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Differential Effects of a *Bacillus megaterium* Strain on *Lactuca sativa* Plant Growth Depending on the Origin of the Arbuscular Mycorrhizal Fungus Coinoculated: Physiologic and Biochemical Traits

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Abstract Coinoculation with plant growth–promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) has been proposed as an efficient method to increase plant growth. In this article we investigate how the interaction between three different AMF isolates (*Glomus constrictum* autochthonous, GcA; *G. constrictum* from collection, GcC; and commercial *Glomus intraradices*, Gi) and a *Bacillus megaterium* strain isolated from a Mediterranean calcareous soil affects *Lactuca sativa* L. plant growth. Inoculation with *B. megaterium* increased plant growth when in combination with two of the AMF isolates (GcA and Gi), but decreased it when in combination with GcC. At the same time, plants inoculated with the GcC fungus alone or in combination with *B. megaterium* (GcC+Bm) showed leaf symptoms of stress injury by accumulating proline and reducing the amount of photosynthetic pigments, whereas the opposite occurred in plants coinoculated with Gi fungus and *B. megaterium* (Gi+Bm). GcC+Bm leaves also presented the highest glucose-6-phosphate dehydrogenase (G6PDH) and the lowest glutamine synthetase (GS) enzymatic activities, whereas Gi+Bm leaves showed the highest GS activity. Results on these enzymatic activities are further discussed in relation to plant growth and performance.

Keywords Arbuscular mycorrhizal fungi · Glucose -6-phosphate dehydrogenase · Nitrogen assimilation · Photosynthetic pigments · Plant growth promoting rhizobacteria · Proline

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Introduction

Plants in nature need to cope with several adverse environmental conditions such as water deficit, high concentration of salts in the soil, extreme temperatures, nutrient deficiency, and pathogen attack (Bohnert and others 2006). However, plants can interact with several soil microorganisms, including plant growth–promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF), that make the plant more tolerant to such stresses (Barea and others 2002, 2005; Vessey 2003). Thus, plants inoculated with PGPR or AMF usually grow better than noninoculated plants under conditions of nutrient limitation (Wu and others 2005; Canbolat and others 2006). Beneficial effects are usually enhanced when both microorganisms are coinoculated, although this depends on the bacterium–fungus pair (Galleguillos and others 2000; Valdenegro and others 2001; Vivas and others 2003, 2006).

There have been several mechanisms described by which PGPR can increase plant growth. These include N_2 fixation, increasing the availability of nutrients in the rhizosphere, enhancing beneficial effects of other symbionts, or reducing ethylene production of the plant (Penrose and Glick 2003; Vessey 2003). It is also known that PGPR induces the production of plant hormones such as IAA, GA, and cytokinin (Glick 1995). Ryu and others (2005) showed that growth promotion by PGPR may be mediated by IAA, brassinosteroid, salicylic acid, and gibberellin signaling pathways. At the same time, it is well documented that an increase in plant growth by AMF is mediated in part by an alteration in the plant's hormone

levels (Goicoechea and others 1997; Shaul-Keinan and others 2002). The positive effect of AMF on plant growth can also be related to improved root nutrients and water uptake and increased defenses against soil pathogens (Filion and others 1999; Smith and others 2001; Marulanda and others 2003). Effects of coinoculation of PGPR and AMF on plant growth and nutrient content have been studied (see Artursson and others 2006). However, as far as we know there is little information available about the effects of PGPR or AMF, either inoculated alone or coinoculated on the primary carbon and nitrogen metabolisms of the plant, both of which are strongly related to plant growth (Amthor 2000).

Ammonium (NH_4^+) is the only inorganic nitrogen compound that plants can assimilate into organic compounds via glutamine and glutamate (Lam and others 1996). Therefore, because nitrate (NO_3^-) is the main inorganic nitrogen ion absorbed by roots, it is necessary to reduce it to NH_4^+ before entering the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle that occurs predominantly in the leaves of most plant species (Andrews 1986; Lam and others 1996; Hodges 2002). The GS/GOGAT cycle needs a carbon skeleton (essentially, 2-oxoglutarate) to complete the cycle and this is provided by aspartate amino transferase (AsAT) among other enzymes (Lam and others 1996; Hodges 2002). Hence, there is a strong link between the metabolism of nitrogen and carbon (Hodges 2002; Stitt and others 2002).

