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Differential interaction between two *Glomus intraradices* strains and a phosphate solubilizing bacterium in maize rhizosphere

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi and phosphate solubilizing bacteria (PSB) have a positive effect on plant productivity primarily through increasing phosphate availability. In order to study the interaction between AM fungi and PSB, we used *Bacillus megaterium*, a PSB isolated from the sterilized surface of AM germinated spores, and two strains of the AM fungus *Glomus intraradices* with different mycelial architecture. A greenhouse experiment was designed with maize as host plant with the addition of tribasic calcium phosphate. We tested the hypothesis that PSB, intimately linked with AM fungi, could interact differentially with the two AM strains. We concluded that inoculation with the PSB positively affected maize mycorrhization. Insoluble phosphate alone did not influence the AM extraradical mycelium (ERM) length and maize mycorrhization when bacteria were not inoculated. The results provide evidence that the adverse effect on infectivity for some AM strains might be caused by solubilized phosphorus release to the rhizosphere by PSB. Differences related to the mycelium architecture of each AM strain were observed: the density of PSB in rhizosphere soil was significantly higher only with the GA8 strain coinciding with the highest values of maize biomass. The density of bacteria associated with GA8 mycelium could be the result of the transfer of photosynthates through the rhizosphere; this close contact would favor the persistence of the intimate relationship between PSB and AM hyphae. In the bacteria-free treatments, soil adherence was not significantly altered. Although the highest development of ERM occurred with GA5, plants inoculated with GA8 showed the highest values for soil adherence. This may be due to the AM mycelium which modifies bacterial persistence in the rhizosphere and consequently soil adherence. Our results show that for potential applications, some characteristics of the AM strains are key in the selection of the AM fungi–PSB combinations. These include the tolerance to soluble phosphorus, the rate of root colonization, and ERM development that favors the persistence of bacteria in rhizosphere soil.

Introduction

Soil microorganisms form a community that depends on the organic compounds that are provided by root exudates (Lynch and Whipps 1991). Therefore, microbial activity in the rhizosphere is particularly high compared to that in bulk soil (Gryndler 2000). Gerhardson and Clarholm (1986) reported that rhizospheric bacteria usually reach a density twenty times higher than bacteria inhabiting bulk soils. Rhizospheric bacteria produce exopolysaccharides that result in significant increases in soil adherence (Kaci et al. 2005). The combined effect of root hairs and mucilage, either produced by roots or by rhizosphere microorganisms (Watt et al. 1994), can lead to the formation of specific structures called rhizosheaths. These structures have been observed across a wide range of plant species, especially in grasses (Hinsinger et al. 2009).

Exudates of AM fungi are also released into the mycorrhizosphere, and may influence selectively the presence of rhizospheric microorganisms (Marschner and Timonen 2005). The extraradical mycelium (ERM) of AM fungi provides a habitat for soil microorganisms, which is different from that provided by roots. Bacterial colonization and formation of biofilm-like structures on the surface of AM hyphae have been reported (Frey-Klett et al. 2007; Silvani et al. 2008). When bacteria are inoculated into the soil they could remain attached on the hyphae of AMs, decreasing their presence in the rest of the soil (Frey-Klett et al. 1999). This close contact could benefit both soil microorganisms, facilitating metabolic interactions and exchange of nutrients (Artursson et al. 2006).

Plants can directly absorb soil phosphate through high-affinity transporters in roots; however, this direct absorption is limited when phosphorus levels decrease in the soil solution near the roots.

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The ERM network grows beyond the depletion zone, absorbing mineral phosphates and translocating them to the AM intraradical structures and then to the root cortex cells. This mechanism is considered the main benefit of the AM symbiosis to the host plant (Smith and Read 1997). Nevertheless, Chiou et al. (2001) suggested that, probably due to the increasing levels of phosphorus in roots, the expression of radical phosphate transporters (MtPT1) in *Medicago truncatula* plants decreased when mycorrhization rates of *Glomus intraradices* and *Glomus versiforme* were augmented.

