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Combined use of beneficial soil microorganism and agrowaste residue to cope with plant water limitation under semiarid conditions

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ABSTRACT

This study investigated the effectiveness of several microorganisms, such as a *Bacillus megaterium* strain and/or an autochthonous consortium of arbuscular mycorrhizal fungi (AMF) on plant growth and drought tolerance in a natural semiarid soil. The effect of treated *Aspergillus niger* residue from sugar beet was also evaluated in non-inoculated and inoculated plants. Results from three successive harvests allowed us to determine the persistence along the time of beneficial effects of these treatments under natural drought conditions. Biomass production and nutrition were more increased by the transformed residue than concomitantly decreased antioxidant enzymatic activities under drought. The microbial inoculants assayed contributed to plant drought tolerance through strategies such as increased nutrition (particularly K^+), hydric content and by decreasing stomatal conductance and antioxidant enzymatic activities. Similar microbial-mediated effects were confirmed at each harvest. The effectiveness of bacterial inoculation under drought conditions in natural soil has been almost unexplored. Here, the interactive effect of these bacteria with an AMF consortium maximized plant growth, water content and C, K, Ca and Mg content. A relevant result is the greater effectiveness of the bacteria when inoculated in residue amended soil that promoted plant growth and hydric content and decreased most antioxidant activities to a greater extent than AMF inoculation. *B. megaterium* (without compost) also affected root growth, physiological and biochemical plant values involved in the adaptative plant drought response. The ability of *B. megaterium* in axenic medium to maintain indole acetic acid (IAA) like molecules and to increase proline production under osmotic stress conditions indicated the drought tolerance of this strain. In this study the management of natural resources, such as selected and drought adapted soil microorganisms and *A. niger* treated agrowaste resulted determinant for enhancing plant performance in an arid degraded soil.

1. Introduction

Desertification processes disturb and limit the re-establishment of the natural plant cover. Nevertheless, the plant association with beneficial soil microorganisms may be critical for plant growth in stressed degraded ecosystems (Bashan and de-Bashan, 2010b; Jeffries and Barea, 2001). The management of selected and appropriate plant growth promoting microorganisms (PGPM) can help plants to grow and to promote the stabilization of a self-sustaining ecosystem under stress conditions. Arbuscular mycorrhizal (AM) fungi enable plants to cope with drought stress not only by alleviating nutrient deficiencies but also by improving drought tolerance overcoming the detrimental effect of water and nutrient limitations (Augé, 2000, 2004; Bashan et al., 2009; Medina et al., 2003). Plant nutrients and water deficit are common stresses affecting plant survival and development in arid and semiarid areas. Thus, the improvement of nutrients and water uptake is important for plants growing under stressed conditions. Detrimental environmental conditions also negatively affect the survival and activity of rhizosphere microorganisms but those autochthonous adapted to adverse conditions (such as water limitation and nutrient deficiencies) may be the best candidates to be used as inocula to compensate in inoculated plants such stress conditions (Bashan et al., 2009; Marulanda et al., 2007; Marulanda-Aguirre et al., 2008).

In fact, ecosystem functioning is largely governed by microbial activity and both soil bacteria and arbuscular mycorrhizal (AM) fungi, particularly autochthonous strains adapted to specific environmental conditions, may be appropriate inoculants since they are able to develop plant-tolerance to cope with these stressful environments (Azcón et al., 2010; Dimkpa et al., 2009; Kim et al., 2012). The role of these microorganisms in alleviating plant drought stress has been previously studied under sterile soil conditions and provided a biological understanding about the plant adaptation to stressed environments (Galleguillos et al., 2000; Marulanda et al., 2009; Valdenegro et al., 2001). The use of these selected microorganisms may result relevant regarding the sustainability of stressed environments (Bowen and Rovira, 1999).

Different effectiveness was found when the contribution of six AM fungi (all of them *Glomus* sp.) to water uptake by colonized plants

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under drought stress conditions was analyzed (Marulanda et al., 2003). Similarly, the particular drought tolerance and plant responses of three indigenous bacterial strains isolated from drought soil were tested, and also the biochemical mechanisms related to bacterial effectiveness in water limited soil were studied (Marulanda et al., 2009). From this previous study under sterile conditions *Bacillus megaterium* resulted the most efficient bacteria in alleviating plant drought symptoms when applied alone or associated with each one of the three different autochthonous AM fungi (Marulanda et al., 2009). Authors concluded that microbial activities of adapted bacterial and fungal strains may exert a beneficial interactive effect on plant growth under drought conditions as also reported Bashan et al. (2009). In fact autochthonous AM fungi and bacteria populations naturally growing in arid soils have developed the ability to survive in water limited soils and are adapted to drought conditions (Bashan et al., 2012).

Arid soils are generally characterized by poor structure, lack of organic matter and low water-holding capacity. The most important factor making the rhizosphere an attractive habitat for saprophyte microorganisms, like many bacteria, is the organic carbon provided by plant roots. Thus, the limited plant growth and C exudation under arid conditions may cause the poor surviving of saprophyte microbial inoculum and it is necessary to assure their establishment to be effective on plant growth in arid soils. In fact, the deterioration of biological properties of arid soils is in part due to their progressive decrease in organic matter content (Bashan and de-Bashan, 2010b). In this respect the application of organic amendments to desertified soil, prior to the microbial inoculation has been recommended (Medina et al., 2004a,b; Trejo et al., 2012). In previous studies the most important effects of organic amendments included not only the improvement of soil quality (nutrients, humus, water-holding capacity) but also an increase of microbial activities (Caravaca et al., 2005b, 2006; Kloepper et al., 1999; López et al., 2013; Trejo et al., 2012).