The initial process in carbon metabolism is atmospheric carbon fixation, which is realized by the ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) enzyme in C_3 plants such as lettuce, whose activity can be regulated by mycorrhizal symbiosis and stresses (Kytöviita and others 1999). Phosphoenolpyruvate carboxylase (PEPC) catalyzes the β-carboxylation of phosphoenolpyruvate to yield oxaloacetate and inorganic phosphate (Andreo and others 1987). In C_3 plants the enzyme is involved in several important metabolic functions (see Latzko and Kelly 1983). The oxidative pentose phosphate pathway is one of the major sources of NADPH in nonphotosynthetic tissues and in photosynthetic tissues under darkness or when photosynthesis is restricted (Neuhaus and Emes 2000). The enzyme glucose 6-phosphate dehydrogenase (G6PDH) is the limiting step of this pathway (Lendzian 1980).

Because the interaction between AMF and PGPR in some cases can be negative in terms of plant growth (Galleguillos and others 2000; Valdenegro and others 2001; Vivas and others 2003, 2006), we decided to also investigate stress markers such as proline content and the amount of photosynthetic pigments. Proline is a free amino acid that accumulates under stress conditions in plant tissues. Proline may act as an osmolyte, stabilizing proteins, scavenging hydroxyl radicals, regulating cytosolic pH, and regulating the NAD/NADH ratio (Matysik and others 2002). Because photosynthetic pigments (chlorophylls and carotenoids) are degraded under leaf senescence, they are also suitable markers for leaf stress (Cabello and others 2006).

The aim of the present research was to evaluate how different AMF isolates interacted with a PGPR (*Bacillus megaterium*; A. Marulanda-Aguirre and R. Azcón, unpublished data) isolated from calcarean Mediterranean soil of southeastern Spain. To characterize the interaction between the bacterium and AMF, plant and fungal growth parameters, plant stress index traits, and some leaf nitrogen and carbon assimilatory enzyme activities were measured.

Materials and Methods

Biological Material and Experimental Design

Seeds of *Lactuca sativa* L. cv. Romana were germinated in sterile sand at 25°C. Seven-day-old seedlings were transplanted to pots containing 500 g of a 5:2 mixture of soil:sand. Soil was collected from Sierra de Baza, Granada, Spain, sieved (mesh diameter = 2 mm), and sterilized by steaming (100°C for 1 h on 3 consecutive days). The soil had pH 7.2 (water), 1.6% organic matter, and the following nutrient concentrations (mg kg^{-1}): N, 2.1; P, 1.7 ($NaHCO_3$-extractable-P); K, 0.8. The soil consisted of 57.8% sand, 19.0% silt, and 23.2% clay (Marulanda and others 2006).

Plants were inoculated with one of the following AMF isolates: *Glomus constrictum* Trappe from collection (Estación Experimental del Zaidín, isolate EEZ 22, GcC); *G. constrictum* autochthonous isolated from Sierra de Baza, Granada, Spain (GcA); and *G. intraradices* (Gi) Shenck and Smith from a commercial company (MYCOSYM, Seville, Spain). Each AMF treatment plant group (including a noninoculated one) was inoculated or not with a *Bacillus megaterium* strain isolated from soil of Sierra de Baza, Granada, Spain (the soil used in the experiments). In total we had 64 plants (one plant per pot), with each plant group having eight plants.

Inoculum culture and application was carried out as described previously by Marulanda and others (2006). Briefly, mycorrhizal inoculums were bulked in an open-pot culture of red clover and consisted of soil, spores, mycelia, and infected root fragments having a colonization of 70%. Ten grams of mycorrhizal inoculum was added to the appropiate pots at transplanting time just below the roots of *L. sativa* seedlings. Nonmycorrhizal pots received the same amount of autoclaved inoculum (to obtain the same soil texture) together with a 2-ml aliquot of an inoculum filtrate. *B. megaterium* culture grown in nutrient broth

medium for 48 h at 28°C was centrifuged at 4500*g* for 5 min. The pellet was suspended in sterilized water, and the suspension contained 10^8 cfu ml^{-1}. *L. sativa* plants were inoculated at transplantation and 15 days later by adding 1 ml of bacterial suspension to each pot. The same amount of autoclaved medium was added to bacteria-free pots.