Several studies have demonstrated a synergistic interaction between AM fungi and phosphate solubilizing bacteria (PSB) (Barea 1997; Kim et al. 1998), with an increase in phosphorus acquisition by the host plant (Toro et al. 1997). The development of the rhizospheric microbial community is involved in plant productivity (Andrade et al. 1997). Therefore, AM fungi and different groups of bacteria, which promote plant growth by different mechanisms, could be considered in the formulation of biofertilizers into the context of sustainable agriculture.

Given this background, the main objective of this study was to analyze the interactions between the PSB *Bacillus megaterium*, isolated from AM propagules, and two strains of *G. intraradices* in the rhizosphere of maize plants. These findings could improve our ability to select beneficial combinations of AM fungi and their associated bacteria.

Considering the number of microorganisms that develop in association with AM structures in soil, our goal was to test the hypothesis that PSB, intimately linked with AM fungi, could synergistically interact with two axenically propagated AM strains.

Materials and methods

Biological material and experimental design

Seeds of maize (*Zea mays*) were surface-sterilized with 70% (v/v) of ethanol solution for 20 min, 20% (v/v) of sodium hypochlorite solution plus Tween 20 (0.1%) for 30 min, rinsed three times with sterile distilled water, and germinated on moist filter paper for 4 days. Maize seedlings uniform in size were transplanted into pots with 500 g of an autoclaved (100 °C for 1 h, three consecutive days), mixture of 1:1:1 perlite, vermiculite and soil (pH 7.1; total C 12.08 and N 1.1 (g kg^{-1}); P 34.2 mg kg^{-1}; K 0.9, Ca 7.5, Mg 1.7 and Na 0.2 (cmol kg^{-1})). Half of the pots were homogeneously amended with 1 g of tribasic calcium phosphate ($Ca_3(PO_4)_2$) per 1000 g of sterile substrate to establish a high insoluble P treatment, while allowing AM symbiosis establishment. Inorganic insoluble phosphates are solubilized by microorganisms due to the release of organic acids (Nautiyal 1999).

Roots of maize seedlings were inoculated at transplanting time separately with one of the *G. intraradices* strains: GA5 (BGIV, http://www.bgiv.com.ar/strains/glomus-intraradices/ga5) or GA8 (BGIV, http://www.bgiv.com.ar/strains/glomus-intraradices/ga8). Previously, differences between the two AM fungal strains in architecture of external mycelium and production of their hyphal structures were observed using monoxenic cultures with transformed carrot roots as the host plant (Silvani 2011). GA5 strain is an extensive and fast colonizer both in soil and *in vitro* conditions. The extraradical mycelium spreads throughout the growth substrate by the development of abundant runner hyphae. In contrast, GA8 strain is characterized by a different mycelial growth pattern. This strain is an intermediate and limited colonizer under both growth conditions; it develops a mycelium network composed of profuse hyphal branches and typical branched absorbing structures (BAS) (Silvani 2011).

Inoculation with AM fungi was carried out by placing 1-cm^3 plugs of 3-month-old *G. intraradices* monoxenic cultures, containing mycorrhizal transformed carrot (*Daucus carota*) roots, approximately 250 spores, and abundant ERM. These cultures were routinely grown in Minimal Medium (MM) (Bécard and Fortin 1988) and incubated in an inverted position at 25 °C in the dark. The non-mycorrhizal control plants were prepared as previously detailed, except that roots were inoculated with 1-cm^3 plugs of MM with non-mycorrhizal transformed carrot roots.

Half of the plants in each AM treatment (*G. intraradices* strain GA5, *G. intraradices* strain GA8 and non-mycorrhizal control) were inoculated with the phosphate solubilizer bacteria (PSB) *B. megaterium* strain SJ5R7 (GenBank accession number JN845569). Two milliliters of concentrated bacterial suspension were added per pot (10^9 cell ml^{-1}) to ensure bacterial survival in the soil in a concentration of at least 10^7 cell ml^{-1}. An association with AM propagules was previously observed for SJ5R7 strain (Silvani et al. 2008). SJ5R7 was initially recovered from the sporosphere of surface-sterilized (15 min in a 5% chloramine-T (Merck) solution) and germinated spores of the AM fungus *Glomus margarita* strain J5 (FCEyN, UBA).

