Mycobization with G*lomus mosseae* and *Aspergillus niger* in *Lycopersicon esculentum* plants

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Velázquez, M.S., Elíades, L.A., Irrazabal, G.B., Saparrat, C.M. and Cabello, M.N. (2005). Mycobization with *Glomus mosseae* and *Aspergillus niger* in *Lycopersicon esculentum* plants. Journal of Agricultural Technology 1(2): 315-326.

The effect of mycobization with both an arbuscular mycorrhizal fungus, *Glomus mosseae*, and a phosphorus-solubilizing microorganism, *Aspergillus niger*, was evaluated on tomato plants grown on steamed perlite-vermiculite-sand substrate added either with or without rock phosphate in six greenhouse treatments. Plant aerial biomass, phosphorus concentration in plant tissue, and P available in the substrate were evaluated upon 60-day-old harvested plants. Mycorrhizal colonization was positive in all treatments inoculated with *Glomus mosseae*, although, lower infection values were found as P content in the substrate increased. A high phosphorus content was observed in plants from the treatments with added phosphoric rock; these levels were independent of the microbial inoculation. The highest values of aerial biomass and height of the plant were seen in the treatments co-inoculated with both microorganisms with or without phosphoric rock. This work confirms the synergic effects between mycorrhizal fungi and phosphorus solubilizing microorganisms. However, the results obtained cannot be extrapolated since each plant-soil-organism system deserves special analysis.

Key words: Arbuscular mycorrhizal fungus, *Lycopersicon esculentum*, mycobization, phosphorus-solubilizing microorganism, rock phosphate

Introduction

Mycorrhizas constitute a symbiotic association between the roots of a wide variety of facultative host plants and obligate symbiotic fungi belonging to the phylum Glomeromycota, class Glomeromycetes (Schüßler *et al*., 2001). Arbuscular mycorrhiza form universal symbiosis which can be established with over 80% of plant species, including most of agricultural crops as well as herbaceous and scrublands species in natural ecosystems (Barea *et al*., 2005).

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In this association, the fungus receives part of the synthates produced by the plant (Sanders and Tinker, 1971) and increases the root absorption area by means of the extension of the extraradical mycelium (Barea, 1991; Tarafdar and Kumar, 1996), thus allowing nutrient absorption, especially in soils with low fertility.

Most agricultural crops are potential host plants for arbuscular mycorrhizal (AM) fungi. AM fungi increase the exploitation of the soil volume by the hyphal network, which increases the active absorption surface and spread beyond the phosphate depletion zone (Martin *et al*., 2001). Mycorrhizal hyphae have a higher affinity for phosphate as expressed in the Michaelis-Menten equation by a lower Km value and absorb P at lower solution concentrations than roots do (Lange Ness and Vlek, 2000).

The combined inoculation of an arbuscular mycorrhiza-forming fungi and a phosphorus-solubilizing microorganism has demonstrated a better uptake both of native P from the soil and of the P coming from the phosphoric rock (Goenadi *et al*., 2000; Cabello *et al*., 2005).

Both the intra and extraradical fungal development are affected by several environmental and genetic factors that influence the symbiotic development. The presence of soil microorganisms is included among one of the major environmental factors. Fungi are frequent and common inhabitants of the rhizosphere, supply the soil with a great microbial biomass, and stand out in their role as decomposers.

The group of microorganisms that affects the availability of P in the soil is formed by P-solubilizing bacteria (Taha *et al*., 1969), actinomycetes (Rao *et al*., 1982) and fungi (Whitelaw, 2000). These organisms have the ability to solubilize the P content in minerals by secreting carboxylic acids, enzymes and bacterial mucilage (Deubel and Merbach, 2005).

The saprobe *Aspergillus niger* is a geofungus widely distributed in the rhizosphere of plant crops; it produces lytic enzymes, organic acids, antibiotics (McAllister *et al*., 1995) and P-solubilizing substances such as phosphomonoesterase (Laskin and Lechevalier, 1973), phosphatases and polymetaphosphatase (Shieh and Ware, 1968).

González Méndez (2005) coined the term microbization to refer to the inoculation of plants with different types of microorganisms; here we propose to use the term mycobization when those microorganisms are fungi.