Plants were grown in a greenhouse under controlled climatic conditions (20–25°C, photoperiod of 16 h, and 50–60% relative humidity). Photosynthetic photon flux density of 350 μmol m^{-2} s^{-1} was supplied by fluorescent lamps. Plants were irrigated daily with tap water to keep the soil at water capacity, and twice a week 10 ml of complete nutrient solution was added. This fertilization treatment was applied based upon preliminary experiments to obtain enough plant biomass for the biochemical assays because the soil had low nutrient content (see above). The nutrient concentration of the solution was as follows: 6 mM $Ca(NO_3)_2$, 6 mM $CaCl_2$, 3 mM KNO_3, 2.3 mM K_2SO_4, 1.5 mM $MgSO_4$, 1.3 mM NaH_2PO_4, 68 μM EDTA-Fe, 13 μM $MnSO_4$, 9 μM H_3BO_4, 1 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM Na_2MoO_4. Plants were harvested 7 weeks after transplanting (56-day-old plants), the same harvest time as previous experiments using lettuce (Marulanda and others 2003). Five plants were used for determination of growth and colonization, whereas the other three plants were immersed in liquid N_2 at harvest and stored at –80°C for biochemical determination.

Plant, Fungal, and Bacterial Growth Measurement

Before harvest, all leaves of each plant were scanned (hp scanjet 5550c, Hewlett Packard, Palo Alto, CA, USA), and the corresponding images were analyzed with Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA, USA). At harvest, shoots and roots of five plants were separated and dried for 48 h at 75°C to obtain dry weights. Fungal colonization was assessed after clearing washed roots in 10% KOH and staining with 0.05% trypan blue (TB) in lactic acid (v/v), according to Philips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980), after counting 100 intersections. Five plates with roots of different plants were analyzed.

Extraradical mycelium production was estimated using the method described by Marulanda and others (2003). Briefly, 1 g of dry soil was treated with sodium hexametaphosphate and stained with TB (0.05%) in lactic acid. The samples were heated in a water bath at 90°C for 30 min and then sieved through 50-μm mesh. The remaining mycelium was mixed with bacteriological agar for quantification using a gridline intersection method of Newman (1966). Five soil samples from different pots were analyzed.

Bacterial density was determined by the dilution plating procedure. One gram of soil from five different pots per treatment was diluted in 9 ml sterilized water, and serial dilutions were performed. Twenty microliters of each dilution was sprayed onto nutrient broth medium plates and bacterial colonies were counted after 24 h incubation at 28°C.

Proline and Photosynthetic Pigment Amounts

Free proline was extracted in 50 mM phosphate buffer (pH 7.8) from 0.3 g leaf tissue from three independent plants (2 leaves per plant) previously immersed in liquid N_2 and stored at –80°C according to Bates and others (1973). The amount of proline was determined by spectrophotometric analysis at 515 nm using the ninhydrin reaction (Bates and others 1973). Photosynthetic pigments were extracted in 95% (v/v) ethanol (Aroca and others 2001) from leaf discs 0.25 cm^2 in diameter from three independent plants (2 leaves per plant). Extinction coefficients and equations reported by Lichtenthaler (1987) were used to calculate the pigment concentrations.

Enzyme Extraction

Leaf tissues (300 mg fresh weight from 3 independent plants) were homogenized as described by Aroca and others (2003). Leaves were homogenized in a cold mortar with 4 ml 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 8 mM $MgCl_2$, 5 mM DTT, and 1% (w/v) PVPP. The homogenate was centrifuged at 27,000*g* for 15 min at 4°C, and the supernatant was used for enzyme activity determination.

Carbon Metabolism Enzyme Assays

Ribulose-1,5-bisphosphate carboxylase–oxygenase (Rubisco, EC 4.1.1.39) activity was determined as described by Lilley and Walker (1974). The reaction was carried out in 2 ml 50 mM Hepes-KOH (pH 7.8), containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.6 mM ribulosebisphosphate, 0.2 mM NADH, 5 mM ATP, 5 mM phosphocreatine, 4.8 units of creatinphosphokinase, 4.8 units glyceraldehyde-phosphate dehydrogenase, 4.8 units of phosphoglycic phosphokinase, and 100 μml of leaf extract. Consumption of NADH at 340 nm was recorded. Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activity was measured following the method of Usuda and others (1984). The