Large amounts of agrowastes are produced during the extraction of sugar from the sugar beet, but this product only can be used as organic amendment after biological transformation processes. Sugar beet residue, because of its lignocellulosic composition, may be mineralized by specific lignocellulosic microorganisms such as *Aspergillus niger*, resulting in a product rich in minerals for plant growth and also in sugars that can be used as energy sources for heterotrophic microorganisms such as plant growth promoting bacteria (PGPB) as suggested by Bashan and Holguin (1998). Nevertheless, the fertilizer ability of this agrowaste can be increased when rock-phosphate (RP) is added to the fermentation medium (Medina et al., 2005). The rock-phosphate solubilization was carried out by the citric acid production by *A. niger* growing on the agrowaste residue. The application of this *A. niger* + RP treated product as amendment improved soil fertility and in previous studies this amendment was used in reclamation strategies of degraded systems particularly associated with AM fungi or yeast (Medina and Azcón, 2010). The application of this amendment, in addition to the nutritional abilities for plants and microorganisms, may affect water uptake by plants (Caravaca et al., 2006). This amendment could be considered as an interesting product to be used for revegetation in water-limited environments improving plant/soil quality and PGPB inocula survival.

We hypothesized that plant responses to drought in an arid soil is a consequence of variations in the level of plant tolerance according to the root microorganisms associated and the amendment applied as Bashan et al. (2012) also suggested. Normally, the combined inoculation of bacterial strains with AM fungi produced growth stimulating effect that surpassed those of individual inoculations (Marulanda-Aguirre et al., 2008). In this study as a next step, we investigate under natural soil conditions the importance of these treatments (amendment, AMF and bacteria) on plant growth, nutrition and drought stress tolerance over time (three harvest) to evaluate the benefits and persistence of these treatments.

In previous studies the effectiveness of this *B. megaterium* strain was assayed in a sterilized Mediterranean soil underlying in this bacteria mechanisms of osmotic stress tolerance (Marulanda et al., 2009). But the persistence in the efficiency of this amendment and/or microbial inocula activity over time under arid natural conditions needs to be evaluated. The present study was carried out under natural (non-sterile) conditions and we determined how the single or combined inoculation of drought adapted selected microorganisms (*B. megaterium* and/or autochthonous consortium of mycorrhizal fungi) in soil amended or not with *A. niger* treated sugar beet residue attenuated the negative effect of water limitation over time. For that three successive harvests were done. As drought stress markers, we analyzed physiological and biochemical plant parameters such as the stomatal conductance and shoot enzymatic antioxidant activities [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR)]. All these values are involved in the plant responses for survival in drought stressed soil.

In addition, the production of proline and IAA-like molecules by *B. megaterium* growing in axenic culture under increasing osmotic stress levels [induced by polyethylene glycol (PEG)] was also evaluated over time to determine the ability of this strain to thrive under drought conditions.

2. Materials and methods

Two independent experiments were carried out in this study. Firstly, in a microcosm experiment we determined both the microbial inocula and *A. niger* treated residue abilities to increase plant growth, nutrition, physiological and biochemical values under natural drought conditions.

In a second experiment the bacteria *B. megaterium* was assayed in axenic medium to test indole acetic acid (IAA-like molecules) and proline accumulation over time under increasing levels of polyethylene glycol (PEG) (0%, 5% and 10%) in the culture medium to induce osmotic stress.

2.1. Experiment I

2.1.1. Fermentation process

The strain of *A. niger* NB2 used in this study was maintained on potato–dextrose agar slants at 4 °C. It was shown to produce only citric acid on complex substrates (Vassilev et al., 1986) and to mineralize lignocellulosic materials (Vassilev et al., 1998). For inoculum preparation, *A. niger* was grown on a slant at 30 °C for 7 days and spores were scraped in sterile distilled water.

Sugar beet waste (SBW) was used as substrate in the fermentation trials. Its characteristics were: cellulose 29%, hemicellulose 23%, lignin 5%, total C 55% and total N 1.7%. This solid residue was dried in a 60 °C oven and ground to pass a 2-mm-pore screen. It was mixed at a concentration of 10% in 40 mL and placed in 250-mL Erlenmeyer flasks with Czape-Dox mineral salt solution. Rock phosphate (Morocco fluorapatite, 12.8% soluble P, 1 mm mesh), was added at a rate of 1.5 g L^{-1}. This culture medium were sterilized by autoclaving at 120 °C for 30 min. Spore suspension of *A. niger* (1.2×10^7), 3 mL, was spread carefully over the surface of the medium. The experiment was carried out in 250-mL Erlenmeyer flasks (in triplicate) in conditions of solid-state fermentations, at 30 °C for 20 days. This amendment was added (5%) to the growing substrate (Medina et al., 2006).

2.1.2. Inocula isolation and production

Rhizosphere soil samples for mycorrhizal inoculum isolation were taken from a natural semiarid soil in the east of Spain (Murcia province). This soil containing colonized roots, spores and mycelia belonging to the native adapted AM fungi was cultivated for inoculum production.

Indigenous AM spores were isolated by wet-sieving and decanting as described by Ruíz-Lozano and Azcón (1995). All the spores obtained were morphologically similar to *Septoglomus constrictum* (EEZ 198), *Diversispora aunantia* (EEZ 199), *Archaeospora trappei* (EEZ 200), *Glomus versiforme* (EEZ 201), and *Paraglomus ocultum* (EEZ 202) compared to those from our current EEZ collection.