Five pots per treatment were established as follows: the AM fungal treatments (GA5, GA8 and non-mycorrhizal) singly or co-inoculated with the PSB, and supplemented with P or non-treated. Pots were placed in a completely random design, and maize plants were grown with natural light and room temperature under greenhouse conditions. During the assay pots were irrigated with 50 ml of Hewitt (1952) nutritive solution without P every 10 days.

Analysis and harvesting

Fungi

The production of extraradical mycelium of both *G. intraradices* strains was determined as the length of ERM from the soil attached to roots following the methods in Graham et al. (1982). Samples of air-dry roots (48 h in the dark at room temperature) were vigorously shaken to remove the soil. Soil particles were washed into a beaker and dried until constant weight. The ERM length (mm) of GA5 and GA8 were quantified after staining with trypan blue in lactic acid (0.02%). Values were calculated for 1 g of the attached dried soil samples (Brundrett et al. 1994). AM colonization of maize roots was observed using the modified Phillips and Hayman (1970) method: roots were cleared with KOH (10%, w/v, 15 min, 90 °C) and stained with trypan blue in lactic acid (0.02%, 10 min, 90 °C). Intraradical colonization was quantified by examination of 50 randomly selected root pieces, in groups of ten, and the frequency (%*F*) of mycorrhizal colonization was calculated as the percentage of root segments containing hyphae, arbuscules or vesicles (Declerck et al. 2004). All measurements were made with a Nikon binocular microscope at 100× magnification.

Soil

A portion of maize roots was removed at harvest time, then dried and vigorously shaken to remove loose soil, as described previously. The layer of adhering soil was washed by immersion in 90 ml sterile distilled water; the soil suspensions were decanted and dried until constant weight. The amount of adhering soil in maize roots was expressed as grams of dry soil/grams of fresh root (Graham et al. 1982).

Bacteria

From rhizospheric soil suspensions previously obtained serial dilutions (1/10) were performed, and 100 μl were uniformly sowed on Petri dishes containing NBRIP solid medium with $Ca_3(PO_4)_2$ (5 g l^{-1}), and incubated at 29 °C in the dark for a week. The density of inoculated PSB on maize rhizospheric soil was determined by counting colonies surrounded by a halo on the medium surface,

due to mineral solubilization (Nautiyal 1999). Bacterial counts were expressed as log 10 CFU g^{-1} in soil (dry weight) (De Leij et al. 1993).

Plants

Maize plants were harvested after forty days of growth; aerial and radical biomass production was separately assessed as dry weight (DW), after drying shoots and roots at 80 °C until constant weight.

Statistical analysis

The experiment was arranged in a completely randomized design with equal replication in each treatment. Five pots were established per treatment. The effects of bacterial inoculation, AM fungal inoculation, and phosphate addition on growth traits of plants (dry weight of roots and shoots), and on AM fungal development (colonization frequency and ERM length) were examined separately by multivariate analysis of variance (MANOVA). Data from bacterial density and soil aggregation were subject to analysis of variance (factorial ANOVA) with bacterial inoculation, AM fungal inoculation, and phosphate addition as variation sources. Assumptions of homoscedasticity and normality were evaluated using Levene test and Shapiro–Wilkes test. Where the three-way interactions were not significant, two-way interactions or main effects were analyzed. Where significant differences were found, comparisons among mean values in each treatment were made using Tukey test (honestly significant difference HSD), calculated at $p < .05$ (Clewer and Scarisbrick 2001). Statistical procedures were carried out with the software package SPSS 15.0 for Windows XP.

