J Plant Growth Regul (2009) 28:115–124
DOI 10.1007/s00344-009-9079-6

Stimulation of Plant Growth and Drought Tolerance by Native Microorganisms (AM Fungi and Bacteria) from Dry Environments: Mechanisms Related to Bacterial Effectiveness

Adriana Marulanda · José-Miguel Barea · Rosario Azcón

Received: 26 May 2008 / Accepted: 19 December 2008 / Published online: 24 February 2009

Abstract In this study we tested whether rhizosphere microorganisms can increase drought tolerance to plants growing under water-limitation conditions. Three indigenous bacterial strains isolated from droughted soil and identified as *Pseudomonas putida*, *Pseudomonas* sp., and *Bacillus megaterium* were able to stimulate plant growth under dry conditions. When the bacteria were grown in axenic culture at increasing osmotic stress caused by polyethylene glycol (PEG) levels (from 0 to 60%) they showed osmotic tolerance and only *Pseudomonas* sp. decreased indol acetic acid (IAA) production concomitantly with an increase of osmotic stress (PEG) in the medium. *P. putida* and *B. megaterium* exhibited the highest osmotic tolerance and both strains also showed increased proline content, involved in osmotic cellular adaptation, as much as increased osmotic stress caused by NaCl supply. These bacteria seem to have developed mechanisms to cope with drought stress. The increase in IAA production by *P. putida* and *B. megaterium* at a PEG concentration of 60% is an indication of bacterial resistance to drought. Their inoculation increased shoot and root biomass and water content under drought conditions. Bacterial IAA production under stressed conditions may explain their effectiveness in promoting plant growth and shoot water content increasing plant drought tolerance. *B. megaterium* was the most efficient bacteria under drought (in successive harvests) either applied alone or associated with the autochthonous arbuscular mycorrhizal fungi *Glomus coronatum*, *Glomus constrictum* or *Glomus claroideum*. *B. megaterium* colonized the rhizosphere and endorhizosphere zone. We can say, therefore, that microbial activities of adapted strains represent a positive effect on plant development under drought conditions.

Keywords Bacteria · Arbuscular mycorrhizal fungi · Drought tolerance

A. Marulanda · J.-M. Barea · R. Azcón (✉)
Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Profesor Albareda no 1, E-18008 Granada, Spain
e-mail: rosario.azcon@eez.csic.es

Introduction

Water deficit is the most common stress affecting plant growth in arid and semiarid regions. Thus, it is necessary to improve the level of efficiency in plant capture and use of water and nutrients. Inoculation of plants with native beneficial microorganisms may increase drought tolerance of plants growing in arid or semiarid areas (Marulanda and others 2007). Plants growing under detrimental environmental conditions, such as those occurring in arid and semiarid soils, undergo water limitation and nutrient deficiencies but have several mechanisms to cope with these adverse factors. Rhizosphere microorganisms are adapted to adverse conditions and may compensate for such detrimental conditions (Ruíz-Lozano and others 1996; Marulanda and others 2008).

Ecosystem functioning is largely governed by soil microbial activity (Kennedy and Smith 1995) because biochemical cycles of major plant nutrients are carried out by microorganisms (Barea and others 2002). Sustainable systems require the understanding of interactions between plants and microorganisms, especially those having a direct influence on plant growth and stress tolerance (Gryndler and others 2000). Both AM fungi and soil bacteria can adapt to specific environmental conditions and develop tolerance to stressful environments (Ruíz-Lozano and

Azcón 2000; Vivas and others 2003b). Abiotic stress tolerance in soil microorganisms has been studied to provide a biological understanding of the adaptation and survival of living microorganisms in extreme environments. Among these microbial groups bacteria and arbuscular mycorrhizal (AM) fungi are ubiquitous in the soil, and there is abundant literature to support the idea that these rhizosphere microbes interact in rather specific ways to influence their relationship with and their effect on plant growth (Galleguillos and others 2000; Marulanda and others 2006). Plant growth-promoting rhizobacteria (PGPR) play an important role as modifiers of soil fertility and as facilitators of plant establishment and development (Barea and others 2002; Caravaca and others 2002). The cooperation of bacteria and AM fungi in nutrient uptake by plants may be due to specific attributes of microorganisms and there is a growing interest in improving our understanding of their involvement not only in nutrient cycling but also in their effect on non-nutritional physiologic values that make the plant more tolerant to environmental stresses, particularly drought stress (Vessey 2003; Barea and others 2005).

Beneficial soil microorganisms such as bacteria and/or AM fungi can adapt to specific environmental conditions and develop tolerance to stressful conditions. The role of these microorganisms in plant stress tolerance to heavy metals, salt, and drought is known and has been studied in the context of providing a biological understanding of the adaptation of living organisms to extreme environments.

The growth effect of PGPR may be mediated by IAA, salicylic acid, and gibberellin signalling pathways (Bent and others 2001; James and others 2002). It is also known that an increase in plant growth caused by AM fungi is mediated in part by an alteration in plant hormone levels. Their effectiveness in drought alleviation has been also reported (Ruíz-Lozano and others 2001; Marulanda and others 2006, 2007). The manipulation of these microorganisms is important with regard to sustainability issues (Bowen and Rovira 1999; Medina and others 2003; Vivas and others 2003a). However, positive results depend on a proper understanding of the system applied and the suitable selection of microbes (Galleguillos and others 2000; Valdenegro and others 2001; Marulanda and others 2003; Vivas and others 2006).