We used as autochthonous mycorrhizal inoculum a mixture of each one of these fungal species. It was bulked in an open pot culture of *Zea mays* and *Trifolium repens* with sterile soil/sand (1:1 v/v) mixture. After six months of plant growth the shoots were eliminated and the under-grown part (mycorrhizal roots plus soil possessing fungal spores and mycelium) was maintained by storage for three to six months in polyethylene bags at 4 °C and used as a stock culture. From this stock culture, 5 g of this fungal consortium was applied as inocula per pot, having an average of 30 spores/g of soil and roots with 70% of AM colonization. This AM inoculum was applied to each one of the appropriate pots at sowing time just below to the seeds. Non-mycorrhizal treatments received the same amount of autoclaved inoculum.

The *B. megaterium* used here was also isolated from the same soil as AM fungi and has been previously reported as very effective under drought conditions (Marulanda et al., 2009). *B. megaterium* was grown in 250-mL flasks containing 50 mL of nutrients broth (8 g L^{-1}) medium (Difco) in shake culture for 48 h at 28 °C. In the appropriate pots, plants were inoculated with 1 mL of the bacterial culture (10^8 cfu mL^{-1}). In control treatments, 1 mL of sterilized bacterial culture was added.

A suspension of *Rhizobium trifolii* was added to each pot (1 mL 10^8 cfu per pot). This was prepared following standard procedure (Azcón, 1993).

2.1.3. Microcosm experiment

The experimental soil used was selected from an area located in the Natural Ecological Park "Vicente Blanes" in Molina de Segura, Province of Murcia (southeastern Spain) (coordinates 38°12′ N, 1°13′ W, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall lower than 270 mm and the potential evapotranspiration (ETP) reaches approximately 1000 mm. The mean annual temperature is 19.2 °C with the absence of frost period. The soil in the experimental area is a Typic Torriorthent, very little developed with a low organic matter content and a silty clay texture that facilitates the degradation of soil structure. The vegetation in the zone was dominated by *Piptatherum miliaceum* L. Cosson., *T. repens* L. and some shrubs of *Thymus vulgaris* L. and *Rosmarinus officinalis* L. growing with patchy distribution.

The main soil characteristics were pH 8.9, P $1.36 \cdot 10^{-3}$ g/kg (Olsen test), organic carbon 0.94%, total N 0.22%, and an electric conductivity of 1.55. The fermentation products (*A. niger* treated agrowaste + RP) prepared as described before, were mixed with a soil-sand mixture (1:1, v/v) supplying 0.005 kg/0.1 kg soil/sand mixture and left for equilibration for 2 weeks at room temperature.

The treatments used in this experiment were as follows: unamended soil/sand (T) or soil/sand amended with *A. niger* treated SB + RP compound (C). The single autochthonous consortium of AM fungi (M) or single *B. megaterium* (B) or dually (M + B) applied to unamended (T) or amended (C) soil. Respective non-inoculated controls were also assayed.

The microcosm experiment was conducted in natural soil under drought conditions (50% of water holding capacity) and replicated five times to give a total of 40 pots. Four surface-sterilized seeds of *T. repens* were grown in each pot (d = 12.2 cm; 0.5 kg capacity).

Plants were grown for four months in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C and 50% relative humidity. The photosynthetic photon flux density (PPFD) was $503 \cdot 10^{-6}$ mol m^{-2} s^{-1}, as measured with a light-meter (LICOR, model LI-188B). Water was supplied daily and loss was compensated by watering every day to reach 50% of water-holding capacity. During the first 2 weeks of plant growth constant soil water content close to water holding capacity was maintained. After this time, plants were allowed to dry until soil water content was 50% of water holding capacity and maintained under these conditions for an additional 13 weeks. To achieve that, the soil moisture in the pots was measured each 24 h and the water was added to reach a maximum of 50% of water holding capacity. However, during the 24-h period comprised between each rewatering the soil water content was progressively decreasing until a minimum value of 40% of water holding capacity. Soil moisture was measured with an ML2× ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil (Allen and Allen, 1986; Roth et al., 1992; White et al., 1994). This volumetric soil moisture is considered to be a normal environmental condition in dry Mediterranean areas.

2.2. Experiment II

The bacterial proliferation (cfu mL^{-1}), proline and IAA-like molecule production by *B. megaterium* growing under increasing stress conditions (0%, 5% and 10% PEG) were determined after 72 h, 96 h, 120 h and 144 h of bacterial growth.

This bacterial strain growing in nutrient broth (8 g L^{-1}) medium without PEG and at two PEG levels (5% and 10%) was replicated four times with a total of 12 flasks. Evaluations were made over time to test the bacterial growth, the permanence of proline and IAA bacterial activities and its evolution with time according to the level of stress.

2.2.1. Parameters measured

2.2.1.1. Stomatal conductance. Stomatal conductance was measured 2 h after the light turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from four different plants from each treatment.

2.2.1.2. Leaf water content. Leaf samples were weighed (fresh weight (FW)) immediately after harvesting. The samples were dried in an oven at 75 °C for a period of 48 h and their dry weights (DW) were obtained. Then WC was calculated using the following equation: (Fresh weight (FW) − Dry weight (DW) / Fresh weight (FW)) × 100: [(FW − DW) / FW] × 100.

2.2.1.3. Biomass production and nutrient acquisition. The plants were sequentially harvested three times. After each harvest shoot fresh and dry weight was recorded after drying at 75 °C. At the last harvest both shoot and root were recorded.

