica*, maintained on potato dextrose agar.

Y. lipolytica was grown in a medium containing (g/l of distilled water): glucose 60, NH_4Cl 3.0, KH_2PO_4 0.4, $MgSO_4 \cdot 7H_2O$ 0.5, yeast extract 0.5. Shake-flask cultivation process and cell biomass preparation for the immobilization procedure were performed as described earlier (Vassileva et al., 2000).

2.2. Immobilization procedure

G. deserticola infected roots of *L. sativa*, cut to 0.1 mm fragments, were sterilized in a solution contained (per 100 ml sterilized distilled water) 40 mg streptomycin, 2 g chloramine-T Trihydrate and 0.1 ml Tween 80. Root fragments and *Y. lipolytica* biomass were mixed in 1.5% solution of sodium alginate which was further poured in a sterile petri dish to form a thin 2-mm-layer. The mixture was hardened by 0.5 M solution of $CaCl_2$ and after 30 min was washed with sterile distilled water. Alginate material was then cut into 3×2 mm pieces.

2.3. Soil–plant system and experimental design

The soil used was the top 0–20 cm of a Granada province (Spain) field soil with a pH of 7.5 containing 8 μg P/g soil (Olsen test), organic carbon 0.46%, total N 0.046%, and a texture of

sand (58.7%), silt (26.4%), and clay (14.9%). The soil was sieved (mesh, 2 mm), mixed with rock phosphate (0.2 g/100 g soil; sedimentary Morocco fluorapatite; 12.8% P; 1 mm mesh); and steam-sterilized.

Tomato was the test plant. Three-day-old seedlings obtained from surface-sterilized seeds grown in petri dishes containing sterile Whatman # 42 paper and 5 ml of water (28 °C, without light) were transplanted to 300-ml-capacity pots (one per pot).

The experiment consisted of six treatments: control (C); free *Y. lipolytica*-inoculated (Fy1); free AM-inoculated (FAM); immobilized AM-inoculated (IAM); immobilized AM-inoculated, supplemented with free cells of *Y. lipolytica* (IAM/FY1); co-immobilized AM + Y1-inoculated (IAM/Y1). Each mycorrhizal treatment received 0.5 g of free or immobilized mycorrhized root fragments per pot. *Y. lipolytica* was applied at a rate of 1.7×10^6 free or alginate-entrapped cells per g soil.

Tomato plants were grown in a greenhouse under a 16-h light:8-h dark (i.e. day/night cycle of 16/8), 25/19 °C, and 50% relative humidity. The pots were weighed and watered to field capacity daily.

2.4. Analytical methods

Plants were harvested after 50 days of growth. Shoot dry weight was recorded after drying at 70 °C to constant weight. Shoot P concentration was measured by the method of Lachica et al. (1973).

The extent of AM root colonization was established by a staining method (Phillips and Hayman, 1970). The percentage of the total root length that became mycorrhizal was calculated by a gridline intersect technique (Giovanetti and Mosse, 1980). Counts of viable yeast cells were performed after serial dilutions of rhizosphere soil samples on acidified malt extract agar with pH 3.7 (Spencer et al., 1996).

Data (five repetitions per treatment; $n = 5$) were processed by analysis of variance and Duncan's test ($P \leq 0.05$).

3. Results

3.1. Behavior of free and alginate-entrapped G. deserticola and Y. lipolytica in soil–plant systems

In order to evaluate the feasibility of using gel-entrapped fungal inoculants in soil–plant systems, five treatments received free and alginate-entrapped mycorrhizal and yeast cultures. All plants, received mycorrhizal inoculum, independently of its form, were mycorrhized (Fig. 1). Free *G. deserticola* and its alginate-entrapped form caused almost equal percentage of plant root mycorrhization while significantly higher level of colonization was observed in plants dualy inoculated with *Y. lipolytica*. The highest level of mycorrhization of 59% was found in root samples of plants inoculated with co-entrapped *G. deserticola* and *Y. lipolytica*.

Y. lipolytica was detected in all yeast-inoculated treatments. Microcosms inoculated with alginate-entrapped yeast cells exhibited significantly higher total culturable counts (log 3.5) compared with free-cell inoculated treatment (log 2.1).