Investigating stress markers such as proline and/or phytohormone synthesis at increasing stress (PEG or salt) levels in the axenic medium of bacterial cultures may aid in selecting the more stress-adapted tolerant strains. Proline may act as an osmolyte, stabilizing proteins, scavenging hydroxyl radicals, and regulating the NAD/NADH ratio. The amount of IAA is also a suitable marker for bacterial effectiveness, particularly under osmotic stress (Boiero and others 2007).

Marulanda and others (2003) analyzed the contribution of six AM fungi to water uptake by plants under drought stress. Recently, Marulanda and others (2006) reported the effect of drought-tolerant AM fungus and *Bacillus* sp. in improving plant tolerance to drought stress and water transport in *Retama sphaerocarpa.* Possible mechanisms involved in the effectiveness of microbial inoculation were analyzed because both AM fungi and soil bacteria can be adapted to specific environmental conditions (Glick and others 1999), and a higher tolerance to stress of indigenous microorganisms in comparison to those nonautochthonous AM fungi from nonstressed sites has been described previously (Vivas and others 2003d; Marulanda and others 2006). Important aspects such as microbial tolerance to stress and the effect of such microorganisms on plant growth must be considered and evaluated.

Only long-term experiments in stressed soil give us the opportunity to study the real detrimental effects of water deficiency and the beneficial effect of inoculated microorganisms for any length of time.

Indigenous bacterial populations may have adapted to stress conditions and developed the capacity to survive in stressed soils. Therefore, three indigenous bacterial strains used here were isolated from a Mediterranean arid soil. We studied the ability of such bacterial strains, isolated from an arid soil, to produce proline or IAA under increasing osmotic stress (by PEG or NaCl supply) in axenic culture. The drought tolerance and effectiveness of these bacteria were determined alone or in association with four autochthonous AM fungi during successive harvests under water limitation in the medium.

Materials and Methods

To test the ability of autochthonous drought-adapted microorganisms (AM fungi and PGPR bacteria) to increase plant growth and to survive and function under osmotic-stress conditions, various independent experiments were carried out in this study.

1. First, noninoculated controls and three autochthonous bacterial strains were assayed in a microcosm experiment under drought conditions. These treatments were replicated five times with a total of 20 pots placed in a random complete block design.
2. In a second bioassay, these three bacterial strains were assayed in interaction with each of the four autochthonous AM fungi isolated from the same test Mediterranean arid soil. Individual mycorrhizal inoculated plants were also assayed. These treatments were replicated five times with a total of 80 pots placed in a random complete block design.
3. Autochthonous bacterial isolates and one reference strain were grown at increasing NaCl concentrations

(0, 150, 300, and 600 mM) to test NaCl tolerance as cfu ml^{-1}. These four treatments were replicated three times for a total of 48 tubes.

4. Bacterial strains were also assayed in axenic medium to test indol acetic acid (IAA) production under increasing levels of polyethylene glycol (PEG) (0, 20, 40, and 60%) or proline production under increasing levels of NaCl (0, 1.5, and 3.0 M) in the culture medium. All these treatments were replicated three times with a total of 63 tubes.
5. Bacterial growth in the rhizosphere and inside root tissues was determined after 15 days of inoculation in maize plants grown in axenic medium (test tubes) giving a total of 15 tubes.

Soil Microorganisms

The soil samples for microbial inocula production were taken from the Mediterranean arid soil used as test soil for these experiments. From this soil, containing the native adapted AM fungi and bacterial populations, these microorganisms were isolated and cultivated for inocula production. The bacterial strains selected were the three most abundant cultivable types. In the same way, mycorrhizal inocula isolated from this soil (the four most abundant species) were morphologically identified as *Glomus* sp. (*G. constrictum*, *G. coronatum*, *G. claroideum*, and *G. mosseae*).

The bacterial isolation was carried out using the conventional procedure: 1 g of homogenized rhizosphere soil was suspended in 100 ml of sterile water (dilution 10^{-2}) and 1 ml of this suspension was serially diluted to reach dilutions of 10^{-4}–10^{-7}. These dilutions were plated in agar nutrient broth Difco medium (8 g L^{-1}) that contained meat extract (3 ml^{-1}) and peptone gelatine (5 g L^{-1}) and cultivated for 48 h at 28°C. Once selected, the most abundant bacterial strains were grown in 250-ml flasks containing 50 ml of nutrient broth medium in a shake culture for 48 h at 28°C. In the appropriate treatments, plants were inoculated with 0.5 ml of the bacterial culture (10^8 cfu ml^{-1}). In control treatments, 0.5 ml of sterilized bacterial culture was added.

Mycorrhizal inoculum from each endophyte was multiplied in an open pot culture of maize and *Trifolium* and consisted of soil, spore, hyphae, and mycorrhizal root fragments. The AM fungal species were *G. constrictum*, *G. coronatum*, *G. claroideum*, and *G. mosseae* according to morphologic identification. Five grams of each AM inoculum, having similar richness (an overage of 30 spores g^{-1} and root fragments with 75% of colonized roots length) was applied below the seed of the *Trifolium* plants. A suspension (1 ml/seed) of the diazotrophic bacterium *Rhizobium leguminosarum* bv *trifolii* (10^8 cfu ml^{-1}) was sprinkled over seeds of all treatments at sowing time.

Test Soil and Growth Conditions

The experimental soil was collected from a Mediterranean arid region of eastern Spain. The calcareous loamy soil was sieved (2 mm), diluted with quartz-sand (<1 mm) (1:1 soil:sand v/v), and sterilized by steaming (100°C for 1 h over 3 days). The soil pH was 7.2 (water) and contained 1.6% organic matter. Nutrient concentrations were 2.1 mg kg^{-1} N; 1.7 mg kg^{-1} P ($NaHCO_3$-extractable P); 0.8 mg kg^{-1} K. The soil texture was made up of 57.8% sand, 19% clay, and 23.2% silt.