At the final harvest, the root system was separated from the shoot and dry weights were measured after drying in a forced drought oven at 75 °C for 2 days.

The nutrients as N, C, Ca, Mg, Na, K and P concentrations in shoots were determined (after Kjeldahl/digestion), by flame photometry inductively coupled plasma atomic emission spectrometry and colorimetry (Olsen and Dean, 1965) respectively.

2.2.1.4. Symbiotic development. Roots were carefully washed and stained. The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples (Phillips and Hayman, 1970), using the gridline intersect method of Giovannetti and Mosse (1980) where the root sample was spread out evenly in dishes that had gridlines marked on the bottom to form 1.27 cm squares. Vertical and horizontal gridlines were scanned under a dissecting microscope at 40 to 100× magnification. The absence or presence of AM colonization was recorded at each point where a root intersected a line and at least 100 gridline intersects were tallied as the authors recommended.

Nodule numbers were estimated by direct observation using a binocular microscope.

2.2.1.5. Antioxidant enzymatic activities. Shoot tissues were homogenized (Aroca et al., 2003) in a cold mortar with 4 mL 100 mM phosphate buffer (pH 7.2) containing 60 mM KH_2PO_4, 40 mM K_2HPO_4, 0.1 mM DTPA and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 18,000 *g* for 10 min at 4 °C, and the supernatant was used for enzyme activity determination. Total SOD activity (EC 1.15.1.1) (Burd et al., 2000) was measured on the basis of SOD's ability to inhibit

the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C. CAT activity (EC 1.11.1.6) was measured as described (Aebi, 1984). Consumption of H_2O_2 (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min was monitored. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 50 μL of enzyme extract in a 2 mL volume. APX activity (EC 1.11.1.11) was measured in a 1-mL reaction volume containing 80 mM potassium phosphate buffer (pH 7.0), 2.5 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH (Carlberg and Mannervik, 1985). The reaction mixture (1 mL) contained 50 mM Tris buffer 3 mM $MgCl_2$ (pH 7.5), 1 mM oxidized glutathione, 50 μL enzyme extract, and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in nmol NADPH oxidized mg $protein^{-1}$ min^{-1}, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}). Total soluble protein amount was determined using the Bradford (1976) method and BSA as standard.

2.2.1.6. Production of indole acetic acid (IAA-like molecules) and Proline by B. megaterium under increasing polyethylene glycol (PEG) concentrations. The bacterial isolates were cultivated at 28 °C in 100 mL sterile nutrients broth (Difco) medium supplemented with 0%, 5% or 10% of PEG in order to induce osmotic stress.

The production of indole acetic acid (IAA-like molecules) by these bacteria under increasing PEG concentrations was determined after 72, 96, 120 and 144 h of culture using the Salper's reagent (Gordon and Paleg, 1957). Three milliliters of fresh Salper's reagent (1 mL 0.5 M $FeCl_3$ in 50 mL 37% $HClO_4$) was added to free-cell supernatant and kept in complete darkness for 30 min at ambient temperature and the optical density at 535 nm was measured in each treatment (Wöhler, 1997). A standard curve was prepared for IAA (Sigma, USA). In the same growing medium the proline accumulation was estimated in the bacterial cell extract by spectrophotometric analysis at 530 nm (Bates et al., 1973). The bacterial extract reacts with ninhydrin and glacial acetic acid during 1 h at 100 °C (the reaction stops by introducing the tubes in ice bath). The reaction mixture is extracted with 2 mL of toluene, shaken vigorously for 20 s. A standard curve was prepared with concentrations of proline known.

2.2.1.7. Statistical analyses. The data results of both experiments were analyzed using SPSS 21 software package for Windows, were subjected to one-way general linear model ANOVA (analysis of variance) and were used to determine the effect of each treatment. The Duncan's (1955) multiple-range test was used for post hoc analysis to determine differences between means. Differences were considered significant at $p < 0.05$. Percentage values were ARC-sine-transformed before statistical analysis.

3. Results

The positive effect of *A. niger* transformed agrowaste was particularly evident on shoot growth. At whatever harvest time this amendment increased shoot biomass over plants without amendment (Fig. 1). The highest plant growth effect was obtained when *B. megaterium* was inoculated in the amended soil. Comparing the total shoot plant biomass yielded in control (T) plants with those amended and inoculated, the highest differences in shoot biomass were of 157% (BC) and 135% (MC). Nevertheless, plants growing in non-amended soil maximized shoot biomass (by 52%) as affected by dual M + B treatments. The single M and dual M + B inoculation resulted the most effective treatments in non-amended soil while single B or M maximized plant growth in amended soil (Fig. 1).

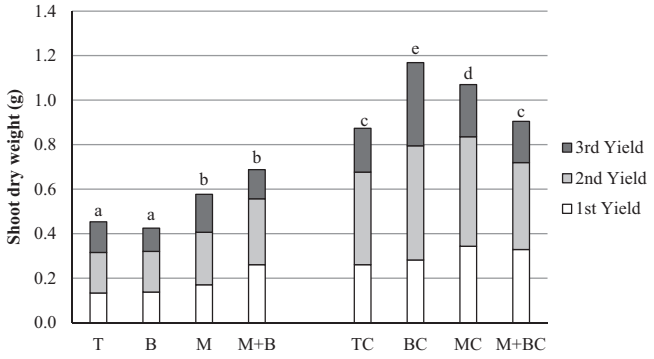

Fig. 1. Shoot dry weight (g) after three successive harvests of noninoculated control *Trifolium repens* plants (T) or inoculated with *Bacillus megaterium* (B), mycorrhizal fungi (M) or both (M + B) inocula under water stress conditions. Natural soil was added or not of treated agrowaste (C). Within each value bars having a common letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

Biomass of plants growing in non-inoculated amended soil (TC) was higher at whatever yield period than those growing with the best inocula (B + M) in non-amended soil. As a result of the applied treatments, the greatest total plant biomass yielded after three successive harvests was reached in bacterial inoculated and amended soil. The high effectiveness of single bacterial inoculum in amended soil under the dry natural conditions used was particularly evidenced in the last two harvests (2nd and 3rd) (Fig. 1).