reaction mixture consisted of 2 ml 50 mM Hepes-KOH (pH 8.0) containing 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.2 mM NADH, 2.5 mM phosphoenolpyruvate, 5 units of malate dehydrogenase, and 100 µml of extract. Consumption of NADH at 340 nm was recorded. Aspartate aminotransferase (AsAT, EC 2.6.1.11) was assayed according to Hatch and Mau (1973). The reaction mixture consisted of 2 ml of 100 mM Tris-HCl (pH 7.8) containing 240 mM aspartate, 0.3 mM NADH, 12 mM α-ketoglutarate, 2 units of malate dehydrogenase, and 100 µml of extract. Consumption of NADH at 340 nm was recorded. Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) was analyzed as described by Fickenscher and Scheibe (1986). The reaction mixture consisted of 2 ml 100 mM Tris-HCl (pH 8.0) containing 0.2 mM $NADP^+$, 3 mM glucose, 2 mM EDTA, and 100 µml of extract. Production of NADPH was recorded at 340 nm.

Nitrogen Metabolism Enzyme Assays

Nitrate reductase (NR, EC 1.6.6.1), glutamine synthetase (GS, EC 6.3.1.2), and glutamate synthase (GOGAT, EC 1.4.1.14) activities were assayed as described by Srivastava and Dwivedi (2003). The NR assay system consisted of 100 mM phosphate buffer (pH 7.5), 30 mM $NaNO_3$, 0.2 mM NADH, and 100 µml of extract, in the final volume of 2 ml. After incubating the tubes at 30°C for 30 min, the reaction was terminated by keeping the tubes in boiling water bath for 5 min. The nitrite produced was measured at 540 nm. The color was developed by adding 1 ml each of 1% sulfanilamide in 1 N HCl and 0.01% N-(1-naphtyl)-ethylene diamine hydrochloride, to a suitable amount of aliquot. The GS reaction mixture consisted of 100 mM Tris-HCl buffer (pH 7.0), 50 mM NH_4Cl, 10 mM ATP, 50 mM $MgCl_2$, 100 mM glutamate, and 100 µml of extract in the final volume of 2 ml. After incubation at 37°C for 15 min, the reaction was stopped by adding 1.1% $FeSO_4$ in 0.3 N H_2SO_4. The Pi liberated was measured at 540 nm using 150 µml of 6.6% ammonium molybdate in 7.5 N H_2SO_4. The GOGAT assay system contained 100 mM Tris-HCl buffer (pH 7.5), 20 mM α-ketoglutarate, 50 mM glutamine, 0.2 mM NADH, and 100 µml of extract in the final volume of 2 ml. Consumption of NADH at 340 nm was recorded. Total soluble protein amount was determined using the Bradford method (Bradford 1976) and BSA as standard.

Statistical Analysis

Means of all treatments of each parameter were analyzed by ANOVA and Fisher's LSD tests.

Results

Plant, Fungal, and Bacterial Growth

We first investigated the effect of a single or double inoculation on lettuce plant growth. Single inoculation with autochthonous *Glomus constrictum* (GcA) or commercial *G. intraradices* (Gi) significantly increased shoot and total dry weights (DW) as well as leaf area in lettuce plants (Table 1). It is noticeable that the increase in shoot DW and leaf area was higher in Gi plants than in GcA plants (Table 1). On the contrary, plants singly inoculated with the collection *G. constrictum* (GcC) showed no significant changes in any of the plant growth parameters measured (Table 1). At the same time, neither GcA nor Gi inoculation had any effect on root DW (Table 1). On the other hand, inoculation with *Bacillus megaterium* (Bm) increased shoot DW and leaf area only in plants without mycorrhiza (NI) as well as in Gi plants (Table 1). Although the total DW increased in NI, GcA, and Gi plants, root DW was increased only by Bm inoculation in Gi plants (Table 1). On the contrary, lettuce plants coinoculated with Bm and GcC were smaller than plants inoculated with GcC or Bm alone (Table 1). The largest plants were those that were coinoculated with Gi and Bm, and the smallest ones were those coinoculated with GcC and Bm (Table 1).

