Journal of Plant Physiology 174 (2015) 87–96

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Functional Biotechnology

Contribution of arbuscular mycorrhizal fungi and/or bacteria to enhancing plant drought tolerance under natural soil conditions: Effectiveness of autochthonous or allochthonous strains

N. Ortiz[a], E. Armada[a], E. Duque[b], A. Roldán[c], R. Azcón[a,*]

[a] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Prof. Albareda 1, 18008 Granada, Spain
[b] Departamento de Proteccion Ambiental, Estación Experimental del Zaidín, CSIC, Prof. Albareda 1, 18008 Granada, Spain
[c] Department of Soil and Water Conservation, Centro de Edafología y Biología Aplicada del Segura, CSIC, P.O. Box 164, Campus de Espinardo, 30100 Murcia, Spain

ARTICLE INFO

Article history:
Received 24 April 2014
Received in revised form 5 August 2014
Accepted 22 August 2014
Available online 20 October 2014

Keywords:
Autochthonous and allochthonous microorganism
Arbuscular mycorrhizal fungi and/or bacteria
Drought
Oxidative stress
Physiological responses

ABSTRACT

Autochthonous microorganisms [a consortium of arbuscular-mycorrhizal (AM) fungi and *Bacillus thuringiensis* (*Bt*)] were assayed and compared to *Rhizophagus intraradices* (*Ri*), *Bacillus megaterium* (*Bm*) or *Pseudomonas putida* (*Psp*) and non-inoculation on *Trifolium repens* in a natural arid soil under drought conditions. The autochthonous bacteria *Bt* and the allochthonous bacteria *Psp* increased nutrients and the relative water content and decreased stomatal conductance, electrolyte leakage, proline and APX activity, indicating their abilities to alleviate the drought stress. Mycorrhizal inoculation significantly enhanced plant growth, nutrient uptake and the relative water content, particularly when associated with specific bacteria minimizing drought stress-imposed effects. Specific combinations of autochthonous or allochthonous inoculants also contributed to plant drought tolerance by changing proline and antioxidative activities. However, non-inoculated plants had low relative water and nutrients contents, shoot proline accumulation and glutathione reductase activity, but the highest superoxide dismutase activity, stomatal conductance and electrolyte leakage. Microbial activities irrespective of the microbial origin seem to be coordinately functioning in the plant as an adaptive response to modulated water stress tolerance and minimizing the stress damage. The autochthonous AM fungi with *Bt* or *Psp* and those allochthonous *Ri* with *Bm* or *Psp* inoculants increased water stress alleviation. The autochthonous *Bt* showed the greatest ability to survive under high osmotic stress compared to the allochthonous strains, but when single inoculated or associated with *Ri or* AM fungi were similarly efficient in terms of physiological and nutritional status and in increasing plant drought tolerance, attenuating and compensating for the detrimental effect of water limitation.

Introduction

Water stress is an environmental factor limiting plant growth and is a major issue in climate change. Desertification processes in arid areas are the result of the degradation of vegetation cover since plant growth is seriously limited in arid sites. Changes in plant physiology, nutrient acquisition and metabolism induced by drought are highly limiting factors for plant growth (Evelin et al., 2009). Nevertheless, plants evolve survival strategies to increase tolerance in changing habitats (Bohnert et al., 2006). Changes in the environment induce alterations in the soil/plant system and associated responses to adapt to the new conditions.

Plants affected by drought show physiological and biochemical changes reducing the normal growth and survival (Marschner, 1995). Nevertheless, several factors activate the resistance response of plants to water stress and changes in the physiological, biochemical, nutritional and hormonal mechanisms in plants can alleviate or increase detrimental effects of drought (Ramoliya et al., 2004). Associated microorganisms can elicit a great variety of nutritional and physiological plant responses to environmental stresses (Benabdellah et al., 2011; Marulanda et al., 2006; Medina and Azcón, 2010). These studies under sterile conditions have demonstrated the great potential of soil microorganism management as a strategy to enhance drought tolerance in plants.

Drought stress mainly affects plant growth and nutrition (Loreto and Centritto, 2008) and it results in reduced photosynthetic activity and an increase in oxidative stress in plants that induces an array of antioxidant enzymes. Several reports have shown that

* Corresponding author. Tel.: +34 958181600.
E-mail address: rosario.azcon@eez.csic.es (R. Azcón).

http://dx.doi.org/10.1016/j.jplph.2014.08.019
0176-1617/

microbial activity in the rhizosphere plays an important role in drought-induced antioxidant responses since it can alleviate effects of drought stress by changing proline and antioxidants accumulation in plant tissues (Ruíz-Lozano et al., 2008).

Soil microorganisms, such as the mycorrhizal soil community, are a very important component in the plant/soil system. Obligate symbionts can improve plant tolerance to this abiotic stress and improve both plant nutrition and protection against the oxidative damage produced by the water stress (Ruíz-Lozano et al., 2012). Symbiosis between arbuscular mycorrhizal fungi (AMF) and most plants provide nutrients, stimulate plant growth, increase plant stress tolerance and thus restore ecosystem functions (Barea et al., 2005). Mycorrhizal colonized plants can also interact with several soil microorganisms including plant growth promoting rhizobacteria (PGPR) that also are able to make the plant more tolerant to these stressed conditions (Azcón et al., 2009; Barea et al., 2004; Dimkpa et al., 2009; Kasim et al., 2013). For these reasons, the interactions between AMF and PGPR might be useful for the development of re-vegetation in soils having water and nutrients limitations (Marulanda et al., 2009). The beneficial effect of these AMF and PGPR under drought conditions in sterile soil have recently been reported (Marulanda-Aguirre et al., 2008; Medina and Azcón, 2012). However, the origin and activities of microorganisms involved is relevant, particularly under natural and drought conditions. Thus, the accurate selection of inoculants is very important for the success of such biotechnology.

Microorganisms such as arbuscular-mycorrhizal fungi (AMF) or PGPR can induce a wide array of effects in plants such as root elongation, improvement of nutrient uptake and increasing resistance to abiotic stresses (Medina and Azcón, 2012). However, few studies have shown the comparative effect and importance of inoculation with selected adapted autochthonous or allochthonous microorganisms to alleviate adverse effects and counteract drought stress in plants grown in natural semi-arid soils.