The effect of applied treatments regarding leaf stomatal conductance (SC), demonstrated that this value was increased in control amended plants and reduced by the microbial treatments irrespective of amendment (Fig. 2). This decreasing microbial effect on such physiological value was particularly important in M inoculated plants irrespective of compost application. Concomitantly, the leaf hydric content was increased in the amended and inoculated plants. The most efficient

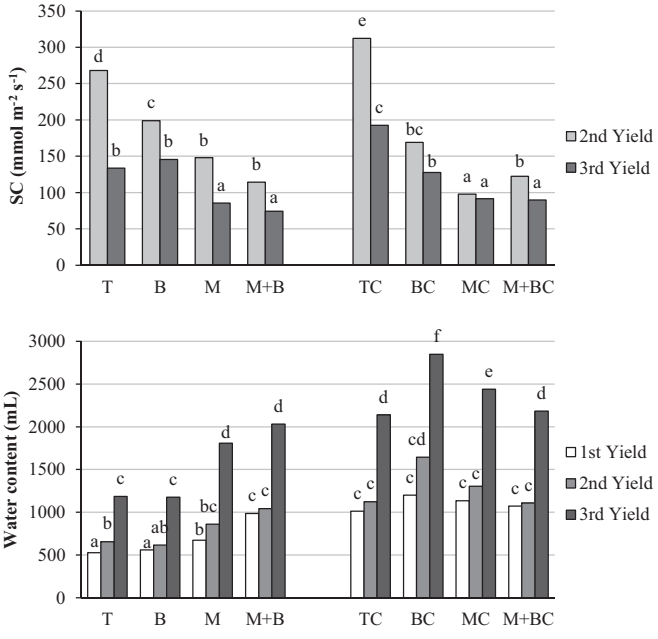

Fig. 2. Stomatal conductance (SC) after 2nd and 3rd yield and water content (mL) (three harvest) of non-inoculated control *Trifolium repens* plants (T) or inoculated with *Bacillus megaterium* (B), mycorrhizal fungi (M) or both (M + B) inocula under water stress conditions. Natural soil was added or not of treated agrowaste (C). Within each yield bars having a common letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

treatment in decreasing SC value was M inoculum that caused a reduction of less than half SC compared to non inoculated plants. In parallel, M application increased plant water content. The effectiveness of amendment was evident on plant water content particularly in 3rd yield (Fig. 2). The effect of BC treatments increasing water content was very relevant for plants growing under drought conditions (Fig. 2). The most effective treatments for improving plant growth (B with compost and M + B without compost) also maximized plant water content (Table 1 and Fig. 1).

Regarding antioxidant activities, it was observed that APX activity was the highest in M and M + B inoculated plants, particularly without compost application; and SOD activity was highly reduced in M and M + B inoculated plants with compost application. For whatever antioxidant activity here measured *B. megaterium* decreased (without compost) each one of these enzymatic values. Whichever antioxidant analyzed was lower in BC than in B. In general, the compost application decreased each of these activities (Fig. 3).

In axenic culture, the greatest IAA-like molecule production by *B. megaterium* was observed after 96 h under 10% of PEG (Fig. 4). Nevertheless, the bacterial IAA-like molecule production over time showed little changes in the presence or absence of PEG (Fig. 4). Proline production by *B. megaterium* was particularly increased after 120–140 h under 10% of PEG (Fig. 5).

Root weight was only increased by single B or M microbial inoculants in the absence and presence of compost, but the amendment reduced root development in non-mycorrhizal plants (Table 1). Mycorrhizal inoculation with the AM fungal consortium produced only a light, non-significant stimulating effect on the percentage of natural mycorrhizal colonization. The microbial inoculations, particularly M fungi in amended soil, highly increased total AM colonization. In contrast the number of nodules formed in the roots was only enhanced by the amendment in control non-inoculated plants (Table 1).

In general, nutrient content in plants resulted highly increased by compost application (Tables 2 and 3). Mycorrhizal inoculation was able to improve Ca, K and C content in plants grown in non-amended soils. The nutrient content did not significantly change in coinoculated plants compared to those single M inoculated irrespective of compost application. Single *B. megaterium* was effective in increasing Ca, Mg and K content only in the amended soil (Tables 2 and 3).

4. Discussion

The inoculation of a drought-tolerant *B. megaterium* strain and a consortium of autochthonous arbuscular mycorrhizal fungi were able to improve plant growth over time and drought stress tolerance. These microorganisms, particularly in composted soil, resulted effective under the drought conditions established here. Plant performance under such stress conditions resulted to be dependent upon the microbial activity of the selected microorganisms to reach the optimum development under successive yields. A stimulating growth effect by the amendment application was also recorded, particularly in interaction with the bacteria. The effectiveness of these treatments was more relevant in increasing plant physiological and biochemical values and water content than in improving the symbiotic developments (nodulation or AMF-colonization) in these plants. Furthermore, the oxidative stress (as index of stress tolerance) produced by drought decreased in treated plants. Antioxidant activities involved in the alleviation of oxidative stress have the ability to protect cells against the detrimental effect of reactive oxygen species (ROS) produced by the drought that are capable of causing cellular damage, such as the peroxidation of membrane lipid components. Here most of the inoculated plants have the possibility to attenuate the oxidative stress generated by drought (Kasim et al., 2013; Kim et al., 2012).