There were no significant differences ($p > 0.05$) in the percentage of mycorrhizal root length found among single mycorrhizal treatments (Figure 1A). Bm inoculation significantly increased the percentage of mycorrhizal root length in Gi lettuce plants (Figure 1A). Gi+Bm plants showed the highest percentage of mycorrhizal root length,

Table 1 Effects of inoculation treatments on lettuce plant growth parameters

	Shoot DW (mg plant^{-1})	Root DW (mg plant^{-1})	Total DW (mg plant^{-1})	Leaf area (cm^2 plant^{-1})
NI	63 a	44 ab	107 ab	35 ab
Bm	96 bc	51 abc	147 de	53 cd
GcA	91 b	53 abc	144 cd	51 cd
GcA+Bm	108 bc	73 c	181 e	64 de
GcC	66 a	45 ab	111 bc	43 bc
GcC+Bm	48 a	26 a	74 a	27 a
Gi	112 c	65 bc	177 de	67 e
Gi+Bm	150 d	103 d	253 f	92 f

Different letters in each column indicate significant differences ($p < 0.05$) between inoculation treatments after ANOVA and Fisher LSD test ($n = 5$)

NI = plants not inoculated; Bm = plants inoculated with *Bacillus megaterium*; GcA = plants inoculated with autochthonous *Glomus constrictum*; GcC = plants inoculated with *G. constrictum* from a collection; Gi = plants inoculated with comercial *G. intraradices*; + = coinoculation; DW = dry weight

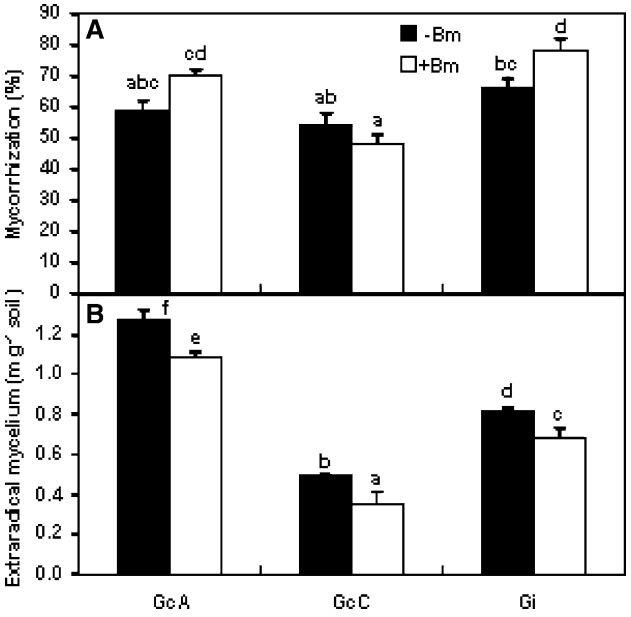

Fig. 1 Percentage of mycorrhizal root length (**A**) and extraradical mycelium growth (**B**) in *Lactuca sativa* plants that were not inoculated (black columns) or inoculated with a *Bacillus megaterium* strain (white columns), and coinoculated either with an autochthonous *Glomus constrictum* fungus (GcA), *G. constrictum* from a collection (GcC), or commercial *G. intraradices* (Gi). Columns with the same letter are not significantly ($p > 0.05$) different using ANOVA and Fisher LSD tests. Columns represent mean ± SE ($n = 5$)

whereas GcC+Bm plants showed the lowest. Regarding extraradical mycelium growth, GcA fungus showed the greatest growth and GcC the lowest; Gi exhibited an intermediate growth (Figure 1B). Coinoculation with Bm decreased the extraradical mycelium growth of the three fungi by a similar degree, although this decrease was proportionally higher in GcC fungus (29%) than in GcA (15%) and Gi (17%) (Figure 1B).

Bacterial density at the end of the experiment was 10^5 ufc g^{-1} soil in the four treatments including bacteria inoculation, with no significant differences ($p > 0.05$) between them.

Proline and Photosynthetic Pigment Amounts

The greatest accumulation of proline was found in plants inoculated with GcC fungus, especially when coinoculated with Bm (Figure 2). The other groups of plants did not show any significant difference ($p > 0.05$) in shoot proline amounts (Figure 2).