Microorganisms living in arid and semiarid soils are often adapted to such detrimental conditions. AMF and PGPR can be found under extreme environmental conditions and can be adapted to these stresses (Kohler et al., 2009; Marulanda et al., 2007; Wilde et al., 2009). In fact, water limitation is detrimental not only to plants but also to microorganisms, reducing growth and activity. However, adapted drought-tolerant microorganisms may compensate for the stress effect and can be active in promoting plant establishment. The use of adapted autochthonous microorganisms to regenerate arid soils is an attractive possibility (Medina and Azcón, 2012).

The inoculation with efficient and tolerant-adapted microorganisms has consistently resulted not only in enhancing growth and nutrition but also in adaptation of plants to survive in arid/semiarid soils (Liddycoat et al., 2009). Nevertheless, the interactions between inoculated microorganisms of different origin may result in a more or less positive plant growth promoting effect and plant establishment depending on abilities and stress tolerance of the microorganisms involved (Benabdellah et al., 2011; Marulanda et al., 2009). The convenience of using adapted/tolerant autochthonous strains is based on these previous results. Thus, we hypothesized that for the optimum plant growth promotion in natural drought-stressed sites, an important characteristic of microbial inoculants applied must be to have resistance and tolerance to this environmental stress in addition to maintaining its PGPR abilities under such conditions.

In a natural soil, rhizosphere competence needs to be considered for successful interactions between introduced and autochthonous microorganisms. In general, transferring rhizosphere microorganisms from one environment to another causes an imbalance in metabolism of bacterial populations, which will decline until the population reaches equilibrium with the environment.

The objective of identifying the PGPR abilities of drought-tolerant (autochthonous) microorganisms for plant establishment in natural arid soils from the southeast of Spain is to preserve local biodiversity without introducing new microbial species. For this to be successful, it is necessary to compare the particular abilities of the microbial inoculants assayed (autochthonous or allochthonous from collection) and to evaluate the benefit in growth, nutrient uptake and hydric status in the host plant, as well as the mechanisms involved and the possibility to use these sustainable strategies to contribute to plant health and productivity.

The lack of water in the growth medium causes limited nutrient diffusion, having detrimental effects on plant growth and the inoculation with efficient and tolerant species of microorganisms has consistently resulted not only in improved growth but also in changing metabolic processes conducting to proline and antioxidant activities in colonized plant. The actions of varied antioxidants acting in synchrony are required to minimize hydroxyl and singlet oxygen produced by the drought stress. The changes in antioxidant responses in drought-stressed plants precludes the role of these enzymatic systems in the protection against oxidative injury. Proline was also evaluated since it is able to increase the activity of enzymes involved in the antioxidant defense system (Apel and Hirt, 2004; Hoque et al., 2007a, 2007b). It is known that major reactive oxygen species (ROS) scavenging enzymes can increase the ability of plants to resist environmental stresses (Alguacil et al., 2003a,b; Azcón et al., 2010; Porcel et al., 2003).

The aim of this study was to compare the PGPR activity of autochthonous and allochthonous inoculants of different origin (but both well adapted to drought conditions) in an attempt to select the most suitable microorganism and also to understand the mechanisms involved in microorganisms-induced tolerance to drought stress in inoculated plants. For this, we used two drought-tolerant bacteria and effective PGPR from collection versus the most representative (abundant) autochthonous bacterial strains. The selected allochthonous bacteria were *Bacillus megaterium* (*Bm*) and *Pseudomonas putida* (*Psp*) (both from Estación Experimental del Zaidín collection) and the autochthonous *Bacillus thuringiensis* (*Bt*) (isolated from the semiarid experimental soil). For allochthonous, AMF was selected as a drought-adapted strain of *Rhizophagus intraradices* (*Ri*) (from EEZ collection) and a consortium of autochthonous mycorrhizal fungi (from the semiarid experimental soil). In this study these microorganisms were single or dually (fungi/bacteria) inoculated in *Trifolium repens* on a natural semiarid soil (from a Mediterranean zone of Spain) to compare the effectiveness of autochthonous and allochthonous microorganisms over non-inoculated control plants under drought conditions.

Growth, nutrition, and physiological parameters such as relative water content (RWC), stomatal conductance (SC), and electrolyte leakages (EL) were evaluated. The antioxidant enzymes such as superoxide dismutase (SOD) glutathione reductase (GR) and ascorbate peroxidase (APX) were assessed in shoot and root tissues for their potential ability to counteract the oxidative damage generated by drought in inoculated and non-inoculated plants.

The particular growth activities that bacteria are able to develop in axenic culture, under osmotic stress conditions, were also analyzed.

In general, we found that alteration in antioxidants seem to be related to the plant protection from dehydration through drought avoidance mechanisms.

Materials and methods

Two independent experiments were performed.

In the first experiment, the bacterial growth characteristics related to drought tolerance were evaluated. For this experiment

each bacteria strain was grown in axenic medium under increasing levels of polyethylene glycol (PEG) (0%, 10%, 20%, 30% and 40%) throughout the time period of the experiment (7 days). This product (PEG) is able to create an osmotic stress and this bacteria exhibiting a highest growth (cfu) at the greatest PEG levels indicated the greatest osmotic stress tolerance (Hamayun et al., 2010; Turkan et al., 2005)

Bacterial axenic growth under increasing PEG levels in the medium

Once tested the bacterial effectiveness on plant development under drought conditions, the growth of the drought tolerant autochthonous bacteria and of those used as reference (from our collection) under increasing PEG levels in the growing medium were assayed. Bacterial strains were cultivated at 28 °C in nutrient broth (8 g L^{-1}) supplemented with increasing PEG (0%; 10%; 20%; 30% or 40%) levels. The number of viable cells was estimated following the conventional procedure at day intervals from 0 to 7 days.

Selection of inocula preparation and identification of drought tolerant microbes

Bacillus megaterium (*Bm*) was used as allochthonous bacteria for reference. It was selected from a semiarid soil in a previous experiment because when compared to other drought-adapted bacterial isolates, it was the most effective under drought conditions (Marulanda et al., 2009). It was isolated from a similar semiarid soil as this zone.

Pseudomonas putida (*Psp*) (BIRD-1 PSC-367), a commercial strain, was used as a reference PGPR in this study. This strain is particularly efficient as a phosphate solubilizing cultivable bacteria (Maillet et al., 2011; Matilla et al., 2011; Vyas and Gulati, 2009). It is able to adhere to plant roots and colonizes the rhizosphere of plants to high cell densities in soil with only 2% moisture. This property seems to be linked to its ability to synthesize trehalose. In addition *Psp* overproduces indole-acetic acid through convergent pathways which influences its ability to stimulate plant growth.