The increased water uptake observed here in inoculated and/or amended plants is crucial to alleviate drought stress symptoms (Long et al., 2008; Martinez-Ballesta et al., 2006). In order to keep tissue water status under hydric stress conditions, plants need to reach a balance between water lost by leaf transpiration and water gained by root uptake. Thus, under water stress conditions the plant drought tolerance resulted of closing their stomata lowering the stomatal conductance in order to avoid leaf dehydration (Aroca et al., 2003), as was observed here in inoculated plants. The lowest stomatal conductance was recorded here in mycorrhizal inoculated plants having the highest water content. Thus, the greatest shoot biomass of *B. megaterium* inoculated plants with compost seems to be related to the increased water uptake. These treatments did not positively affect AM colonization but increased plant Ca and Mg uptake that are involved in the regulation of plant response to drought stress (Knight et al., 1997).

In general, the highest hydric content found in whatever inoculated plants with compost and in mycorrhizal inoculated plants without compost indicated the better status in these plants to support drought conditions. These results evidenced the effectiveness of the treatments applied as strategies for improving plant drought tolerance.

In this study, not only microbial inocula but also the amendment attenuated the adverse effects of drought-induced oxidative stress by regulating the antioxidant activities. The recorded values of enzymatic systems such as SODs, CATs, GRs and APXs suggest variations in the level of plant stress-tolerance according to the applied treatments. In fact, nutrients and water limitations were overcame by the treatments applied (AMF, *B. megaterium* and/or amendment) helping plants to attenuate the adverse effects of drought-induced stress. The reduction and regulation of antioxidant activities in inoculated plants are the result of a diminution of oxidative damage in these plants (Marulanda et al., 2007). These values could be related to the observed increment in water and nutrient uptake.

We know that APX, CAT and SOD antioxidant activities interact with active forms of oxygen and keep them at low levels (Smirnoff, 1993). These antioxidant systems in plant cells counteract the toxic levels of ROS and they determine the extent of oxidative stress. SOD is the first enzyme of defense against ROS and in this study inoculated plants, particularly those AM-inoculated plus compost, showed the lowest SOD activity. In a next step, CAT and APX act to detoxify the H_2O_2 produced by SOD. Here APX was particularly activated by AM inoculation. Contrasting, *B. megaterium* inoculated plants exhibited the lowest CAT and GR activities. These enzymatic values indicated the effectiveness of the treatments applied for decreasing the need of such enzymes to maintain

Table 1
Root fresh weight (mg), percentage and total mycorrhizal colonization and nodule number of non-inoculated control *Trifolium repens* plants (T) or inoculated with *Bacillus megaterium* (B), mycorrhizal fungi (M) or both (M + B) inocula under water stress conditions. Natural soil was added or not of treated agrowaste (C).

Microbial treatment	–				C			
	Root	AM colonization		Nodule number	Root	AM colonization		Nodule number
		%	Total			%	Total	
T	1780b	19ab	338.2a	71a	1440a	24b	345.6a	148b
B	2420d	15a	363.0a	47a	2080c	27b	561.6b	56a
M	2160c	24b	518.4b	70a	2200c	33c	726.0c	60a
M + B	1340a	21ab	281.4a	54a	1550ab	33c	511.5b	53a

Within each parameter values having a common letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

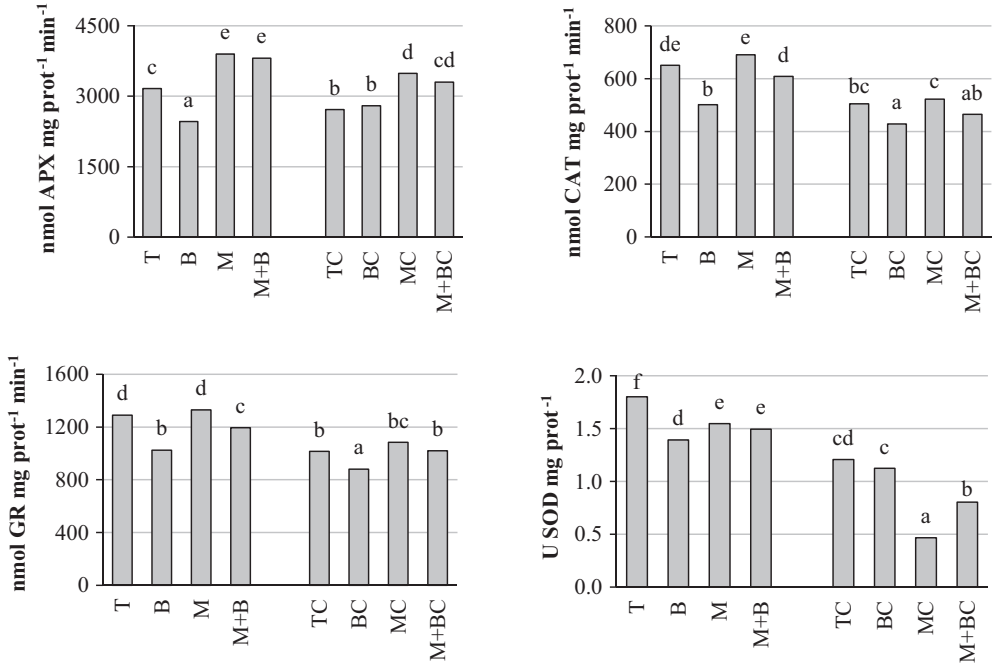

Fig. 3. Ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) antioxidant activities in shoot after the 2nd yield of noninoculated control *Trifolium repens* plants (T) or inoculated with *Bacillus megaterium* (B), mycorrhizal fungi (M) or both (M + B) inocula under water stress conditions. Natural soil was added or not of treated agrowaste (C). Within each value bars having a common letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

the cellular physiology. Thus, changes detected in these antioxidant activities can be considered as markers of plant strategies for drought tolerance.