3.2. Plant growth and P acquisition

Plant dry weights were affected by the presence of *G. deserticola* and *Y. lipolytica* in the rock-phosphate-supplemented soil microcosms (Fig. 2). Plant dry weights of mycorrhizal plants were gen-

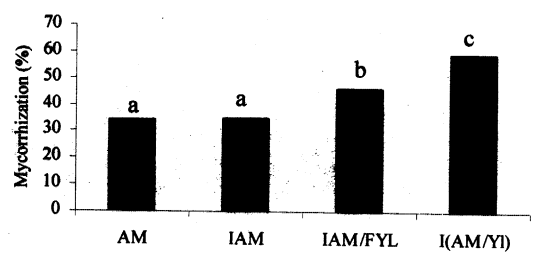

Fig. 1. AM colonization of roots of tomato grown in soil inoculated with free and entrapped *G. deserticola*. In the latter case, *Y. lipolytica* was co-inoculated into soil microcosms in free form or co-entrapped with *G. deserticola*. *FAM, free mycorrhizal inoculum; IAM, alginate-entrapped mycorrhizal inoculum; IAM/FY1, alginate-entrapped mycorrhizal inoculum introduced with *Y. lipolytica* cell suspension; I(AM/Y1), mycorrhizal inoculum co-entrapped with *Y. lipolytica*.

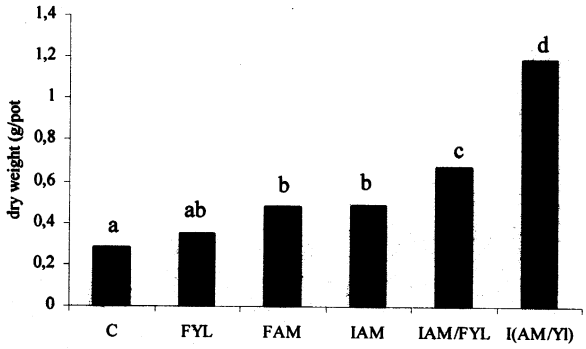

Fig. 2. Shoot dry weight of tomato plants grown in soil inoculated with free and entrapped *Y. lipolytica* and *G. deserticola*. In the latter case *Y. lipolytica* was co-inoculated into soil microcosms in free form or co-entrapped with *G. deserticola*. *C, control without microbial inoculation; FY1, free *Y. lipolytica*; FAM, free mycorrhizal inoculum; IAM, alginate-entrapped mycorrhizal inoculum; IAM/FY1, alginate-entrapped mycorrhizal inoculum introduced with *Y. lipolytica* cell suspension; I(AM/Y1), mycorrhizal inoculum co-entrapped with *Y. lipolytica*.

erally higher than those of non-mycorrhizal plants. Shoot dry weights were comparable for both mycorrhizal treatments inoculated with either free or alginate-entrapped *G. deserticola*. Mycorrhizal plants grew better in treatments where *Y. lipolytica* was introduced into soil compared with mycorrhizal plants that did not receive yeast inoculum. However, tomato plants responded significantly better to inoculation with co-entrapped *G. deserticola* and *Y. lipolytica* than plants inoculated with alginate-entrapped AM fungus and freely suspended yeast cells.

Accordingly, there was a large variation in plant P acquisition, depending on the fungal inoculum form applied. Generally, inoculation with either free or alginate-entrapped *G. deserticola* resulted in higher levels of shoot P content than those measured in non-mycorrhizal control plants (Fig. 3). Inoculation with only *Y. lipolytica* or dual inoculation with both *G. deserticola* and *Y. lipolytica* enhanced P acquisition the latter reaching its maximum of 3.1 mg per pot in the treatment inoculated with alginate-entrapped combination of the fungal inoculants.

4. Discussion

It is well known that immobilized microbial cells can be used in industrial, environmental and agricultural applications (Vassilev and Vassileva, 1992; Federici, 1993; Van Elsas and Heijnen, 1990; Cassidy et al., 1996; Bashan, 1998).