Single inoculation with GcA or Gi had no effect on the total amounts of either chlorophylls or carotenoids compared with NI plants (Figure 3). However, single inoculation with GcC decreased the amount of both kinds of pigments (Figure 3). Coinoculation with Bm had no effect on pigment amounts, except in those plants inoculated with Gi, in which

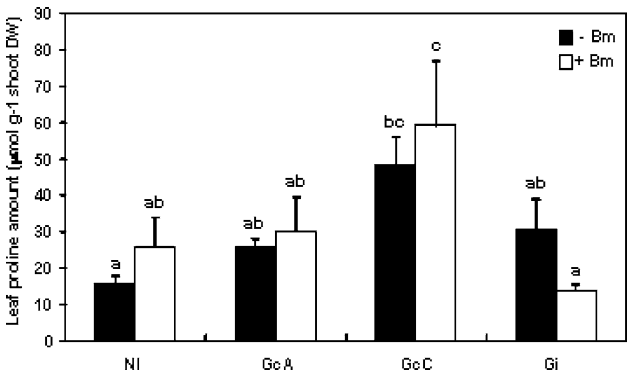

Fig. 2 Leaf proline amount of *Lactuca sativa* plants not inoculated (black columns) or inoculated with a *Bacillus megaterium* strain (white columns), alone (NI) or in combination with either an autochthonous *Glomus constrictum* fungus (GcA), *G. constrictum* from a collection (GcC), or commercial *G. intraradices* (Gi). Otherwise as for Figure 1

a rise in both total chlorophyll and carotenoids was observed (Figure 3). Thus, plants coinoculated with Gi fungus and Bm bacterium were the ones that had the highest amount of photosynthetic pigments, whereas those inoculated with GcC had the lowest amount (Figure 3).

Carbon and Nitrogen Assimilatory Enzyme Activities

Finally, we measured leaf carbon and nitrogen assimilatory enzyme activities to correlate them with plant growth. No

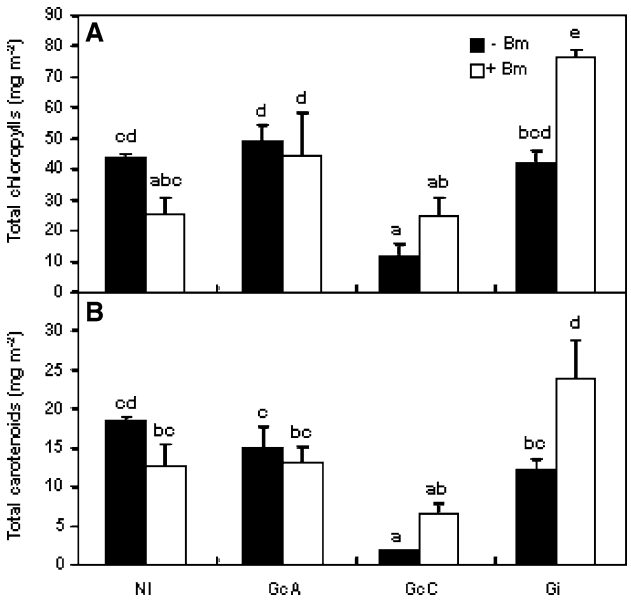

Fig. 3 Total chlorophylls (**A**) and carotenoids (**B**) in leaves of *Lactuca sativa* plants not inoculated (black columns) or inoculated with a *Bacillus megaterium* strain (white columns), alone (NI) or in combination with either an autochthonous *Glomus constrictum* fungus (GcA), *G. constrictum* from a collection (GcC), or commercial *G. intraradices* (Gi). Otherwise as for Figure 1

Table 2 Effects of different inoculation treatments on some C and N assimilatory enzymes

	G6PDH (nmol $NADP^+$ mg^{-1} protein min^{-1})	AsAT (nmol NADH mg^{-1} protein min^{-1})	NR (nmol NO_3 mg^{-1} protein min^{-1})	GS (nmol ATP mg^{-1} protein min^{-1})
NI	14.2 d	139 a	51 a	378 ab
Bm	7.1 c	480 ab	62 a	407 b
GcA	6.3 bc	445 ab	361 abc	301 ab
GcA+Bm	4.0 ab	461 ab	367 abc	458 bc
GcC	3.1 a	517 b	541 bc	299 ab
GcC+Bm	18.8 e	847 c	747 c	209 a
Gi	2.9 a	378 ab	101 ab	375 ab
Gi+Bm	6.6 bc	259 ab	49 a	601 c