The combination of phosphoric rock and mycorrhizal fungi presents an attractive and alternative possibility in the intensive use of fertilizers and in the survival of crops in the world development (Antunes and Cardoso, 1991).

The aims of this study were both to evaluate the effects of an AM fungus *Glomus mosseae* (Nicolson and Gerdemann) Gerdemann and Trappe and a P-

solubilizing fungus *Aspergillus niger* van Tieghem on the growth of *Lycopersicon esculentum var*. *platense* Mill and to study their interactions in a soil less substrate with rock phosphate (RP), in greenhouse conditions.

Materials and methods

The AMF strain used was *Glomus mosseae* [La Plata Spegazzini Herbarium (LPS) culture number SB1]. Inocula consisted of rhizospheric soil from *Sorghum vulgare* L. plant pot culture that contained spores (110 per g dry soil), mycelia and colonized root fragments. These inocula were mixed with growth substrate.

The phosphorus-solubilizing microorganism (PSM) was the fungus *Aspergillus niger* [LPS culture number 845], isolated from soil from Magdalena, Buenos Aires Province, Argentina (Elíades *et al*., 2004). Its phosphorus-solubilizing capacity was tested according to Pikovskaya (1948). *Aspergillus niger* was grown in a medium containing 50 g barley seeds, 15 ml distilled water and 5- ml solution of 0.1 g aspargine and 3 g glycerol per 100 ml distilled water. This medium was autoclaved at 121°C for 1 hour. *Aspergillus niger* spore suspension was inoculated in 5 g of the above described medium, and cultured at 25°C for a week.

Tomato seeds (*Lycopersicon esculentum var plantense* Mill.) were surface-sterilized with sodium hypochlorite (10% v/v) for 10 min and thoroughly rinsed with sterilized water. After germination, fifteen-day-old tomato plants, obtained as previously described, were selected regarding uniform size, and then transplanted into pots according to the following six treatments: (1) vermiculite-perlite-sand substrate; (2) vermiculite-perlite-sand substrate inoculated with *G. mosseae*; (3) vermiculite-perlite-sand substrate inoculated with both *G. mosseae* and *A. niger*; (4) vermiculite-perlite-sand substrate with rock-phosphate (RP) inoculated with *G. mosseae*; (5) vermiculite-perlite-sand substrate with RP inoculated with *A. niger*; (6) vermiculite-perlite-sand substrate with RP inoculated with both *G. mosseae* and *A. niger*. One plant per pot (250cm3, filled with 315 g steamed vermiculite-perlite-sand substrate mixture) was used in each treatment. Each treatment was replicated five times. These pots were placed in a greenhouse at 24 ± 1°C day/ 20 ± 1°C night, and a 16-h photoperiod was provided by incandescent and cool-white lamps; the plants were fertilized with a nutritive solution without P (Cabello, 1999) twice a week. Contamination was avoided as follows: each pot stood on a 2-cm deep plate in an indoor greenhouse. The substrate corresponding to treatments 4, 5 and 6 was supplemented with 22.7 mg of rock phosphate (RP of marine and sedimentary origin formed at the

Cenozoic period Bahía de Camarones, Chubut province, Argentina). It was supplied as a 100 µm mesh-size fine powder, containing 13.1% total P, which was present as chloroapatite nodules in a silt-clayed matrix. According to treatment 3, each pot was supplemented with 1.5 g *G. mosseae* inoculum and 20 g *A. niger* inoculum. Plants were harvested at 60 days.

Leaves and stems were used to determine aerial plant biomass through dry weight at 70ºC until constant weight. P content in leaves and substrate samples was estimated according to Bray and Kurtz (1945). The root system of tomato was cleared and stained (Phillips and Hayman, 1970), and the colonization percentage was calculated Giovannetti and Mosse (1980). For spore extraction, 100 g substrate was treated by the wet sieving and decanting method (Gerdemann and Nicolson, 1963). The resulting material was centrifuged in 80% sucrose gradient (Walker *et al*., 1982). The microbial inoculation effect was calculated according to Bagyaraj (1992):

Microbial inoculation effect (MIE)

$$\frac{\text{DW inoculated plant* - DW non-inoculated plant}}{\text{DW inoculated plant*}} \times 100$$

DW: dry weight
* indicates all microbial inoculations

The presence of unit P-solubilizing fungal colonies (UFC) from substrate samples was calculated by using the substrate adhering to the roots. This substrate was serially diluted, and 0.1 ml of the appropriate dilution was spread on Pikovskaya medium plates. The plates were incubated at 30ºC for 1 week. At the end of the incubation, the colonies showing a clear zone of $Ca_2(PO_4)$ dissolution were counted.