These autochthonous microorganisms were isolated from rhizosphere of plants naturally grown in the experimental semiarid soils. Bacterial strains were isolated following serial soil dilutions, 1 g of homogenized rhizosphere soil was suspended in 100 mL of sterile water (dilution 10^{-2}) and this suspension was further diluted to reach dilution 10^{-4}–10^{-7}. These dilutions were plated in agar nutrient broth Difco medium (8 g L^{-1}) and cultivated for 48 h at 28 °C. We selected the most abundant autochthonous bacterial strain (based on morphological characteristics) as well as the allochthonous assayed (*Bm* and *Psp*) and each one was separately grown in the corresponding 250-mL flasks containing 50 mL of this nutrient broth medium, in shake culture for 24 h at 28 °C for inocula production.

Autochthonous AM inoculum also was isolated from the rhizosphere of plants growing in this semiarid area. Spores, hyphae and mycorrhizal root fragments were multiplied in an open pot culture of maize and *Trifolium repens* for ten months. After this time, the AM inoculum from this stock culture, as consortia of indigenous AM fungi having spores, hyphae and AM root fragments were obtained. Similarly, the inoculum of the allochthonous mycorrhizal fungus *Rhizophagus intraradices* (accession no. EEZ 195) was prepared. It also consisted of spores, hyphae and AM root fragments.

The identification of autochthonous bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were extracted, diluted, lysed, and directly used as a template in the PCR reactions.

All reactions were conducted using a 25 μL volume containing PCR buffer 10×, 50 mM $MgCl_2$, 10 μM each primers 27FA (AGAGTTTGATCCTGGCTCAG) and 1492RA (GGTTACCTTGTTACGACTT), 5 U/μL of Taq polymerase (Platinum, Invitrogen).

The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 °C, followed by 30 cycles of 45 s at 95 °C, 45 s at 44 °C and 2 min at 72 °C, and finally, one cycle of 10 min at 72 °C. The products of PCR were analyzed by 1% agarose gel electrophoresis.

For extraction of DNA bacterial we used a QIAquick Gel extraction kit (QUIAGEN). Each sequence was compared with the database of 16S rDNA, the NCBI/BLAST and EMBL-EBI program. Database searches for 16S rDNA sequence similarity unambiguously identify the bacterium as *Bacillus thuringiensis* (Accession NR 043403.1, similarity 98%).

Autochthonous spores found in the autochthonous AM inoculum 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. The AM inoculums applied consisted of a mixture of them.

Soil–plant bioassay

The second experiment consisted of soil-plant bioassays. 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 1.000 mm. The mean annual temperature is 19.2 °C with no 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. *Trifolium repens* L., and some shrubs of *Thymus vulgaris* L. and *Rosmarinus officinalis* L. growing with patchy distribution. From rhizospheres of these autochthonous plants the microbial inocula were selected.

The topsoil (0–20 cm) from this soil was used in a soil-sand mixture (5:2, v/v). The main soil characteristics were pH 8.90, P 1.36×10^{-3} g/kg (Olsen test), organic carbon 0.94%, total *N* 0.22%, and electric conductivity of 1.55 dS m^{-1}.

In this bioassay with *Trifolium repens* plants, the effects of microorganisms on growth, nutrition, physiological parameters and antioxidant enzymes were determined after four months of plant growth under drought conditions.

The microbial treatments used in this experiment were as follows: the autochthonous AMF inocula consortium or *Ri* from collection were singly or dually applied with autochthonous bacterias *Bt*, or of collection (*Bm* or *Psp*). Plants non-inoculated with AM fungi or bacteria as controls were also assayed. Each treatment was replicated five times for a total of 60 pots. All the plants were inoculated only with *Rhizobium trifolii.*

Microbial inoculation

In the appropriate pots, plants were inoculated with 1 mL of the corresponding bacterial culture (10^8 cfu mL^{-1}). In control treatments, 1 mL of sterilized bacterial culture was added.

Five grams of autochthonous AMF or *Ri* were inoculated per pot, having an average of 30 spores/g of soil and roots with 70% of AM colonization. These were applied to each one of the appropriate pots at sowing time just below to the seeds.

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).

Plant growth conditions

Four surface-sterilized seeds of *Trifolium repens* were grown in each pot (0.3 kg capacity) containing a mixture of natural soil and sterilized quartz sand in the ratio 5 soil: 2 sand (v/v). Water was supplied daily to maintain constant soil water content close to water holding capacity during the first 3 weeks of plant growth. At this time, plants were allowed to dry until soil water content was 50% of water holding capacity and maintained under these conditions for additional 20 weeks.

Plants were grown in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C and 50% relative humidity. The range of photosynthetic photon flux density (PPFD) was 1100–500 $\mu mol\ m^{-2}\ s^{-1}$, throughout the duration of the experimental study, as measured with a light-meter (LICOR, model LI-188B). Each fifteen days this was checked and also coinciding with the time of measurement of physiological parameters in plants. Water loss was compensated by watering every day to reach 50% of water-holding capacity (WHC).

To achieve that, the soil moisture in the pots was measured each 24 h and water added to reach a maximum of 50% of water holding capacity. However, during the 24-h period comprised between each re-watering the soil water content was progressively decreasing until a minimum value of 40% of water holding capacity. Soil moisture was measured with an ML2 X 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.

Parameters measured

Plant biomass production

At harvest (six months after sowing), the root system was separated from the shoot and the plant biomass (shoots and roots) was determined.

The shoot and root tissues were separated in 0.5 g aliquots and frozen in liquid nitrogen for future determination of antioxidant enzymatic activities (GR, APX and SOD) and proline content.

Leaf relative water content

Leaf relative water content (RWC) was calculated in plants as follows: (Fresh weight − Dry weight)/(Turgid weight − Dry weight) × 100 (Aroca et al., 2003).

Stomatal conductance

Stomatal conductance was measured in one young leaf in five plants of each treatment 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 young leaf of each plant at midday the day before harvest. Plants were watered early in the morning and brought back at 50% WHC. At midday, SC measurements were performed in full-developed young leaves.

Electrolyte leakage

One young leaf of the same size from the first primary branch toward the distal end was collected from three plants for each treatment and washed thoroughly with deionized water to remove surface-adhered electrolytes. The samples were placed in closed vials containing 10 mL of deionized water and incubated at 25 °C on a rotary shaker for 24 h, and the electrical conductivity of the solution (L_t) was determined using Seven Easy conductivity (Metler Toledo AG 8603, Switzerland). Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (L_0) was obtained after cooling at 25 °C. The electrolyte leakage was defined as follows:

$$\text{Electrolyte leakage}\,(\%) = \left(\frac{L_t}{L_o}\right) \times 100$$

Nutrients and metals shoot concentrations

After drying in a forced-drought oven at 70 °C for two days shoot elements concentration were determined by flame photometry and colorimetric (Olsen and Dean, 1965) and plasma atomic emission spectrometry respectively.