Trifolium repens plants were grown in 500-ml pots in a greenhouse under controlled climatic conditions (18-24°C, with a 18/6 h light/dark period and 50% relative humidity). A photoperiod of 16 h at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} as measured with a light meter (model LI–188B; Licor Inc., Lincoln, NE, USA) was maintained during the experiment by supplementary light to compensate natural illumination.

Water was supplied daily to maintain constant soil water close to field capacity (17% volumetric soil moisture) during the 2 weeks after sowing. After this time, and during a period of 15 weeks, these plants were allowed to dry until the soil water content was 75% of field capacity (13% volumetric soil moisture). Pots were subjected to drought conditions (0.17 MPa) after 2 weeks of sowing. The two microcosm experiments were subsequently carried out using the same soil, plant, and pot and under the same environmental conditions.

Molecular Identification of the Bacterial Strains

Bacterial identification was carried out by 16S rDNA cloning and sequencing as previously described (Vivas and others 2003d). Database searches for 16S rDNA sequence similarity unambiguously identified the bacteria as *Pseudomonas putida*, *Bacillus megaterium*, and *Pseudomonas* sp. (*Pseudomonas plecoglossicida* being its closest relative with 98% of sequence identity).

Production of Indole-3 Acetic Acid by Bacterial Strains Under Increasing Polyethylene Glycol (PEG) Concentrations

The bacterial isolates were cultivated at 28°C in medium supplemented with 0, 20, 40, or 60% of PEG to induce drought stress. The production of indole-3 acetic acid (IAA) by these bacteria under increasing PEG concentrations was measured. For gas chromatography-mass

spectrometry (GC-MS) analyses bacterial strains were grown at 28°C in liquid minimal medium MMAB (Vanstockem and others 1987) containing 0.5% malate as a carbon source and supplemented with 0.1 μM tryptophan (Trp) as a precursor for IAA biosynthesis and 10 mM arabinose as inducer for the plant-inducible promoter. Samples of the culture (5 ml) were taken and made cell-free by centrifugation. Samples were stored at −20°C until analysis. IAA was purified from 5 ml of cell-free supernatant by solid-phase extraction using [phenyl-$^{13}C_6$]-IAA as internal tracer and analyzed by GC-MS (Prinsen and others 2000) as pentafluorobenzyl ester (Epstein and Cohen 1981). All data are averages of three replicates.

Proline Production by Bacterial Strains Under Increasing NaCl Concentrations

Proline accumulation by these bacteria under increasing osmotic stress produced by NaCl application was determined by a colorimetry method (Bates and others 1973).

Bacterial Growth Under NaCl Levels in the Medium

The growth of autochthonous bacteria under increasing NaCl levels was tested in comparison to *Bacillus thuringiensis* from an arid zone (previously proved as PGPR) (Marulanda and others 2006). This bacterium was used as a control for comparison with the strains assayed in this study. Bacterial strains were cultivated at 28°C in nutrient broth (8 g L^{-1}) supplemented or not (control) with increasing NaCl levels (150, 300, or 600 mM NaCl). The number of viable cells was estimated at 12-h intervals from 0 to 24 h following the conventional procedure: 1 ml of suspension was plated in agar nutrient broth medium.

Rhizosphere and Endorhizosphere Bacterial Colonization After Inoculation on Maize Plants

The autochthonous bacteria were also assayed in test tubes under axenic conditions to study the endophytic colonizing abilities of these rhizosphere microorganisms. For that, maize seeds under sterile conditions were suspended or not (control) for 1 h in each one of the bacterial suspensions (10^8 cfu ml^{-1}). They were placed in sterilized test tubes (40 ml) containing vermiculite and 25 ml of Murashige and Skoog (1962) medium lacking phytogel and glucose. Maize plants were grown for 15 days under growth chamber conditions (16/8 h light/dark and 24°C, minimum 200 μmol s^{-1} m^{-2}). Sterile water was supplied daily to maintain constant water content.

After 15 days, bacterial growth in the rhizosphere and inside the root tissue of the maize plants was determined (five repetitions by each bacterial strain). The bacterial growth in the rhizosphere was carried out following the conventional procedure: 1 g of homogenized rhizosphere was suspended in 100 ml of sterile water (dilution 10^{-2}) and was serially diluted to reach 10^{-7}. The dilution was plated (0.2 ml) in agar nutrient broth medium (8 g L^{-1}) and cultivated at 28°C. The bacterial growth inside the roots (endorhizosphere) was determined by selecting 1 g of fresh root, which was cleaned and surface disinfected (shaking 20 min in 30% [v:v] H_2O_2 and washed in five changes of distilled water). After grinding, 100-μl aliquots were suspended in 10 ml of sterile water (dilutions 10^{-2}) and 1 ml of this suspension was serially diluted to each dilution of 10^{-4}–10^{-7}. Dilutions were plated in agar nutrient broth medium (8 g L^{-1}) and cultivated for 48 h at 28°C.

Results

These autochthonous bacteria were identified as *Pseudomonas* sp., *Pseudomonas putida*, and *Bacillus megaterium*. Inoculation of each of these autochthonous bacterial strains increased shoot and root biomass compared to the control plants under water stress conditions (Figure 1). The bacterial effect on the water content (WC) of plants was also evident. A similar positive effect on WC was observed in the inoculated plants irrespective of the bacteria involved (Figure 1).