Nevertheless, in this study single mycorrhizal inoculated plants kept, irrespective of amended treatments, higher APX, similar CAT and lower SOD than control plants. But the amendment or *B. megaterium* application decreased whichever of these activities which evidenced their role in alleviating plant stress and in the defense against the lower oxidative stress induced in these treated plants by water limitation. The protective action of these enzymatic activities and the contribution of each one in the protection against oxidants are not easy to elucidate (Kasim et al., 2013). Moreover, the balance between these activities is a critical factor involved in ROS metabolism. Activities like SOD, CAT and APX usually act as the first line of defense against ROS and subsequently GR detoxifies the H_2O_2 and O_2^- produced by SOD. Thus, in plant tissues to maintain a right balance between them is very important to understand steady state level of ROS (Apel and Hirt, 2004; Azcón et al., 2010). Nevertheless, results from this study suggested that the SOD, CAT, GR and APX activities were not the single mechanism involved in sustaining plant growth and drought tolerance under water limiting conditions.

A high level of proline as was produced by *B. megaterium* under the highest stress conditions (10% PEG) enables the bacterial cells to maintain an osmotic balance under low water potential. Consistent with this, circumstantial evidence the beneficial effects of osmolyte-producing rhizobacteria were more important when the stress conditions were more severe. Proline protects *B. megaterium* cells against osmotic stresses by adjusting osmotic pressure and by stabilizing membranes and proteins (Mäkelä et al., 2000). The drought adaptation of this bacterium is evidenced by this measurement that resulted a useful biochemical marker able to control the osmotic stress in this bacterium. Thus, proline and other osmolytes can be used for bacterial selection under drought.

The specific microorganisms used here (consortium of autochthonous AM fungi and *B. megaterium*) are involved in processes such as nutrient and water acquisition and consequently in plant growth promotion. But each one of these plant growth promoting processes could be differently regulated according to the particular microbial abilities. In this study, the beneficial effects of the inoculation with *B. megaterium*, without compost, were evidenced by activities as the root growth improvement, the changes in stomatal conductance that represents a physiological adaptation to counteract drought stress, and by a decrease of whatever antioxidant

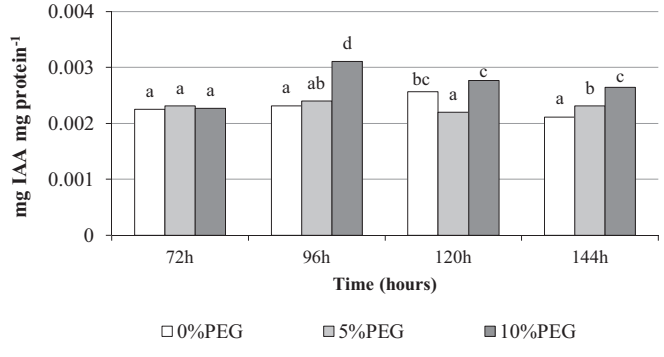

Fig. 4. Indole acetic acid-like molecule (IAA) accumulation by *Bacillus megaterium* with time (hours) as affected by polyethylene glycol (PEG) levels (0%, 5% or 10%).

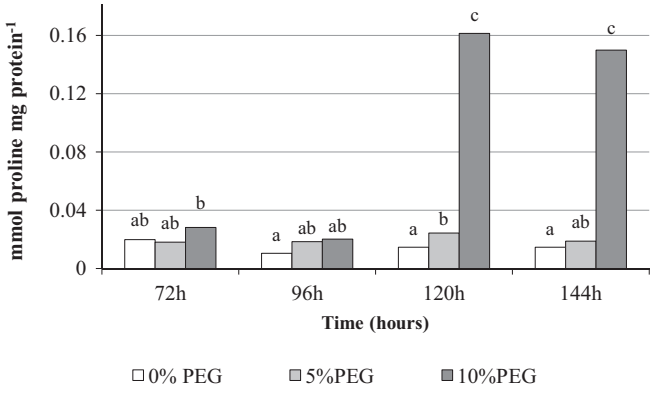

Fig. 5. Proline accumulation by *Bacillus megaterium* with time (hours) as affected by polyethylene glycol (PEG) levels (0%, 5% or 10%).

Table 2
N, P and K content (mg) in shoot (average of three harvests) in non-inoculated control *Trifolium repens* plants (T) or inoculated with *Bacillus megaterium* (B), mycorrhizal fungi (M) or both (M + B) inocula under water stress conditions. Natural soil was added or not of treated agrowaste (C).

Microbial treatments	–			C		
	N	P	K	N	P	K
T	0.928ab	0.169b	4.204a	1.529c	0.494d	6.858b
B	0.715a	0.211b	3.862a	1.747c	0.477d	9.189c
M	1.209b	0.219b	5.973b	1.977 cd	0.340c	9.751c
M + B	1,290b	0.126a	6.921b	2.000d	0.329c	9.966c

Within each parameter values having a common letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

activity. A higher root development normally has positive effects on water acquisition and nutrient uptake (Ferdose et al., 2009; Long et al., 2008; Marulanda et al., 2010), but any bacterial effect in enhancing plant nutrition was observed in the absence of compost.