Results

AM fungal and bacterial development

No significant triple interaction for MANOVA of AM symbiosis development and length of ERM was observed among the combined inoculations of AM fungi, bacterial, and P treatment. The two-way analysis revealed significant interactions among treatments (Table 1a). When bacteria were co-inoculated with GA5 without addition of phosphate, root colonization increased to more than 80%; and development of ERM increased to 233 mm/g dry rhizospheric soil. However, a significant decrease of mycorrhization (to 30%) and ERM length (to 70 mm/g dry rhizosphere soil) occurred when bacteria and phosphate were also added to pots. Without bacterial co-inoculation, GA5 strain always showed intermediate mycorrhization and ERM values (Fig. 1a). A similar effect of PSB inoculation was observed with the GA8 strain. When GA8 and bacteria were co-inoculated, there was an increase in root colonization values (from 17% GA8 alone to 45%) and in the length of ERM (from 64 to 240 mm/g dry rhizosphere soil). This effect of bacteria on GA8 colonization was not significantly modified when pots were amended with phosphate (30% of mycorrhization), but addition of phosphate eliminated this increase in the ERM that reached a length of less than 100 mm/g dry weight rhizosphere soil (Fig. 1a). AM colonization and ERM development were not observed on roots of uninoculated control plants.

A significant interaction between the inoculation of AM fungal strains and phosphate was observed on bacterial density (Table 2a). The number of PSB on rhizosphere was significantly elevated (25×10^7) when GA8 and phosphate were added to pots with bacteria. No other treatments significantly altered bacterial density. CFU of PSB in rhizosphere soil of mycorrhizal plants with GA5 did not show differences (36×10^6 and 58×10^6) with those treatments without AM fungi (15×10^6 and 75×10^6) (Fig. 2a). No PSB colonies were detected in control treatments.

Rhizosphere soil

No significant interactions between the tested factors were observed on the amount of adhering soil in maize roots, and bacterial inoculation was the only factor that had a significant effect on this variable (Table 2b). When bacteria were not inoculated the amount of dry soil attached to maize roots was not significantly different between treatments, and it was lower than 10 g/g of fresh weight root. Dry soil attached to roots of plants co-inoculated with bacteria and GA5 or GA8, and fertilized with phosphate, was similar to plants inoculated with bacteria alone and also reached a lower value than 10 g/g of fresh weight root. The largest amount of dry soil attached was observed in roots of plants co-inoculated with bacteria and GA5 without phosphate, and was approximately of 15 g/g of fresh weight root (Fig. 2b).

Plant growth

MANOVA of root (DWr) and shoot dry weight (DWs) yielded a significant three-way interaction among the combined inoculations of AM fungi, bacterial and P treatment (Table 2b). Compared to uninoculated and unamended plants addition of GA5 reduced root mass regardless of other treatments. DWr resulted in the lowest values obtained when SJ5R7 and GA5 were co-inoculated. GA8 also reduced root mass except when plants were co-inoculated with PSB and also amended with phosphate, this treatment produced plants with the largest DWr (Fig. 2b).

Little differences were observed for DWs between plants colonized by AM fungi and the non-mycorrhizal controls, when *B. megaterium* was not co-inoculated. Co-inoculation of GA5 and SJ5R7 resulted in a DWs value equivalent to plants with bacteria and phosphate. Shoot mass was significantly increased when maize plants were co-inoculated with GA8, SJ5R7 and phosphate (Fig. 2b).

Table 1
MANOVA results.

Effect	(a) %M/ERM				(b) DWr/DWs			
	df		Pillaïs trace		df		Pillaïs trace	
	Num	Denom	*F*	*p*	Num	Denom	*F*	*p*
AMF	2	31	160.46	<.001	4	96	21.76	<.001
B	2	31	29.55	<.001	2	47	23.68	<.001
P	2	31	81.16	<.001	2	47	46.90	<.001
AMF × B	2	31	20.45	<.001	4	96	8.44	<.001
AMF × P	2	31	93.10	<.001	4	96	8.70	<.001
B × P	2	31	115.48	<.001	2	47	26.82	<.001
AMF × B × P	2	31	6.73	.040	4	96	10.13	<.001

Effect of AM fungal inoculation (AMF), PSB inoculation (B) and phosphate addition (P) on: AM mycorrhization (%M) and ERM length (a), and dry weight of roots (DWr) and shoots (DWs) (b). Main effects and interaction terms are indicated.