Proline content in Trifolium shoot and root

The proline was extracted in 100 mM phosphate buffer (pH 7.8) from 0.5 g of fresh leaves and root, previously immersed in liquid N_2 and stored at −80 °C according to Bates et al. (1973). The homogenate was centrifuged at 18.000 × *g* for 10 min at 4 °C and the supernatant was used for quantification of proline content. Proline was estimated by spectrophotometric analysis at 520 nm using the ninhydrin reaction (Bates et al., 1973).

Antioxidant enzymatic activities in Trifolium shoot and roots

Plant tissues were homogenized as described by 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) 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) (Beyer and Fridovich, 1987) 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. 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. 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 in 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 $MgCl_2$, 1 mM oxidized gluthathione, and 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^{-1} protein min^{-1}, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH ($\varepsilon_{340} = 6.22\ mM^{-1} \times cm^{-1} \times L$). Total soluble protein amount was determined using the Bradford method (Bradford, 1976) and BSA as standard.

Mycorrhizal development

The percentage of mycorrhizal root infection was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% Trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). Quantification of the root colonization was performed according to the grid-line intersect method (Giovannetti and Mosse, 1980). Five replicates per treatment were used.

Statistical analyses

The 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) was used to determine

Table 1
Shoot and root dry weight (mg) of control non-inoculated plants (C), plants inoculated with single bacteria, single AM fungi [*autochthonous consortium* (AM) or *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions.

	C	*B. megaterium*	*B. thuringiensis*	*Ps. putida*
Shoot dry weight (mg)				
C	104a	116ab	140b	107ab
AM	209c	082a	227c	296d
Ri	264d	306d	123b	289d
Root dry weight (mg)				
C	220b	160a	250b	980f
AM	340c	240b	260b	640d
Ri	300c	270bc	270bc	680d

Within each plant organ values having a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

the effect of each treatment. The Duncan's (Duncan, 1955) multiple-range test was used for post hoc analysis to determine differences between means. Differences were considered significant at $p \leq 0.05$.

Results

The allochthonous bacteria used were less tolerant to the osmotic stress applied, particularly *Bm*. At the highest (40%) PEG concentrations, they were more sensitive to surviving in this medium than the autochthonous *Bt* (Fig. 1). This *Bt*, autochthonous isolate, showed a greater number of viable cells (cfu) under the highest osmotic stress conditions than the reference strains used after 4, 5, 6 and 7 days of growth.

Shoot and root weights were increased by the microbial inoculants except the shoots of plants treated with the dual *Bm* and autochthonous fungi and the roots of single *Bm* inoculated plants (Table 1). In parallel, nutrition and physiological values in *Trifolium repens* plants were increased by most of the microbial treatments applied under the drought conditions (50% water holding capacity). Plants enhanced macro and micronutrient contents with most of the microbial inocula applied (Table 2). Particular effectiveness on nutrients uptake showed plants inoculated with the autochthonous consortium of AM fungi and the autochthonous bacteria *Bt* or *Psp*. Dually inoculated plants with autochthonous microorganisms increased the P content by 89%, K by 217%, Ca by 131%, and Mg by 79%. Similarly, the micronutrients (Zn) were enhanced by 62% (Zn), while B increased by 131 (B). The P, Ca, Mg and Zn contents were significantly depressed in *Bm* + AM inoculated plants. The single inoculation with allochthonous *Ri* also was very effective in increasing all of these nutrients (Tables 2 and 3), and physiological values such as relative water content (RWC) (Fig. 2A). It was also effective to decrease electrolyte leakage (Fig. 2C). As Fig. 1A shows, the RWC increased in most of the inoculated plants under these drought stress conditions. However the RWC enhancement was particularly pronounced in plants coinoculated with autochthonous microorganisms (*Bt* + AM) or *Psp* associated at whatever mycorrhizal inocula here used (Fig. 2A).

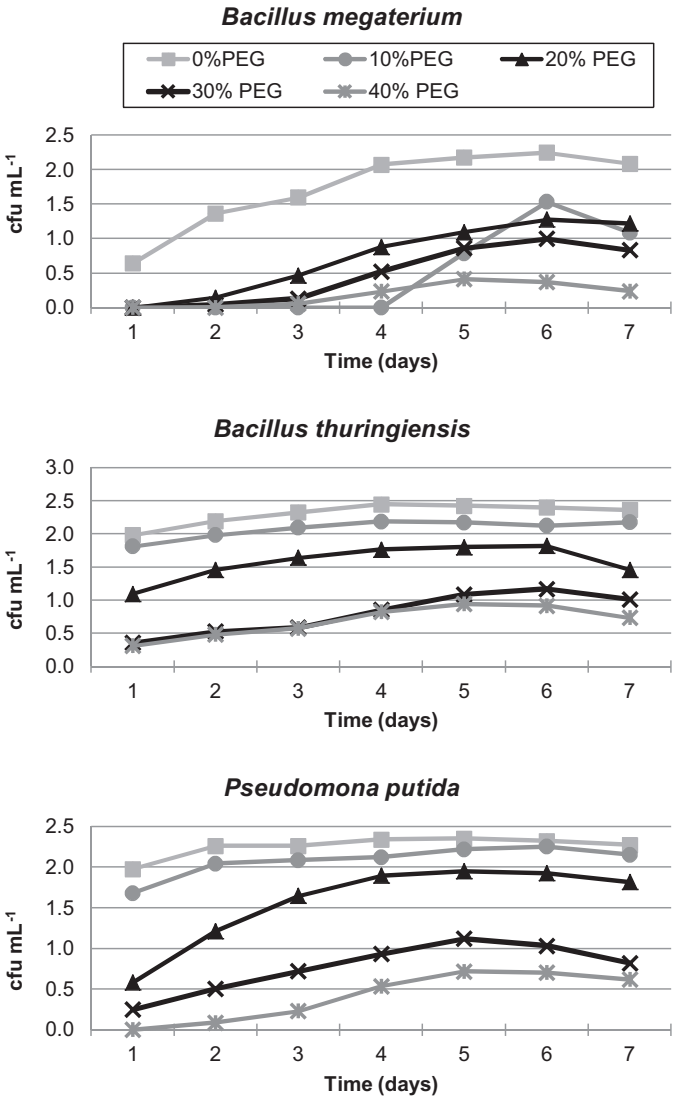

Fig. 1. Viable cells (cfu mL^{-1}) of bacterial strains growing in nutrient medium supplemented with increasing levels of polyethylene glycol (PEG) at different time intervals.