Statistical analysis

One-way ANOVA with LSD was performed to study differences ($P \leq 0.05$) among groups.

Results

Microscopic observations of stained roots showed mycorrhizal presence in AMF-inoculated treatments and mycorrhizal absence in pots that did not receive mycorrhizal inoculum. The AMF spore number in 100 g dry soil was significantly different: treatment 2 (inoculated with *G. mosseae* without RP)

presented the highest number of spores (1,496 in 100 g of dry soil), while treatment 4 (inoculated with *G. mosseae* with RP) presented the lowest number (421 spores in 100 g of dry soil) (Figure 1).

Figure 2 shows the colonization percentage reached in the treatments inoculated with AMF. The highest percentage of colonized root was observed in the treatment inoculated with *G. mosseae* and *A. niger* without rock phosphate. This treatment differed significantly from that inoculated with both microorganisms and added with rock phosphate. No differences were observed in the treatments inoculated only with *G. mosseae* either with or without rock phosphate.

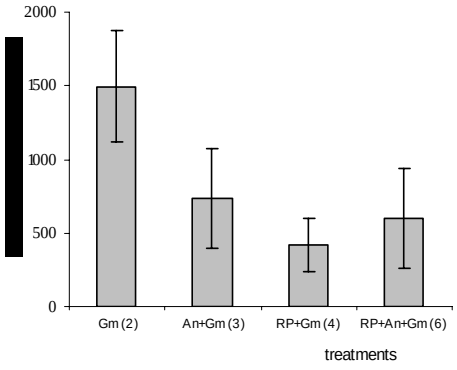

Figure 1. Number of spores in 100 g dry substrate. Gm, *Glomus mosseae*; An, *Aspergillus niger*; RP, rock phosphate. Treatment number between brackets. Values are means ± SD for five pots. The same letter above the bar indicates that values do not significantly differ between treatments according to ANOVA and LSD test ($P<0.05$).

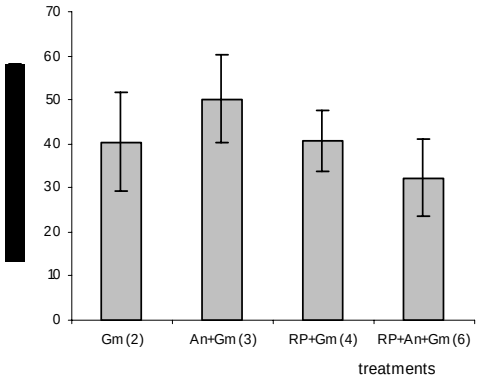

Figure 2. Percentage of root colonization. Same notations as in figure 1.

The microbial inoculation effect (MIE) is observed in Figure 3. The treatment inoculated only with *G. mosseae* (treatment 2) presented the lowest MIE value. Treatments 3, 4, 5 and 6 differed significantly from the non-inoculated control.

Table 1 summarizes both the growth parameters evaluated for tomato plants and P content in plant tissues. The values of aerial biomass and plant height were significantly higher in treatments 3 and 6, which were inoculated with *G. mosseae* and *A. niger* either with or without rock phosphate

Table 1. Effect of inoculation and RP on the different parameters evaluated in tomato. Gm, *Glomus mosseae*; An, *Aspergillus niger*; RP, rock phosphate. Treatment number between brackets. Values are the means of five replicates. Means in the same column followed by the same letter do not significantly differ between treatments according to ANOVA and LSD test ($P \leq 0.05$).

	Plant height (cm)	Aerial dry weight (g)	Dry weight root (g)	P content (µg/g plant)
Control (1)	32.3 a	0.5 ab	0.4 c	2800 a
Gm (2)	30.6 a	0.4 a	0.2 a	3500 a
An+Gm (3)	42.3 b	0.9 e	0.2 abc	3500 a
RP+Gm (4)	35.8 ab	0.6 bc	0.2 ab	4700 b
RP+An (5)	35.9 ab	0.7 cd	0.3 bc	5000 b
RP+An+Gm (6)	43.4 b	0.8 d	0.2 abc	4400 b

respectively. The highest dry weight root was observed in the non-inoculated control, showing significant differences in relation with treatment 2 and 4 (inoculated with *G. mosseae* with or without RP respectively). Values of P content in aerial biomass from treatments 4, 5 and 6 with rock phosphate were significantly different from those in which plants grew without rock phosphate in the substrate.