The dual inoculation of any of these bacterial strains with each of the AM fungi assayed proved the effectiveness of each AM fungus in increasing shoot biomass (Figure 2). Nevertheless, as the results of the first harvest shown, an effect of the bacterial inoculation was evidenced only in mycorrhizal plants by *G. coronatum* and *G. claroideum* fungi.

The dual inoculation of these autochthonous microorganisms was critical for plants to reach optimum growth. These results also indicate that the most efficient fungus in enhancing biomass development (*G. constrictum*) obtained the poorest response to bacterial inoculation. *G. constrictum*-colonized plants increased plant growth by 48% when co-inoculated with *B. megaterium*. However, the effectiveness of *B. megaterium* in increasing the biomass of AM-colonized plants was in terms of percentage: 95% (*G. coronatum*), 75% (*G. claroideum*), and 121% (*G. mosseae*) (Figure 2).

The bacterial inoculation also increased root weight compared to the control plants, but the bacterial effect was more evident in shoot improvement (Figure 1). The bacterial inocula increased plant water content; this bacterial effect is relevant for plants grown in soils with water limitation.

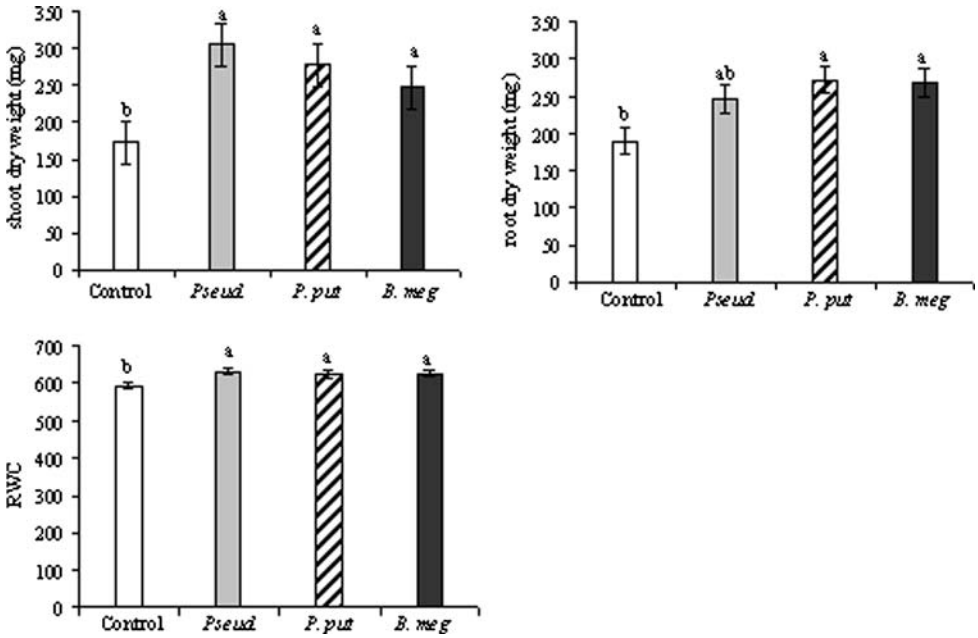

Fig. 1 Shoot and root dry weight (mg) and relative water content (RWC) of *Trifolium* plants not inoculated or inoculated with each of the autochthonous drought-adapted bacterial strains [*Pseudomonas* sp. (*Pseud.*), *P. putida* (*P. put*), and *B. megaterium* (*B. meg*)]. Within each value, bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 3$)

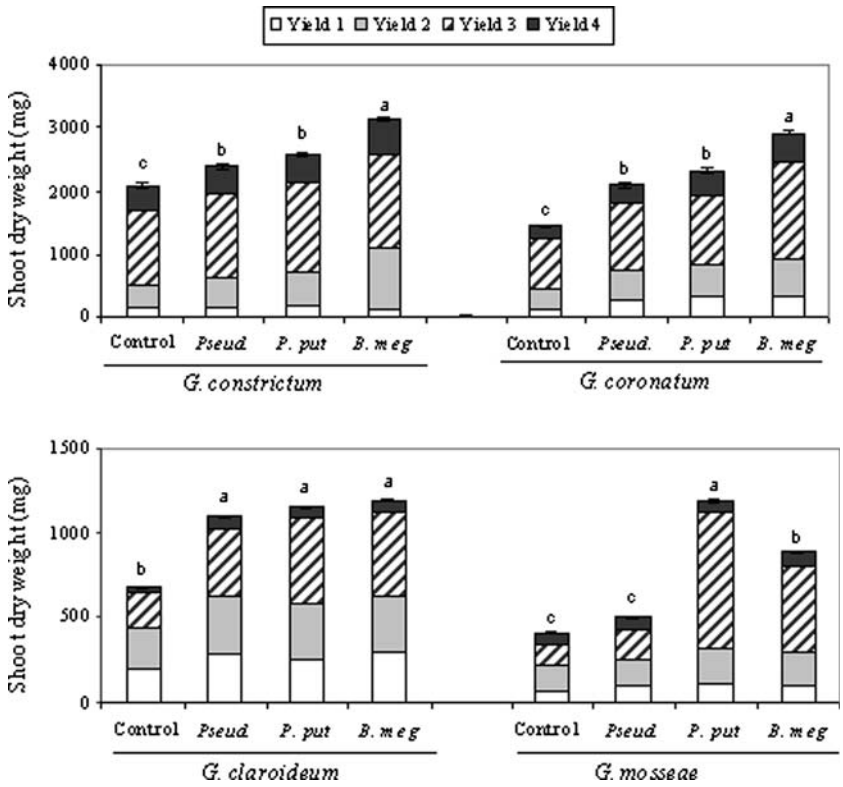

Fig. 2 Shoot dry weight (mg) of *Trifolium* plants inoculated with AM only (control) or inoculated with each of the autochthonous drought-adapted bacteria strains [*Pseudomonas* sp. (*Pseud.*), *P. putida* (*P. put*), and *B. megaterium* (*B. meg*)] and AM fungus after four harvests. Within each value, bars having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 3$)