The interactions of autochthonous microorganisms AMF and *Bt* increased RWC at 80%, was twice that of the uninoculated control at 40%. In parallel, microbial treatments in which *Bt* and *Psp* were involved highly decreased the stomatal conductance (Fig. 2B) and concomitantly electrolyte leakage (Fig. 2C). The decrease of stomatal conductance and electrolyte leakage were more pronounced in *Bt* inoculated plants (Fig. 2B and C). This decrease seems to be related to a high accumulation of proline in shoot and root of the plants (Fig. 3). In contrast, non-inoculated control plants showed

Table 2
Nutrients (P, K, Ca and Mg) content (mg $plant^{-1}$) in non-inoculated control plants (C), plants inoculated with single bacteria [*Bacillus megaterium*, *Bacillus thuringiensis* or *Pseudomonas putida*], single AM fungi [autochthonous consortium (AM) or *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions.

Bacterial treatments	P			K			Ca			Mg		
	C	AM	*Ri*	C	AM	*Ri*	C	AM	*Ri*	C	AM	*Ri*
C	1.9b	2.7c	3.7d	23a	60c	82d	26b	37c	55d	5.2b	7.7c	9.8d
B. megaterium	2.5c	1.4a	3.1cd	27b	19a	80d	29b	17a	60d	5.8b	3.3a	10.0d
B. thuringiensis	2.5c	3.6d	2.8cd	28b	73cd	26b	37c	60d	37c	5.8bc	9.3d	7.4c
Ps. putida	2.6c	3.5d	2.9cd	21a	81d	73cd	29b	58d	54d	6.6bc	11.0d	10.0d

Values having a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

Table 3
Elements (Zn and B) content (μg plant^{-1}) in non-inoculated control plants (C), plants inoculated with single bacteria [*Bacillus megaterium*, *Bacillus thuringiensis*, *Pseudomonas putida*], single AM fungi [autochthonous consortium (AM), *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions.

Bacterial treatments	Zn			B		
	C	AM	*Ri*	C	AM	*Ri*
C	580b	690bc	850cd	260a	480c	610d
B. megaterium	600b	290a	750c	310b	210a	620d
B. thuringiensis	760c	940d	790c	340b	600d	380b
Ps. putida	720c	880cd	490c	300b	640d	550cd

Values having a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

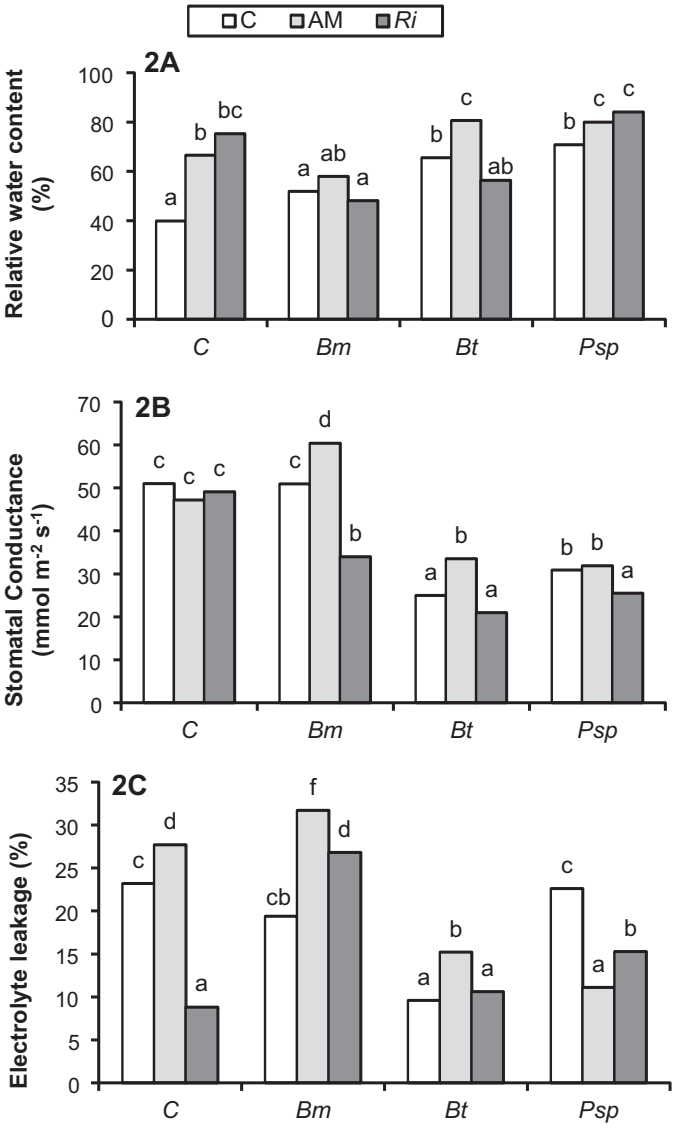

Fig. 2. Relative water content (A), stomatal conductance (B) and electrolyte leakage (C) in shoot of non-inoculated control plants (C), plants inoculated with single bacteria [*Bacillus megaterium* (*Bm*), *Bacillus thuringiensis* (*Bt*) or *Pseudomonas putida* (*Psp*)], single AM fungi [autochthonous consortium (AM) or *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions. Within each graph, values having a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