Different letters in each column indicate significant differences ($p < 0.05$) between inoculation treatments after ANOVA and Fisher LSD test ($n = 4$)

GcA = *Glomus constrictum* autochthonous; GcC = *G. constrictum* from collection; Gi = *G. intraradices* all alone or in combination with Bm = *Bacillus megaterium*; G6PDH = glucose-6-phosphate dehydrogenase; AsAT = aspartate aminotransferase; NR = nitrate reductase; GS = glutamine synthetase. Otherwise as for Table 1

significant differences ($p > 0.05$) between treatments in Rubisco, PEPC, and GOGAT were found (data not shown). G6PDH activity decreased in all AMF-inoculated plants compared to NI plants (Table 2). Inoculation with Bm bacterium alone also caused a decrease in G6PDH activity, whereas no changes were observed in GcA leaves, while an increase in GcC and Gi leaves occurred (Table 2). The highest G6PDH activity was recorded in GcC+Bm leaves and the lowest in Gi ones (Table 2). Aspartate aminotransferase (AsAT) and nitrate reductase (NR) activities showed a similar trend to each other. No significant differences ($p > 0.05$) in their activity were found between treatments, except for plants inoculated with GcC fungus that showed higher activities, being even higher in those coinoculated with Bm (Table 2). Finally, no significant differences between treatments were found for leaf glutamine synthetase (GS) activity, except in plants coinoculated with Gi and Bm. Bm and GcA+Bm plants had higher activity than GcC+Bm ones (Table 2). Therefore, Gi+Bm leaves showed the highest activity of GS enzyme and GcC+Bm the lowest (Table 2).

Discussion

There is usually a synergistic effect on plant growth under several environmental conditions when PGPR and AMF are coinoculated (Vivas and others 2003, 2006; Artursson and others 2006). Here, coinoculation by both microorganisms increased lettuce plant growth when the autochthonous (GcA) or commercial (Gi) fungi were involved but decreased it when the collection fungus (GcC) was the partner. It is known that the effect of coinoculation of PGPR and AMF on plant growth depends on the pair inoculated (Galleguillos and others 2000; Valdenegro and others 2001; Vivas and others 2003, 2006). A positive effect has been frequently seen when both partners have the same origin and they are grown in the original soil (Vivas and others 2003, 2006). Here, we investigated some plant biochemical traits that could explain the different results observed. At the same time, we confirmed that the *Bacillus megaterium* strain isolated here can also act as a PGPR, as described for other strains (de Freitas and others 1997; Kishore and others 2005).

First, we investigated how the bacterium could influence fungal growth and colonization. Plants with the highest root fungal colonization (Gi) also showed maximum growth. In contrast, the lowest growth was found in those plants that had the lowest fungal colonization (GcC). This correlation between percentage of mycorrhizal root length colonized and plant growth has not always been observed (Garcia-Garrido and others 2000; Galleguillos and others 2000; Marulanda and others 2003). On the other hand, GcC exhibited the lowest extraradical mycelium production which was even more reduced by Bm coinoculation. Taking together the lowest values of percentage of mycorrhizal root length colonized and extraradical mycelium growth in GcC+Bm treatment, it is not surprising that these plants showed the lowest growth. However, no relationship between external mycelia growth and plant growth was found, as already shown in other studies (Garcia-Garrido and others 2000; Marulanda and others 2003). This lack of relationship may be due to differences in the activity of the microorganisms in the rhizosphere, ranging from beneficial to detrimental depending on the environmental conditions and the host plant (Kurek and Jaroszuk-Ścisel 2003; Mehnaz and Lazarovits 2006). Hence, the results showed that the combination of GcC fungus and Bm bacterium had a deleterious effect on the growth of lettuce in the soil used. However, this same

bacterium alone or in combination with one of the other fungal isolates had a positive effect on plant growth. It is possible that the increase in plant biomass induced by the bacterium in combination with GcA and Gi fungi was caused by the liberation of nutrients after extraradical mycelium degradation by the bacterium. However, the bacterium decreased extraradical mycelium in GcC pots which had a negative effect on plant biomass. Therefore, it is possible that there is a critical extraradical hyphal length required to enhance plant growth by the *B. megaterium* strain used here. Ravnskov and others (2002) found that some bacterium strains of *Burkholderia cepacia* also diminished extraradical mycelium growth of *G. intraradices*, although this had no effect on hyphal phosphate uptake. Because the *B. megaterium* treatment decreased the extraradical hyphal length of the three AM fungi in our experiment, it is possible that under field conditions where interaction with other microorganisms takes place, a negative effect on plant growth could occur. This possible effect needs to be tested in future experiments because extraradical mycelium helps the plant to take up water and nutrients and increases defenses against soil pathogenic microorganisms (Filion and others 1999; Smith and others 2001; Marulanda and others 2003).