In axenic culture, *B. megaterium* exhibited the highest tolerance to the osmotic stress caused by NaCl. The reference strain of *B. thuringiensis* as *Pseudomonas* sp. was sensitive to 600 mM NaCl, reducing widely the bacterial growth (Figure 3). Indol-3 acetic acid (IAA) production by *B. megaterium* was higher than that by *Pseudomonas* strains at whatever PEG concentration in the culture medium (Table 1). As a consequence of salinity, proline concentration by *P. putida* and *B. megaterium* increased (Table 2), reaching optimum production at 1.5 mM NaCl. At this NaCl level, proline production ranged from 0.01 (*Pseudomonas* sp.) to 0.03 μg ml^{-1} (*B. megaterium*).

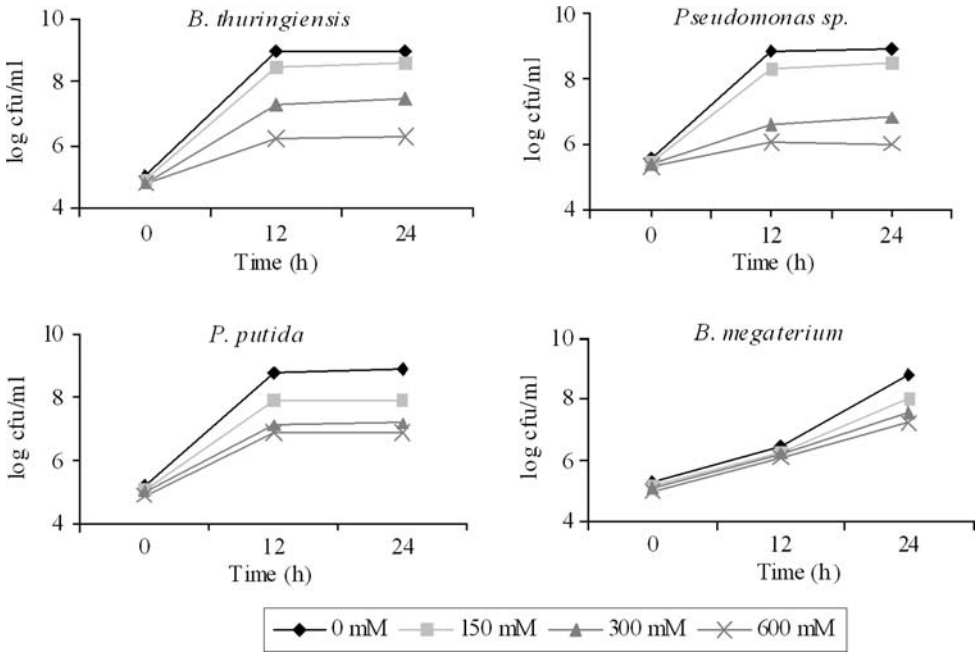

Fig. 3 Number of viable cells (log cfu ml^{-1}) at different time intervals (h) of *B. thuringiensis* (reference strain), *Pseudomonas* sp., *P. putida*, or *B. megaterium* grown in nutrient broth supplemented with 0, 150, 300, or 600 mM NaCl. Data correspond to the average value ($n = 3$)

Table 1 Indole acetic acid (IAA) production (μg ml^{-1}) by autochthonous drought-adapted bacterial strains growing under increasing polyethylene glycol (PEG) concentrations in the culture medium

% PEG	μg IAA ml^{-1}		
	Pseudomonas sp.	*P. putida*	*B. megaterium*
0	1.0d	0.8d	2.5b
20	0.9d	0.9d	2.3bc
40	0.8d	1.2c	2.5b
60	0.3e	2.6b	3.0a

Values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 3$)

Table 2 Proline accumulation (μg ml^{-1}) by autochthonous drought-adapted bacterial strains under increasing levels of NaCl in the culture medium

NaCl (mM)	*Pseudomonas* sp.	*P. putida*	*B. megaterium*
0	0.009c	0.006d	0.010c
1.5	0.010c	0.014b	0.030a
3	0.005d	0.011c	0.016b

Values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 3$)

The colonizing niche and abilities of these bacteria are given in Table 3. *B. megaterium* was the only bacterium colonizing the root tissue. Thus, it is characterized as endophytic bacterium. Its growth inside the root was 6×10^6 cfu g^{-1} fresh root. The presence of both *Pseudomonas* strains was detected only in rhizosphere medium (8.8×10^6 cfu g^{-1} fresh soil) (Table 3).

Table 3 Autochthonous drought-adapted bacterial strains (cfu 10^6 g^{-1} root or soil) after 15 days of inoculation in maize plants

	Pseudomonas sp.	*P. putida*	*B. megaterium*
Soil	8.6a	8.6a	8.0a
Root	0.0b	0.0b	6.0a

Values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 3$)

Discussion

The underlying mechanism of enhancing plant tolerance to drought (in terms of growth) by bacterial inoculation could be the ability of these plants to increase their water content, because plants with a well-developed root system have the greatest ability to take up water. The enhancement of root growth by bacterial inoculation could be due to IAA produced by bacteria.