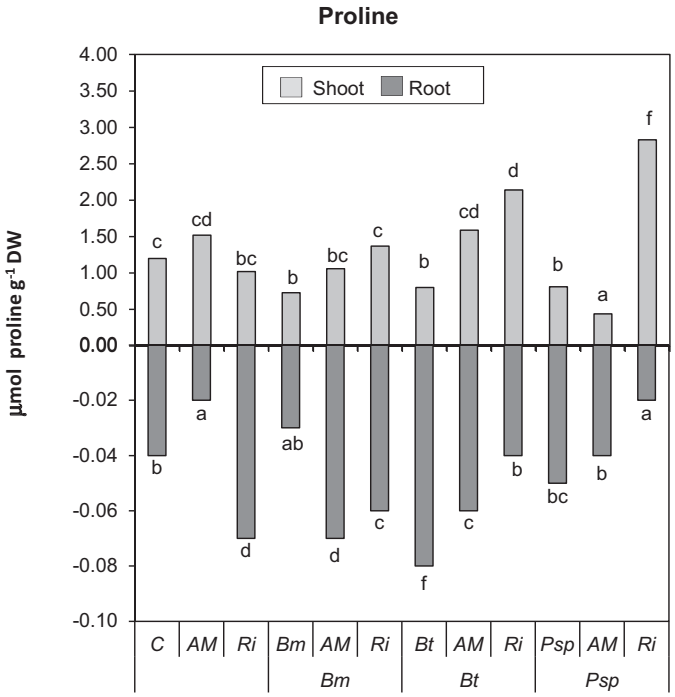

Fig. 3. Proline accumulation in shoot and root in non-inoculated control plants (C), inoculated plants with single bacteria [*Bacillus megaterium* (*Bm*), *Bacillus thuringiensis* (*Bt*) or *Pseudomonas putida* (*Psp*)], single AM fungi [autochthonous consortium (AM) or *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions. Within each graph, values having a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

lower RWC and low proline content values but high stomatal conductance and electrolyte leakage (Figs. 2A–C and Fig. 3).

Proline is accumulated in cells under drought stress conditions. It may act as osmolyte and stabilizing protein. In this study, the greatest proline accumulation in shoots was found in plants dually inoculated with *Ri* and *Psp* (Fig. 3). By contrast, in roots, the lowest proline accumulation values were observed under these treatments. Nevertheless, the amount of proline accumulated in the shoot was considerably higher than in roots (Fig. 3).

With respect to antioxidant activities (APX, GR and SOD) directly involved in the adaptive response for plant survival under drought, important changes were observed in shoot and root tissues according to the microbial inoculants applied (Figs. 4–6). These enzymatic values show the potential ability of plants to counteract the oxidative damage caused by drought. APX activity was decreased, particularly in roots of *Bt* inoculated plants irrespective of mycorrhizal associations (Fig. 4) while GR activity increased in shoots of these plants (Fig. 5). In contrast, APX and GR activities in non-inoculated control plants showed opposite tendencies (Figs. 4 and 5). Single *Ri* greatly increased GR in shoots and single *Bm* enhanced APX in shoots (Figs. 4 and 5) but dual inoculation of these microorganisms highly depressed both GR and APX activities, mainly in shoots (Figs. 4 and 5).

SOD activity was particularly increased in shoots of controls plants. The autochthonous AM fungi when associated to whatever *Bacillus* strain increased SOD values in shoot and also in root and *Psp* decreased SOD in root irrespective of single or dual inoculation (Fig. 6).

The inoculation of AMF or *Ri* and particularly *Psp* + AM enhanced the symbiotic mycorrhizal development (Fig. 7). Nevertheless, a low mycorrhizal colonization was observed in the non-inoculated plants growing in the natural soil. While *Ri*, the greatest colonizer, did not increase this value when associated with any bacteria, the

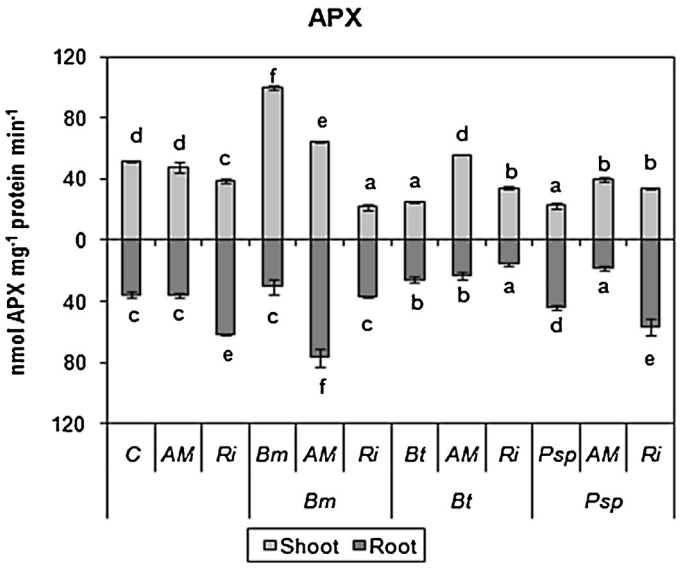

Fig. 4. Ascorbate peroxidase (APX) activity in shoot and root of non-inoculated control plants (C), plants inoculated with single bacteria [*Bacillus megaterium* (*Bm*), *Bacillus thuringiensis* (*Bt*) or *Pseudomonas putida* (*Psp*)], single AM fungi [autochthonous consortium (AM) or *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions. Within each graph, values having a common letter (in shoot or root) are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

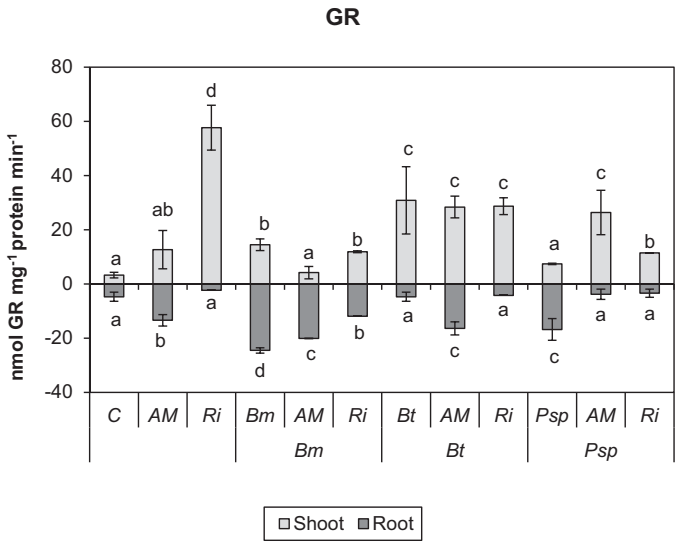

Fig. 5. Glutathione reductase (GR) activity in shoot and root of non-inoculated control plants (C), plants inoculated with single bacteria [*Bacillus megaterium* (*Bm*), *Bacillus thuringiensis* (*Bt*) or *Pseudomonas putida* (*Psp*)], single AM fungi [autochthonous consortium (AM) or *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions. Within each graph, values having a common letter (in shoot or root) are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

autochthonous AM fungi expressed its optimum colonizing potential when associated to *Bm* or *Psp* (Fig. 7).