Figure 4 shows the results of the concentrations of P present in the substrate. The treatments that were not supplemented with rock phosphate (1, 2 and 3) showed significantly lower concentrations than those with rock phosphate. Among the latter, treatment 5 (inoculated with *A. niger)* showed the highest P content in the substrate.

At the end of the experiment, no UFC were formed in the treatments that were not inoculated with *A. niger*, whereas $58.8 \times 10^7 \pm 6.58$, $60.62 \times 10^7 \pm 3.27$, and $54.5\ 10^7 \pm 2$ UFC of *A. niger* were counted in the inoculated treatments (i.e. 3, 5 and 6), respectively.

Discussion

The high sporulation rate of *G. mosseae* in treatment 2 without rock phosphate correlates with a low nutritional state of the host plant, reflected in the growth parameters evaluated and the low content of phosphorus in the aerial biomass. It has been observed that under these conditions of nutritional stress, carbohydrate flow from the plant to the fungus decreases and, as a consequence, sporulation is stimulated. Toro (1984) has observed that high

levels of fertilization inhibit *G. mosseae* sporulation in fertile soils of the Cauca Valley.

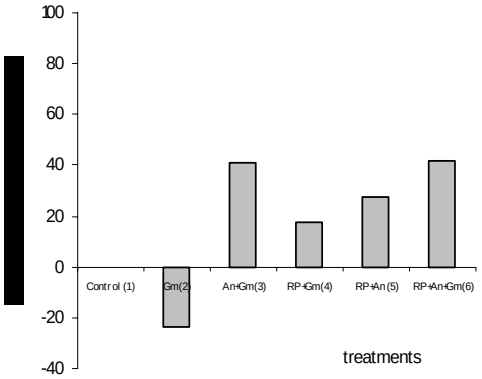

Fig. 3. Microbial inoculation effect on tomato plants by two microorganisms: *G. mosseae* and *A. niger*. Same notation as in fig. 1.

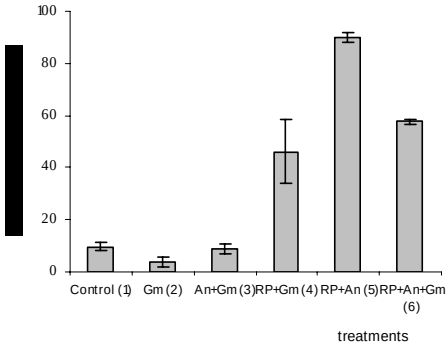

Fig. 4. P extracted from the substrate. Same notations as in fig. 1.

The colonized root percentage did not show decreasing tendencies with the increase of P in the substrate; however, the infection was significantly lower in the treatment inoculated with both microorganisms that received phosphoric rock. A negative correlation between P availability and mycorrhizal colonization has been well documented (Smith and Read, 1997). In our experiment, significant differences in the colonization in the treatments co-inoculated with *A. niger* and *G. mosseae* were observed when the addition of rock phosphate was assessed.

The greatest colonization values were obtained in the treatment without rock phosphate (treatment 3). When both microorganisms were co-inoculated and rock phosphate was added (treatment 6), we recorded the lowest colonization value. Similar results have been observed by Singh and Singh (1993), who attributed this fact to changes in the radical morphology and in the physiology of the host plant. On the other hand, McAllister *et al*. (1995) have observed that in axenic crops *A. niger* inhibits the germination and pre-symbiotic development of *G. mosseae*. Soluble substances present in the exudates of *A. niger* decrease the percentage of spore germination and hyphal growth in *G. mosseae*. These noxious effects of *A. niger* metabolites were not observed in our experiments, where a positive interaction between both microorganisms is clearly demonstrated.