Some autochthonous bacterial strains such as *P. putida* and *B. megaterium* isolated from dry soils had a higher tolerance to NaCl than *B. thuringiensis* (reference strain) or *Pseudomonas* sp. because these latter two strains did not grow under certain NaCl concentrations and appeared to be sensitive to the highest NaCl concentration used. Whereas the most sensitive strains (*Pseudomonas* sp. and *B. thuringiensis*) greatly reduced their growth in media supplemented with 600 mM NaCl, the most tolerant strains were less affected by such NaCl concentration. These results agree with the highest proline accumulation of these two strains under moderate or strong osmotic stress caused by NaCl in the medium. Moreover, the ability of these strains to increase the production of IAA as much as the

increased osmotic stress (PEG) in the growing medium would account for their osmotic tolerance. On the other hand, *Pseudomonas* sp. produced lower amounts of IAA, particularly under the highest levels of osmotic stress (40 and 60% PEG).

The positive effect on plant growth after the inoculation of these bacterial strains was evident not only in single inoculations but also in dual inoculations with whatever autochthonous AM fungi here assayed. These bacterial strains were compatible and effective in increasing the benefits of AM symbiosis on plant growth with each fungal isolate under the drought conditions assayed here.

Both AM fungi and the inoculated bacterial strains came from Mediterranean arid soil and were assayed (as inocula) under natural soil conditions in microcosms. This is important from a practical point of view because these microorganisms were able to survive and multiply to reach a sufficient population to express their activities in the natural soil through time (four harvests at least). Inconsistent plant responses by inoculation of selected microorganisms under axenic conditions have been reported (Bowen and Rovira 1999). In this study we investigated some bacterial biochemical traits that could explain the positive results observed in plant growth after being inoculated under water-limiting conditions. We confirmed in axenic culture that *B. megaterium* exhibited the highest tolerance to water deficit caused by osmotic stresses (PEG or NaCl), increasing proline and IAA production as much as the increase in the osmotic stress in the growing medium.

The increasing proline accumulation in axenic culture of the native bacteria *B. megaterium* under increasing stress conditions could induce the adjustment of cell osmotic potential, indicative of osmotic cellular adaptation. This is a mechanism by which microbial cells can cope with drought stress (Paleg and others 1984). According to our results, inoculation with *B. megaterium* increased shoot and root biomass and water content in plants. The highest IAA production by this bacteria under stressful conditions may explain its effectiveness in promoting shoot and root growth by 86% and water content respectively. This PGPR capacity and the proline accumulation may enhance its competitive advantage under dry environments. This bacterium also was the most effective in interactions with three of the four AM fungi assayed, resulting in particular effects on plant performance depending on the AM fungus involved.

This is the first time that some physiologic bacterial mechanisms related to drought tolerance and effectiveness when inoculated in dry environments have been tested. We observed that the highest IAA and proline production by *B. megaterium* in axenic culture are directly correlated with the greatest root growth. As well, co-inoculation with most of the native AM fungi resulted in the most effective interaction. Therefore, both compounds (proline and IAA) were useful markers of bacterial performance in plants growing in water-stressed soils.

Marulanda and others (2003) reported that a rhizosphere bacterium isolated from an arid soil and identified as *Bacillus* sp. positively influenced the development and activity of two *Glomus* sp., stimulating not only the activity of intraradical mycelium from *Glomus* sp. but also the development of extraradical mycelium from *G. intraradices*. In a recent (unpublished) study, we found that *B. megaterium* increased the amount of photosynthetic pigments in *G. intraradices*-colonized plants in which an increase of both total chlorophyll and catorenoids was observed. The enhancement of the photosynthetic rate in dually inoculated plants affects the translocation of soluble sugars to host roots, thus increasing fungal growth and activity in the roots. Nevertheless, co-inoculation of this bacterium with *G. mosseae* did not increase plant gas-exhange values. The effects of specific interactions between the bacterium and each *Glomus* sp. on plant physiology and metabolism were independent of drought stress. Specific microbe-microbe interactions that modulate the effect of microbial associations on plant physiology can occur (Marulanda and others 2008).

The advantages of a well-developed and persistent bacterial community include better survival and effectiveness in plant development in osmotically stressed environments because the activity of such microbial communities may be essential in the establishment and nutrition of plants in such environments. Whereas plant root and shoot biomass were limited by osmotic stress, the microbial inoculation attenuated the negative effect of such detrimental factors.

The PGPR are associated with plant roots (inside and/or outside) and either directly or indirectly stimulate plant growth, but there is a gradient of root proximity and intimacy depending on the niche. There are bacteria living in the soil near the roots, bacteria colonizing the rhizoplane (root surface), and bacteria residing in root tissue (inside cortical cells) (Gray and Smith 2005). These aspects are important for intimacy with the associated plant, from almost casual to extremely regulated and housed in specialized structures.

In general, for an effective growth stimulation a close interaction between microorganisms and host plants is a prerequisite for utilization of plant assimilates and microbial metabolites, respectively, by the partners (Grayston and others 1996). Particular and specific interactions between plants and microbial groups need to be compatible at a physiologic level (Marulanda and others 2006). Combined inoculation of bacterial strains with AM fungi produced growth-stimulating effects that surpassed those of individual inoculations (Galleguillos and others 2000).

The fungal isolates used were indigenous drought-tolerant isolates but the effectiveness of each AM fungus in increasing plant growth was different and depended on the AM endophyte involved. One study reported that different AM fungi are highly variable in their tolerance to drought stress (Marulanda and others 2003).