A number of recent studies carried out on the application of immobilized, mainly entrapped and encapsulated, cells of soil microorganisms undoubtedly shows their advantages over traditionally used free cells in processes such as rock phosphate solubilization or nitrogen fixation. Entrapment of endomycorrhizal fungi was also tested in laboratory conditions (Declerck et al., 1996; Strullu and Plenchette, 1991). However, while inoculation of plants with nitrogen-fixing bacteria is used on a large scale, the agroindustry is still waiting for better solutions in preparation of formulations containing plant-growth-promoting microorganisms, mycorrhizal fungi and biocontrol microorganisms (Bashan, 1998).

The results of this study prove the applicability of immobilization methods in preparation of microbial inoculants. The potential of infectivity of mycorrhizal inoculum consisting of gel-entrapped *G. deserticola*-bearing roots was comparable to

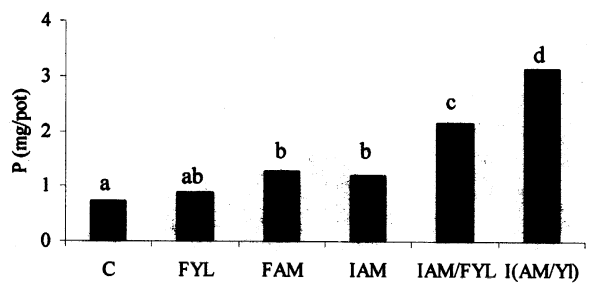

Fig. 3. P accumulation in plants (shoots) grown in soil inoculated with free and entrapped *Y. lipolytica* and *G. deserticola*. In the latter case *Y. lipolytica* was co-inoculated into soil microcosms in free form or co-entrapped with *G. deserticola*. *C, control without microbial inoculation; FY1, free *Y. lipolytica*; FAM, free mycorrhizal inoculum; IAM, alginate-entrapped mycorrhizal inoculum; IAM/FY1, alginate-entrapped mycorrhizal inoculum introduced with *Y. lipolytica* cell suspension; I(AM/Y1), mycorrhizal inoculum co-entrapped with *Y. lipolytica*. ∗For each response variable, values not sharing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test).

that of free mycorrhizal inoculum thus confirming the effectiveness of the immobilization technology as reported by other authors (Declerck et al., 1996; Calvet et al., 1996). However, the major benefit of this technique was obtained with the inclusion of *Y. lipolytica* in the cell–alginate system. It appears that the presence of yeast cells strongly stimulated the level of mycorrhizal-root colonization.

Live yeast cells of *Saccharomyces cerevisiae* have been reported to increase root–AM infection and nodulation of forage legumes (Singh et al., 1991; Ravnskov et al., 1999). However, this is the first report, where a P-solubilizing yeast culture, co-immobilized with an AM fungus, demonstrated its stimulating AM-root colonization effect. What is the mechanism of such stimulation is still unclear. Increases in the level of inorganic nutrition after decomposition of yeast inoculum and enhanced biologically derived CO_2 production were proposed to explain partly the multiple effects of dry yeast culture (Larsen and Jakobsen, 1996). It is also generally believed that microorganisms exert their mycorrhiza helper effect by specialized metabolic activities such as production of vitamins, amino acids and hormones (Barea et al., 1997).

The P-solubilizing ability of immobilized *Y. lipolytica* was proven in an earlier study but in conditions of repeated-batch liquid fermentation (Vassileva et al., 2000). In the conditions of this study, *G. deserticola* readily absorbs the phosphate solubilized by *Y. lipolytica* thus improving the use of rock phosphate by tomato plants. It is accepted that microbial solubilization of rock phosphate can take place in discrete microhabitats, thus the released ions would be taken up by the mycorrhizal hyphae reaching these microenvironments. This would diminish P sorption by soil particles and result in a synergistic microbial interaction. By the latter, we can explain the higher effect of inoculation of *G. deserticola*/*Y. lipolytica* on plant growth and P uptake compared with all other treatments.

In conclusion, our results show that alginate immobilization technology present an effective approach for preparing inoculant formulations containing both arbuscular mycorrhizal fungi and P-solubilizing microorganisms. Further studies are needed to develop efficient inoculant system that may be stored before application.

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