Here the effectiveness of the amendment on plant drought tolerance may be ascribed to various mechanisms such as nutritional and physiological plant improvements by direct and higher water and nutrient uptake, since no effect of the amendment on the promotion of root growth was found. In addition, the amendment application and AM colonization were able to change soil structure by increasing aggregate formation and stabilization, as previously reported by Medina et al. (2010). As a result, plants growing in amended soil could be less damaged by drought (Caravaca et al., 2005a; Medina et al., 2004b).

Previously, it was confirmed that the *B. megaterium* strain used here was able to modify salt response in maize plants in terms of leaf relative water content. The modification in plants of PIP aquaporin expression and abundance by the inoculation of this strain of *B. megaterium* could be involved in the plant responses observed (Marulanda et al., 2010). Results reported in this previous study using similar bacteria but different environmental conditions (as different plant, sterile-conditions and fertile soil) would validate those results found here. Nevertheless, an interesting and new result from the present study is that *A. niger* treated agrowaste used as amendment positively interacted with both inocula, particularly with *B. megaterium*. In this natural and poor dry soil the amendment was required to optimize *B. megaterium* activity which positively affected plant growth and increased the availability of nutrients (K, Ca and Mg) to plants. This amendment is probably required for the bacteria as C and energy source, which lead to an enhancement of bacterial growth, survival and activities. As a result, the main effect of *B. megaterium* in the amended soil was to enhance plant hydric content being more effective under these dry conditions than mycorrhizal inocula at whichever harvest as also happened in trees in the desert (Bashan et al., 2012). In fact, water stress was more compensated by the inoculation of *B. megaterium* than by AM fungi in amended soil. The greater plant hydration induced by *B. megaterium* might be attributable to increased water use efficiency and/or enzymatic lowering of plant ethylene concentration (Saravanakumar and Samiyappan, 2007).

Table 3
C, Ca, Mg content (mg) in shoot (average of three harvests) in non-inoculated control *Trifolium repens* plants (T) or inoculated with *Bacillus megaterium* (B), mycorrhizal fungi (M) or both (M + B) inocula under water stress conditions. Natural soil was added or not of treated agrowaste (C).

Microbial treatment	–			C		
	C	Ca	Mg	C	Ca	Mg
T	17.58b	5.66a	0.97a	29.19d	7.30b	1.49c
B	13.86a	5.33a	0.88a	33.94df	9.31d	1.68d
M	23.16c	6.37b	0.99a	35.09f	7.70b	1.48c
M + B	24.14c	8.36bd	1.28b	36.34f	7.16b	1.43c

Within each parameter values having a common letter are not significantly different ($p < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

The ABA production by this strain of *B. megaterium* was tested in axenic conditions. As it is well-known this phytohormones are able to induce stomatal closure in plants in response to drought as was observed here. It resulted an adaptative response to cope with drought stress (Porcel et al., 2014). The ACC-deaminase production by these bacteria was also maintained under drought.

The high effect of *B. megaterium* in the amended soil, where it developed its maximum PGPB potential, can be also explained by the enhancement of activities previously exhibited in maize plants such as increasing root hydraulic conductance and plasma membrane type two (PIP2) aquaporin amount in maize roots under osmotic stress conditions (Marulanda et al., 2010). In addition, the plant root biomass was increased in the *B. megaterium* inoculated plants. The mechanisms underlining these results may be related to all of these coordinated effects resulting in an improved ability to acquire water under water limited conditions as was reported before (Marulanda et al., 2010). Thus, according to this previous information this *B. megaterium* strain influenced two crucial components of plant osmotic stress tolerance such as root development, root hydraulic characteristics, PIP aquaporin regulation (expression and abundance) and hormone (ABA and IAA) synthesis. Consistent with the beneficial PGPB effect, *B. megaterium* resulted to be an osmolyte-producing bacterium under stress conditions and its effectiveness was prominent in improving plant physiological and biochemical values under drought. The ability of *B. megaterium* to increase the IAA-like molecule production at 10% of PEG inducing osmotic stress in the medium represents an efficient mechanism to resist drought and to enhance plant drought tolerance. The ability of this osmo-tolerant bacterium to produce IAA-like molecules improved root proliferation in plants (by 36%) induced by this hormone (Yuwono et al., 2005). Similarly under water deficiency, *Azospirillum brasilense* displayed improved relative and absolute water contents in rice plants (Ruíz-Sánchez et al., 2011) and it also affects many others plant growth improvements as reported by Bashan and de-Bashan (2010a). Hydric content in this study is correlated with plant growth stimulation as found before (Casanovas et al., 2002). The most important detrimental effect of water limitation is the reduction of plant biomass, as control non-treated plants did. In the opposite way, plants having the highest biomass, as those inoculated with *B. megaterium* plus amendment, seem to be less affected by the water stress imposed and showed the highest water content.

The effectiveness of the treatments applied, involving saprophytic and symbiotic soil microorganisms, resulted successful for enhancing plant performance under water limited conditions. Thus, the present results evidence the suitability of these treatments for improving and thereby preserving plant physiological and biochemical traits in stressful environmental conditions. The management of natural resources as PGPB microorganisms and *A. niger* treated agrowaste assured plant growth and establishment under water and nutrient limitations, providing a valuable tool for the recovery of degraded semiarid areas.

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