Recently, Marulanda and others (2007) reported that the selection of effective AM fungal strains for inoculation could lead to the enhancement of plant growth in drought soils. The most efficient AM fungus exhibited greater extraradical mycelium (glomalin) and plant water uptake probably as a result of an improvement in root conductivity to water flow and/or via a mycelium able to transport water to the AM-colonized root system (Ruíz-Lozano and Azcón 1995; Bryla and Duniway 1997). As a result, the increased root growth due to effective AM colonization increased plant water content and concomitantly decreased antioxidant compounds such as glutathione, ascorbate, and H_2O_2 which have an important role in plant protection and metabolic functions under water-deficit conditions (Marulanda and others 2007).

In a recent study, Marulanda and others (2008) determined that the *B. megaterium* strain studied was effective in co-inoculation with *G. coronatum*, the same autochthonous strain used here, but it did not improve plant growth when it was associated with *G. coronatum*. Physiologic and biochemical traits of these AM fungi-bacteria associations were discussed. We cannot rule out the changes in IAA content as affected by each of the bacterial inoculants under stress conditions; however, this may be relevant in the growth effect observed.

In this study, the proline accumulation under NaCl-induced stress is an indication of the adjustment of bacterial cells to the osmotic potential required for enhanced intracellular osmotic balance. Stressed cells increased osmotic resistance by maintaining high levels of proline. This and other processes are mechanisms by which cells can cope with osmotic stress (Paleg and others 1984). According to the results of the present research, each of the selected bacteria had different abilities to accumulate proline under osmotic stress conditions. Without salt slight differences in proline were found between bacterial strains; however, under high (3 mM NaCl) and moderate (1.5 mM NaCl) salinity only *P. putida* and *B. megaterium* increased proline production. The highest values were observed under moderate salinity (1.5 mM NaCl) and these results correlated with the highest growth presented by such strains in medium with high NaCl concentrations. In fact, proline accumulation could be used here as an osmotic tolerance index on these autochthonous bacteria and on the effect on plant growth under drought conditions.

Despite the fact that two of the bacterial isolates belong to the same genus (*Pseudomonas*), high variability in IAA production was observed, particularly at the highest PEG level (Table 1). We found IAA amounts ranging from 0.3 (*Pseudomonas* sp.) to 3.0 (*P. putida*) μg ml^{-1} under 60% PEG. Without PEG in the culture medium, such differences were lower. These results indicate that the autochthonous bacteria assayed were able to increase plant growth in non-mycorrhizal and mycorrhizal plants. However, the best compatibilities between AM fungi and these bacteria were evidenced using *B. megaterium*, except when associated with *G. mosseae*. The fact that these bacteria showed differences only in plant growth when associated with AM fungi suggests that they produced some substances responsible for AM functioning (Souchie and others 2007). Bacteria called "mycorrhiza helper bacteria" stimulate AM root colonization (Vivas and others 2006) and mycelial growth from *G. mosseae* spores in vitro culture (Azcón 1987; Vivas and others 2006), but here these values were not determined.

Previously, we reported (Marulanda and others 2006) that co-inoculation of microorganisms such as *Bacillus thuringiensis* (the bacterial strain used in this study as a reference to test proline accumulation) and *G. intraradices* (a drought-tolerant fungal specie) reduced by 42% the water required to produce 1 mg of shoot biomass compared with an uninoculated control. These results were the first evidence of the ability of a rhizosphere bacterium to increase plant water-use efficiency.

B. megaterium acted as an endophyte bacteria. The water content of plants was enhanced by bacterial inoculation which represents a positive bacterial effect on plant development under drought conditions. This bacterial activity is very important for preventing damage and enhancing plant survival in semiarid and arid areas. Knowledge about the characteristics of indigenous bacteria involved in stress adaptation requires further study.

The effect of bacterial strains on root development is fundamental for root function (Barea and others 1996). In fact, the PGPR ability of these bacterial strains can be envisaged as an addition to the mycorrhizal helping activity involved in mycorrhizal plant responses (Vivas and others 2003c; Germida and Walley 1996). Aspects of rhizosphere biology emphasize the ecologic and practical importance of soil microorganisms for plant development under stressed environmental conditions and the positive interactions developed with members of soil microbiota such as AM fungi and PGPR bacteria.

References

Azcón R (1987) Germination and hyphal growth of *Glomus mosseae* in vitro. Effect of rhizosphere bacteria and cell-free culture media. Soil Biol Biochem 19:417–419

Barea JM, Tobar RM, Azcón-Aguilar C (1996) Effect of a genetically modified *Rhizobium meliloti* inoculant on the development of

arbuscular mycorrhizas, root morphology, nutrient uptake and biomass accumulation in *Medicago sativa*. New Phytol 134: 361–369

Barea JM, Azcón R, Azcón-Aguilar C (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie Van Leeuwenhoek 81:343–351

Barea JM, Pozo MJ, Azcón R, Azcón-Aguilar C (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56:1761–1778

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Bent E, Tuzun S, Chanway CP, Enebak SA (2001) Alterations in plant growth and in root hormone levels of lodgepole pines inoculated with rhizobacteria. Can J Microbiol 47:793–800

Boiero L, Perrig D, Masciarelli O, Pena C, Cassán F, Luna V (2007) Phytohormone production by strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl Microbiol Biotechnol 74:874–880

Bowen GD, Rovira AD (1999) The rhizosphere and its management to improve plant growth. Adv Agron 66:1–102

Bryla DR, Duniway JM (1997) Water uptake by safflower and wheat roots infected with arbuscular mycorrhizal fungi. New Phytol 136:591–601

Caravaca F, Barea JM, Palenzuela J, Figueroa D, Alguacil MM, Roldán A (2002) Establishment of shrubs species in a degraded semiarid site after inoculation with native or allochthonous arbuscular mycorrhizal fungi. Appl Soil Ecol 22:103–111