Discussion

One interesting and new result found in the present study under natural conditions is the intrinsic ability of the microorganisms assayed (autochthonous or allochthonous) to cope with drought and to tolerate stressful conditions. In addition, not only autochthonous, but also allochthonous inoculants proved very

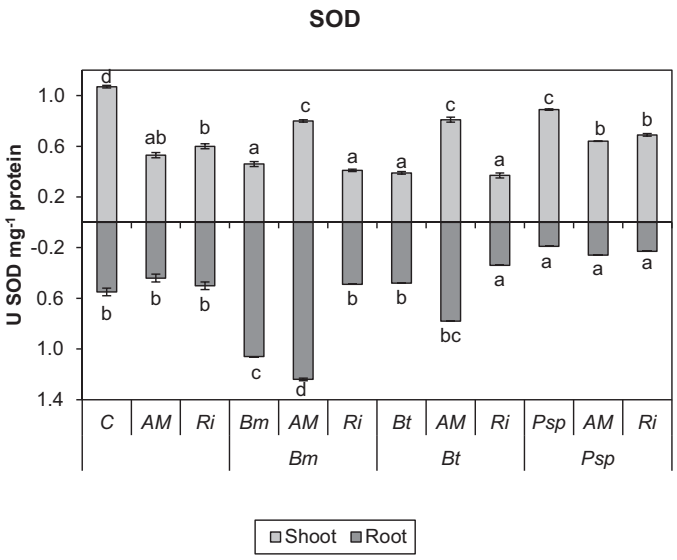

Fig. 6. Superoxide dismutase (SOD) activity in shoot and root in non-inoculated control plants (C), plants inoculated with single bacteria [*Bacillus megaterium* (*Bm*), *Bacillus thuringiensis* (*Bt*) or *Pseudomonas putida* (*Psp*)], single AM fungi [autochthonous consortium (AM) or *Rhizophagus intraradices* (*Ri*)] or dual bacteria/AM fungi combinations in a natural soil under drought conditions. Within each graph, values having a common letter (in shoot or root) are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

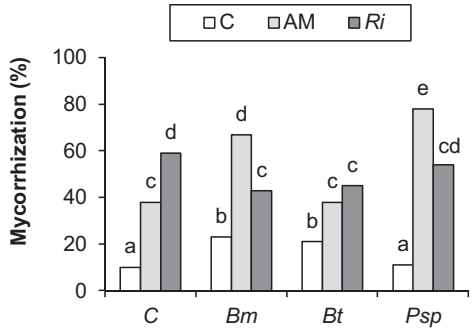

Fig. 7. Percentage of mycorrhization in non-inoculated control plants (C), plants inoculated with single bacteria [*Bacillus megaterium* (*Bm*), *Bacillus thuringiensis* (*Bt*) or *Pseudomonas putida* (*Psp*)], single AM fungi [autochthonous consortium (AM) or *Rhizophagus intraradices* (*Ri*)] or dually bacteria/AM fungi combinations under drought conditions. Values having a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test ($n = 5$).

effective in helping inoculated plants to attenuate the detrimental water stress effects on growth, nutrition and oxidative stress regulating antioxidant activities. The assayed bacteria (autochthonous and allochthonous strains) showed a high level of osmotic stress tolerance (growing at 40% PEG) in the "*in vitro*" experiments. It is evident the greatest tolerance of *Bt* compared with the allochthonous drought tolerant strains. It would be worth further investigating the mechanisms involved in such drought tolerance.

In this study, water content was increased by most of the inocula applied that impaired nutritional, physiological and biochemical mechanisms caused by drought. Microbial inoculants applied evoke various natural processes to help plants to sustain their development under drought.

An important element K (the main inorganic osmolyte) is involved in plant response to drought and alleviation of water stress. The lowest K accumulation in non-inoculated control plants evidenced their poor ability to cope with drought in an efficiently way. Here, plants colonized by specific allochthonous or autochthonous bacteria and the mycorrhizal inocula increased the

plant K content by 217% (*Bt* + AM) or by 348% (*Psp* + AM or *Bm* + *Ri*). This nutrient is able to maintain higher photosynthetic CO_2 fixation and to protect chloroplast from the oxidative damage (Romheld and Kirkby, 2010). K also affects the root water uptake level (Porras-Soriano et al., 2009; Querejeta et al., 2007).

The autochthonous bacteria *Bt* associated with the autochthonous mycorrhizal inoculum increased the macronutrients P by 89%, Ca by 131%, Mg by 79% and micronutrient Zn by 62% respectively and physiological RWC value. Under the water stress, the allochthonous *Ri* proved very effective in increasing macro and micronutrients and decreased stomatal conductance when associated with any bacteria assayed but the dual inoculations of this effective fungus with bacteria did not significantly improve growth and nutrient assimilation over single *Ri* in *Trifolium* plants.

The *Pseudomonas* strain used, a good auxins producer, increased root growth. This is a common reported plant response mediated by bacterial inoculation in several plant species (Lucy et al., 2004). This bacteria highly decreased the shoot/root ratio. A large root surface area is normally related to improvement of nutrients acquisition and water content. Strains of the species *Psp* are acknowledged as one of the most efficient phosphate solubilizing cultivable bacteria (Vyas and Gulati, 2009) and among them, this allochthonous strain here used (*Psp* BIRD-1) resulted particularly efficient. Dual coinoculations of allochthonous *Bm* + *Ri* and *Psp* + *Ri* or *Psp* + AM resulted the most effective in increasing growth and nutrients uptake.

The response of plants to water deficit has been evaluated based on physiological values as the electrolyte leakage and stomatal conductance that in this study were the lowest in plants single inoculated with autochthonous *Bt*. The low electrolyte leakage is an indicator of cell membranes stability and tolerance to water stress (Berglund et al., 2004). Cell membranes are very important in controlling the effectiveness of microbial-root associations (Bashan et al., 2004). Most of the inocula applied reduced the membrane damage in these stressed plants.