Although the differences in height and biomass production of plants inoculated with *G. mosseae* without rock phosphate were not significant as compared to controls, a smaller size of inoculated plants was evident. These results agree with those observed by Cardoso *et al*. (1986) and could be explained as a competence between the mycorrhizal fungus and the plant for carbohydrates (So and Smith, 1988). On the other hand, evidence from several independent sources points to the *Glomeromycota* fungi as the cause of plant stunt disease (Hendrix *et al*., 1995).

In the substrate without rock phosphate, no significant differences were observed in plant growth in plants inoculated either with or without *G. mosseae*, while significant differences were observed between these treatments, and in the treatment in plants inoculated with *A. niger*. The co-inoculation with *G. mosseae* and *A. niger* improved tomato biomass both in the substrate with and in the substrate without rock phosphate.

In the treatment where plants were inoculated with both microorganisms and that were not fertilized with rock phosphate, the production of aerial biomass was significantly higher that in the other treatments; this additionally supports the synergic effect of *G. mosseae* and *A. niger*, which turned out to be beneficial for plant growth. On the other hand, the lower biomass production in the treatments inoculated with the AM fungus *G. mosseae* and fertilized with phosphoric rock could indicate that the plant-fungus interaction has important consequences for the physiology of the plant that are not always immediately related with the total production of dry mass (So and Smith, 1988).

Plants with AM fungi increase the exploitation of the soil volume by the hyphal network, which increases the active absorption surface. This increase in soil volume explored by arbuscular mycorrhizal fungi is evident by a lower production of radical biomass (Sieverding, 1991). Our results confirm this statement. Radical biomass values found in the treatments inoculated with *G. mosseae* significantly differ from the non-inoculated control, where the plant had to produce a greater radical biomass to satisfy its nutritional demands.

P-solubilizing microorganisms dissolve non-available forms of this nutrient by excretion of organic acids and quelant substances (Kucey *et al*., 1989; Kapoor, 1995). Reddy *et al*. (2002) have pointed out the capacity of *Aspergillus tubingensis* and *A. niger* of biosolubilizing rock phosphate. In treatment 5, where rock phosphate and *A. niger* were used, we obtained the highest values of substrate P concentration. The other values of P found in the substrate, support previous findings (Gianninazi-Pearson *et al*., 1981; Cabello *et al*., 2005) that establish that arbuscular mycorrhizal fungi do not solubilize phosphoric rock but do favour the uptake of slow motion P ions from soil solution.

P solubilization due to abiotic factors has been reported by He *et al*. (1999). The plant can also solubilize P by producing radical exudates (Dakora and Phillips, 2002). Grimal *et al*. (2001) also point out the solubilizing function by means of the production of radical mucilages and root-associated bacteria. These mechanisms can explain the presence of P in the substrate and in plant tissues from treatment 4, which were not inoculated with P-solubilizing microorganisms.

Our work confirms the synergic effects between mycorrhizal fungi and P-solubilizing microorganisms already reported (Buscot and Varma, 2005; Cabello *et al*., 2005). Our studies show that mycorrhizas are a necessary part of a P-solubilizing system and therefore that a significantly more efficient system that increases the capacity of radical systems to absorb P can be obtained. However, it is important to consider that the solubilizing capacity of the microorganisms tested *in vitro* not always reflects the solubilizing potential in field conditions and the mechanisms involved, since the substrates used do not possess an effective buffer system, and the acidification that is produced in them could be the main solubilizing mechanism (Villegas and Fortín, 2002; Whitelaw, 2000).

We conclude that soil cultures with nutritional deficit can be improved by mycobization with arbuscular mycorrhiza-forming fungi and P-solubilizing saprobes, which would allow plants to obtain P in insoluble forms such as phosphoric rock. However, more studies are necessary in order to evaluate the potential of co-inoculation and the application of phosphoric rock. under different agroclimatic conditions and particularly in field conditions, since each plant-soil-microorganism system deserves special analysis.

Acknowledgements

The authors wish to thank Lic. Bucsinszky, A. M. (CONICET technician) who is in charge of the culture collection at Spegazzini Institute. Velázquez, S. is a recipient of a scholarship from Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC); Eliades, L. is a recipient of a scholarship from La Plata University, Irrazabal, G. and Saparrat, M. are recipients of scholarships from CONICET; Cabello, M. is researcher from CIC. A grant from CIC and ANPCyT No 13404 BID 1201/OC-AR, Argentina, have supported this research.

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(Received 19 October 2005; accepted 1 December 2005)