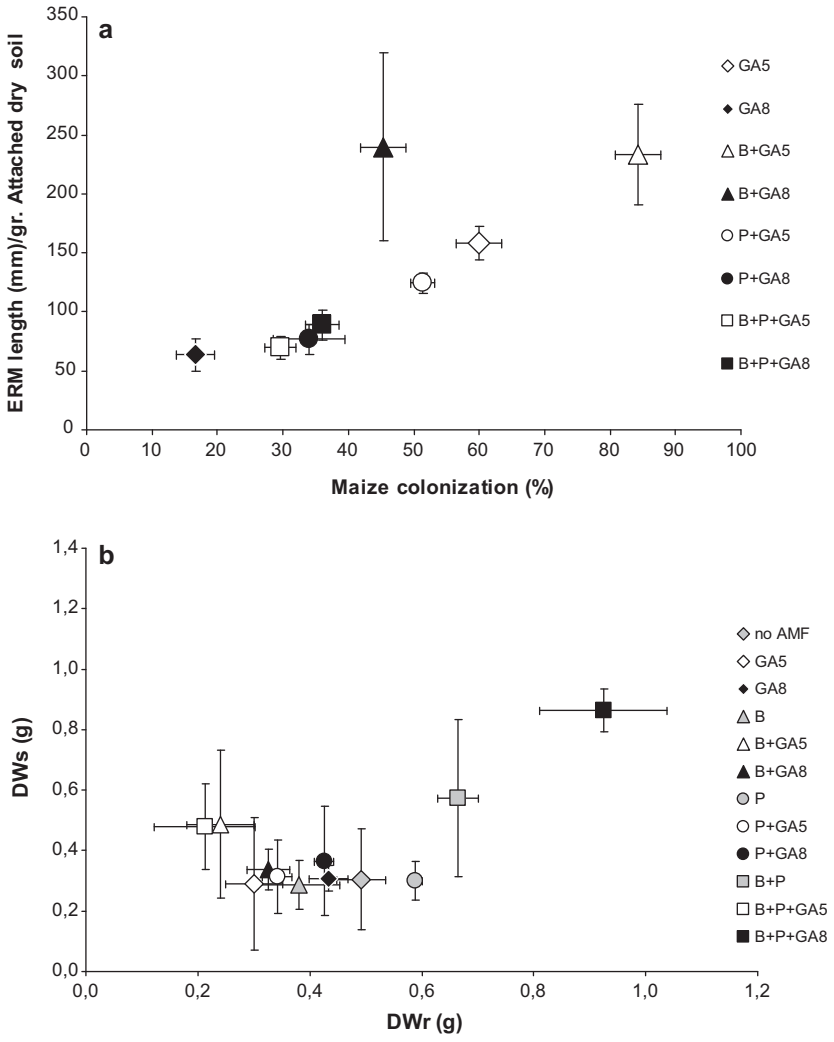

Fig. 1. (a) Root colonization frequency (%) and development of ERM of *G. intraradices* (strains GA5 and GA8) in maize rhizosphere, with and without addition of *B. megaterium* SJ5R7 (B) and tribasic calcium phosphate (P). (b) Growth of maize plants expressed as roots (DWr) and shoots dry weight (DWs). Values are the mean of five observations (±standard error).

Table 2
Factorial ANOVA results.

	(a) CFU			(b) Agg		
Effect	df	*F*	*p*	df	*F*	*p*
AMF	2	0.87	.074	2	1.95	.164
B	–	–	–	1	24.89	<.001
P	1	19.18	<.001	1	0.92	.348
AMF × B	–	–	–	2	2.13	.141
AMF × P	2	7.91	<.001	2	2.96	.071
B × P	–	–	–	1	0.32	.579
AMF × B × P	–	–	–	2	2.54	.100

Effect of AM fungal inoculation (AMF), PSB inoculation (B), and phosphate addition (P) on: bacterial density (CFU) (a), and rhizospheric soil aggregation (Agg) (b). Main effects and interaction terms are indicated.