Epstein E, Cohen JD (1981) Microscale preparation of pentafluorobenzyl esters: electron-capture gas chromatographic detection of indole-3-acetic acid from plants. J Chromatogr 209:413–420

Galleguillos C, Aguirre C, Barea JM, Azcón R (2000) Growth-promoting effect of two *Sinorhizobium meliloti* strains (a wild type and its genetically modified derivative) on a non-legume plant species in specific interaction with two arbuscular mycorrhizal fungi. Plant Sci 159:57–63

Germida JJ, Walley FL (1996) Plant growth-promoting rhizobacteria alter rooting patterns and arbuscular mycorrhizal fungi colonization of field-grown spring wheat. Biol Fertil Soils 23:113–120

Glick B, Patten C, Holguin G, Penrose D (1999) Biochemical and genetic mechanisms used by Growth Promoting Rhizobacteria. Imperial College Press, London

Gray EJ, Smith DL (2005) Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. Soil Biol Biochem 37:395–412

Grayston SJ, Vaughan D, Jones D (1996) Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. Appl Soil Ecol 5:29–56

Gryndler M, Hrselová H, Stríteská D (2000) Effect of soil bacteria on hyphal growth of the arbuscular mycorrhizal fungus *Glomus claroideum*. Folia Microbiol 45:545–551

James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PPM, Olivares FL, Ladha JK (2002) Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Mol Plant Microbe Interactions 15:894–906

Kennedy AC, Smith KL (1995) Soil microbial diversity and the sustainability of agricultural soils. Plant Soil 170:75–86

Marulanda A, Azcón R, Ruíz-Lozano JM (2003) Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress. Physiol Plant 119: 526–533

Marulanda A, Barea JM, Azcón R (2006) An indigenous drought-tolerant strain of *Glomus intraradices* associated with a native bacterium improves water transport and root development in *Retama sphaerocarpa*. Microb Ecol 52:670–678

Marulanda A, Porcel R, Barea JM, Azcón R (2007) Drought tolerance and antioxidant activities in lavender plants colonized by native drought-tolerant or drought-sensitive *Glomus* species. Microb Ecol 54:543–552

Marulanda A, Azcón R, Ruíz-Lozano JM, Aroca R (2008) 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. J Plant Growth Regul 27:10–18

Medina A, Probanza A, Manero FJG, Azcón R (2003) Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). Appl Soil Ecol 22:15–28

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Paleg LG, Stewart GR, Bradbeer JW (1984) Proline and glycine betaine influence protein solvation. Plant Physiol 75:974–978

Prinsen E, Van Laer S, Öden S, Van Onckelen H (2000) Auxin analysis. In: Tucker GA, Roberts JA (eds) Methods in molecular biology, vol 141. Plant Hormone Protocols. Humana Press, Totowa, NJ, pp 49–65

Ruíz-Lozano JM, Azcón R (1995) Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. Physiol Plant 95:472–478

Ruíz-Lozano JM, Azcón R (2000) Symbiotic efficiency and infectivity of an autochthonous arbuscular mycorrhizal *Glomus* sp. from saline soils and *Glomus deserticola* under salinity. Mycorrhiza 10:137–143

Ruíz-Lozano JM, Azcón R, Palma JM (1996) Superoxide dismutase activity in arbuscular mycorrhizal *Lactuca sativa* plants subjected to drought stress. New Phytol 134:327–333

Ruíz-Lozano JM, Collados C, Barea JM, Azcón R (2001) Cloning of cDNAs encoding SODs from lettuce plants which show differential regulation by arbuscular mycorrhizal symbiosis and by drought stress. J Exp Bot 52:2241–2242

Souchie EL, Azcón R, Barea JM, Saggin-Júnior OJ, Ribeiro da Silva EM (2007) Indolacetic acid production by P-solubilizing microorganisms and interaction with arbuscular mycorrhizal fungi. Acta Sci Biol Sci 29:315–320

Valdenegro M, Barea JM, Azcón R (2001) Influence of arbuscular-mycorrhizal fungi, *Rhizobium meliloti* strains and PGPR inoculation on the growth of *Medicago arborea* used as model legume for re-vegetation and biological reactivation in a semiarid Mediterranean area. Plant Growth Regul 34:233–240

Vanstockem M, Michiels K, Vanderleyden J, Van Gool AP (1987) Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: physical analysis of Tn*5* and Tn*5*-mob insertion mutants. Appl Environ Microbiol 53:410–415

Vessey KV (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586

Vivas A, Azcón R, Biró B, Barea JM, Ruíz-Lozano JM (2003a) Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity. Can J Microbiol 49:577–588

Vivas A, Biró B, Campos E, Barea JM, Azcón R (2003b) Symbiotic efficiency of autochthonous arbuscular mycorrhizal fungus (*G. mosseae*) and *Brevibacillus* sp. isolated from cadmium polluted soil under increasing cadmium levels. Environ Pollut 126:179–189

Vivas A, Marulanda A, Ruíz-Lozano JM, Barea JM, Azcón R (2003c) Influence of a *Bacillus* sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress. Mycorrhiza 13:249–256

Vivas A, Vörös A, Biró B, Barea JM, Ruíz-Lozano JM, Azcón R (2003d) Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mosseae* associated with a Cd-adapted strain of *Brevibacillus* sp. in improving plant tolerance to Cd contamination. Appl Soil Ecol 24:177–186

Vivas A, Biró B, Ruíz-Lozano JM, Barea JM, Azcón R (2006) Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn-toxicity. Chemosphere 62:1523–1533