Because plant growth results indicated that plants (especially those inoculated with GcC fungi) may be suffering from stress, we analyzed shoot proline content to identify stress symptoms within the plants (Ruiz-Lozano and others 1995). Plants inoculated with the GcC fungus, especially those coinoculated with Bm bacterium, had the highest amount of shoot proline. These data may indicate a greater stress injury in those plants because levels of proline can correlate with stress damage in lettuce plants (Ruiz-Lozano and others 1995; Porcel and others 2004). At the same time, GcC and GcC+Bm plants also had the lowest amount of photosynthetic pigments in their leaves, whereas Gi+Bm had the highest. A correlation between levels of photosynthetic pigments and plant growth performance was found here as shown in others studies (Kristjansdottir and Merker 1993). These results are not unusual because photosynthetic pigments are the first molecules to convert light energy into NADPH and ATP in leaf tissues (Wollman 2001).

Reduced energy (NADPH) in healthy leaves comes mainly from photosynthetic reactions. However, when photosynthesis is restricted, NADPH comes predominantly from the oxidative pentose phosphate pathway, in which G6PDH is the main regulatory enzyme (Wakao and Benning 2005). Here, we found that those plants that grew poorest (GcC+Bm) also had the highest G6PDH activity. G6PDH activity has been seen to rise when photosynthesis is inhibited (Hauschild and von Schaewen 2003). Hence, because GcC+Bm plants presumably had low photosynthetic activity, it is not strange that they also had higher G6PDH activity.

Aspartate aminotransferase (AsAT) catalyzed the transformation of glutamate and oxaloacetate into 2-oxoglutarate and aspartate (Hodges 2002). The 2-oxoglutarate produced is later used by GOGAT to catalyze the synthesis of two molecules of glutamate, one of which is used by the GS enzyme to form one molecule of glutamine by incorporating NH_4^+ into the GS/GOGAT cycle (Hodges 2002). AsAT activity has been seen to rise under conditions of nitrogen limitation (Bedell and others 1999). Here, we saw an increase of AsAT activity in the leaves of GcC plants, especially in those coinoculated with Bm bacterium. Thus, it is possible that these plants were suffering from nitrogen limitation because they were the smallest and had the lowest amounts of chlorophylls, common symptoms of nitrogen starvation (Debouba and others 2006). Because GcC and GcC+Bm plants had the highest NR activities, they would not be limited in this step of nitrogen assimilation. They also had the lowest GS activity and could be accumulating NH_4^+ (the end product of the pathway initiated by NR activity) as well as incorporating less ammonium into amino acids via glutamine. Lettuce plants belong to the Asteraceae family, members of which are sensitive to NH_4^+ accumulation (Britto and Kronzucker 2002). In fact, lettuce plants usually grow much better when nitrogen is supplied as nitrate than when it is supplied as ammonium (Demsar and Osvald 2003). However, Gi+Bm plants showed the highest GS activity and were the biggest plants. Miflin and Habash (2002) proposed a strong correlation between GS activity and crop yield, which is in agreement with our results.

We cannot rule out that changes in hormonal contents caused by the inoculants employed here could be influencing the biochemical traits studied (Goicoechea and others 1997; Ryu and others 2005) and this hypothesis would need to be checked in future experiments.

As a summary, the same bacterium strain had contrasting effects on plant performance depending upon the AMF involved. This is the first time that metabolic enzyme activities have been measured in plants coinoculated with PGPR and AMF. We found that G6PDH and GS activities were inversely and directly correlated to plant growth, respectively. G6PDH showed the highest activity in the negative interaction between GcC+Bm, whereas GS activity was greatest in the positive interaction between Gi+Bm. Therefore, these two enzyme activities could be used as markers of plant growth performance in lettuce plants.

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