Discussion

Inoculation with the PSB *B. megaterium* SJ5R7 positively affected root colonization by *G. intraradices* strains (GA5 and GA8) on maize plants. Our results agree with those of Marulanda et al. (2008) who observed the same effects when plants of *Lactuca sativa* were co-inoculated with *B. megaterium* and *G. intraradices*. While there are no reports of adverse effects of *B. megaterium* on AM fungi, this bacterium could have differential effects on AM infectivity depending on the fungal species involved (Marulanda et al. 2008). When other PSBs such as *Pseudomonas* (Kohler et al. 2006) and *Enterobacter* (Kim et al. 1998) were inoculated, there were no increases in the percentage of root mycorrhization by *G. intraradices.*

In our study the amount of GA5 ERM in the rhizosphere of maize was related to the percentage of mycorrhization achieved. Both variables were higher when GA5 and bacteria were co-inoculated without phosphate addition. Insoluble phosphate did not influence ERM length and the mycorrhization values when bacteria were absent. These findings demonstrate that the adverse effect on infectivity of certain AM fungi might be caused by the

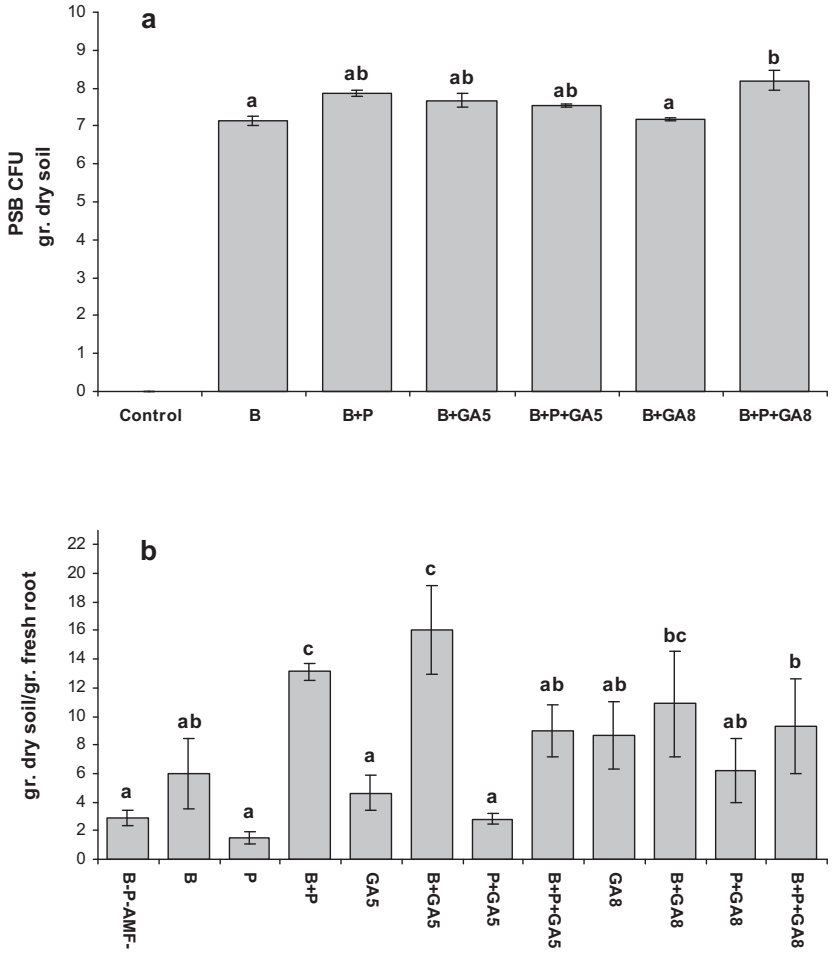

Fig. 2. (a) Bacterial density with and without addition of *G. intraradices* (strains GA5 and GA8) and tribasic calcium phosphate (P). (b) Rhizosphere soil adherence. Values are the mean of five observations (±standard error). Bars with different letters are significantly different (HSD test, $p < .05$).

solubilized phosphorus released into the rhizosphere by the PSB. Previous studies showed that high levels of phosphorous available in the soil solution could have negative effects on mycorrhization (Liu et al. 2000; Maldonado-Mendoza et al. 2001).

In spite of these findings, infectivity of GA8 increased when bacteria were co-inoculated, regardless of phosphate addition. In this treatment ERM length in the rhizosphere and root mycorrhization were also positively related. But at similar values of ERM, the percentage of mycorrhization of GA8 did not increase as much as that of GA5. When bacteria and GA8 were co-inoculated and phosphate was not added, the two variables reached their highest values, but given the mycelium architecture of this strain, the length of external hyphae increased proportionally more than mycorrhization. *In vitro* GA8 developed a large number of ramifications in the mycelium, but a reduced amount of runner hyphae, which would be responsible to contact and infect the roots (Friese and Allen 1991). This is related to the fact that despite of having an ERM development similar to that of GA5, GA8 mycorrhization is almost 50% lower.

In our study, only when GA8 was co-inoculated and phosphate was added, was the density of bacteria in the rhizosphere soil significantly increased, compared with the control treatment (bacterial single inoculation). In this treatment also the highest values of maize biomass were observed, suggesting that the density of bacteria linked to GA8 mycelium could be associated with the transfer of photosynthates through the rhizosphere. Toro et al. (1997) observed that when rock phosphate was added and *G. intraradices* with a PSB were co-inoculated in *Allium cepa*, the phosphorus absorbed by plants was preferentially the remaining phosphorus available from rock phosphate by the action of microorganisms. The uptake of phosphate ions released from rock phosphate by the AM mycelium, takes place in soil microhabitats where the rock particles are attacked by bacteria. This close contact would favor the persistence of the intimate relationship between PSB and AM hyphae.

In the bacterial-free treatments, the root-adhering soil was not significantly altered either by inoculation with AM fungi or by phosphate fertilization. Despite of GA5 ERM length was higher; the amount of adhering soil in roots inoculated with GA8 had high values. These results show that qualitative differences in the external mycelium of these strains could influence variation in soil adherence.

In the treatments where *B. megaterium* was co-inoculated, GA5 and GA8 significantly increased their ERM development; this rise was associated with the increase of rhizosphere soil adherence for both AM strains. Kohler et al. (2006) reported a similar enhancement in soil adherence when bacteria and phosphorus were combined. These authors did not observe a significant increase in bacterial density in the rhizosphere, related to the increase in soil adherence. The carbohydrates released from plant roots can promote the development of microbial communities (Kohler et al. 2006). Bacterial populations exude exopolysaccharides and roots produce mucilage that together affect soil structure increasing soil adherence (Jastrow et al. 1998; Kaci et al. 2005). AM mycelium also modifies bacterial persistence in the rhizosphere and consequently soil adherence.

Several studies reported a synergistic effect on plant growth when specific combinations of AM fungi and bacteria were co-inoculated (Vivas et al. 2003; Artursson et al. 2006). In spite of the negative effect of GA5 on root biomass production, GA8 strain significantly increased maize biomass when PSB and phosphate were also added to plants. Other studies with maize plants under greenhouse conditions showed that the application of *Glomus* species and bacteria, including a PSB strain of *B. megaterium*, significantly increased plant growth (Wu et al. 2005). With other plant hosts *G. intraradices–B. megaterium* co-inoculation also increased plant biomass, although the combination of this bacterium with *Glomus constrictum* had the opposite effect (Marulanda et al. 2008).

Under particular conditions AM fungi–PSB co-inoculation could have a synergistic effect on plant growth. Boomsma and Vyn (2008) found that productivity of host plants like maize benefits from the AM symbiosis, especially in infertile and drought environments. Testing to determine the exact combinations of beneficial microorganisms could be related with characteristics as: AM strain tolerant to phosphorus soluble, with a moderate root colonization rate and an ERM architecture that favors the persistence of bacteria in the rhizosphere soil.

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