The water and nutrients limitation highly damage plant physiology, which leads to the production of reactive oxygen species (ROS) (Zgallai et al., 2005) that may cause cellular damage (Menconi et al., 1995). Thus, it has been used as an indicator of plant stress. To counteract the adverse effects of reactive oxygen species the plants activated various enzymatic antioxidant defenses to control and to repair damage caused by the imbalanced redox status. As this study shows the microbial inoculants are involved in the plant response to drought stress by regulating oxidative reactions and inducing antioxidant defense (Koussevitzky et al., 2008). Proline is able to change plant drought responses by regulating cytosolic pH and NDA/NDAH rate, stabilizing proteins and scavenging hydroxyl radicals protecting cells from the adverse effect of ROS. Therefore, proline is normally used as a suitable index of drought and oxidation stress (Marulanda et al., 2009). The accumulation of proline in shoots and roots of inoculated plants was affected by the inoculants applied. Allochthonous *Ri* markedly increased proline accumulation in shoot when associated to *Bt* or *Psp*, which indicates a greater plant response to the stress, but concomitantly decreased this value in roots tissue. On the contrary, when less proline is accumulated in shoots as in single *Ri* or dual *Bm* + *Ri*, the plant required more proline in root tissue in order to cope with the low water potential of drying soil and to keep a water potential gradient favorable to water entrance into the roots, as previously observed (Porcel and Ruíz-Lozano, 2004).

The total proline accumulation and changes in the shoot/root ratio in inoculated plants could be involved in the drought alleviation. In general, the greater proline accumulation observed in most of the dually inoculated plants led to a decline in cell osmotic potential and, as result, increased water uptake to maintain osmotic balance (Dong et al., 2002). It is an indicator of the enhanced osmoprotective capacity and of the ability to maintain water uptake under drought conditions in inoculated plants. But here, some results did not totally support the involvement of this osmolyte in drought alleviation. In this study, the inoculation reduced the oxidative damage under stress compared to control.

Antioxidant enzymes such as APX, GR and SOD in shoot and root tissues are important components in preventing the oxidative stress in plants. In general, APX and SOD (in roots) increased more in plants treated with the less effective treatment as *Bm* + AM. The high APX level may protect plant cells from free radical oxidation presumably produced in a highest amount in these plants. *Bm* + AM inoculated plants showed the lowest growth and nutrient content but the highest electrolyte leakages and stomatal conductance. In parallel, the dual inoculation of the microorganisms that highly improved plant growth and nutrition decreased this APX activity. Thus, the inoculants applied were able to regulate oxidative reactions and antioxidant defense.

Plants with greater adaptation or tolerance to the drought conditions did not require high protection against the stress since they are less affected by drought. This down regulation of the APX enzymatic activity observed in plants inoculated with the most effective microorganisms is consistent with the greatest proline accumulation and the lowest stomatal conductance determined in these plants.

The balance between these enzymatic activities and their distribution in plant tissues is important for suppressing toxic ROS formed in each plant tissue (Apel and Hirt, 2004).

Under drought conditions, plant requires that antioxidant systems be able to work in a coordinate form to provide the best scavenging systems and plant defence (Blokhina et al., 2003).

The plants dually inoculated (particularly the autochthonous AM fungi and *Bt* or those from collection *Ri* plus *Psp*) have the better water status and would be less damaged by the water stress imposed. Thus, they would need to have lower expression of particular antioxidant activities. In general, the highest levels of some of these activities may be the result of increased cell damage since these activities contributed to the osmotic adjustment mainly required in the non-inoculated plants.

SOD activity is normally accompanied by an increase in the APX activity as H_2O_2 scavenging enzyme. SOD catalyzes the dismutation of superoxide into molecular oxygen (O_2^-) and H_2O_2 that will be subsequently dismuted into H_2O and oxygen by APX (or CAT). The APX activity was more reduced in the plants associated with the most beneficial microbial treatments and this suggests that APX is important in H_2O_2 scavenging and it may be the result of a higher reduction in the oxidative stress (Maheshwari and Dubey, 2009). The overall results indicate that dually inoculated plants (with the exception of *Bm* + AM) were better protected against the drought stress imposed. They suggest changes of the defense against ROS to avoid oxidative damage under drought stress in the plant tissues distribution according to the microbial treatments applied. To maintain the right balance among SOD, APX, and GR activities is crucial to determine the steady state level of ROS (Apel and Hirt, 2004).

However, the coordination between different antioxidant activities for ROS removal network of plants is complex (Mittler et al., 2004) and the cooperation between them may be critical in the stress response. Microbial inoculants applied play an important role in orchestrating antioxidant activities in shoot and roots of associated plants in the process of drought tolerance.

These results indicate the different capacity to efficiently detoxify ROS in the production site according to microbial inoculants applied.

The effectiveness of dual inocula was not related to the colonization level as previously was also noted (Marulanda et al., 2009).

Overall, our results clearly suggest that synergistic or additive mechanisms are involved in the improvement of plant growth, nutrient uptake and adaptation to unfavorable drought soil conditions. Most of the inoculated plants with autochthonous or allochthonous microorganisms maintain an adequate water status under drought through the control of stomatal conductance, nutrition and the improved osmotic adjustment as results of the enhancement of compatible solute as proline and the regulation of antioxidants systems. This can be regarded as an efficient biotechnology to improve plant development in drought environments. But the understanding of the adaptation and functioning of living plant and microorganisms to extreme conditions requires additional studies.

Acknowledgments

This study was supported by "Plan Nacional I+D" Spain (project AGL-2009-12530). We thank Domingo Álvarez for the morphological identification of autochthonous mycorrhizal fungus and the Instrumentation Service (EEZ) for the plant analysis. We thank Shaun Smith (native English teacher) for their assistance in the revision of this work.

References

Alguacil MM, Caravaca F, Azcón R, Pera J, Díaz G, Roldán A. Improvements in soil quality and performance of mycorrhizal *Cistus albidus* L. seedlings resulting from addition of microbially treated sugar beet residue to a degraded semiarid Mediterranean soil. Soil Use Manage 2003a;19:277–83.

Alguacil MM, Hernández JA, Caravaca F, Portillo B, Roldán A. Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil. Physiol Plant 2003b;118:562–70.

Allen EB, Allen MF. Water relations of xeric grasses in the field: interactions of mycorrhizas and competition. New Phytol 1986;104:559–71.

Amako K, Chen GX, Asada K. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol 1994;35:497–504.

Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 2004;55:373–99.

Aroca R, Irigoyen JJ, Sánchez-Díaz M. Drought enhances maize chilling tolerance. II. Photosynthetic traits and protective mechanisms against oxidative stress. Physiol Plant 2003;117:540–9.

Azcón R. Growth and nutrition of nodulated mycorrhizal and non-mycorrhizal *Hedysarum coronarium* as a results of treatments with fractions from a plant growth-promoting rhizobacteria. Soil Biol Biochem 1993;25:1037–42.

Azcón R, Medina A, Roldán A, Biró B, Vivas A. Significance of treated agrowaste residue and autochthonous inoculates (arbuscular mycorrhizal fungi and *Bacillus cereus*) on bacterial community structure and phytoextraction to remediate soils contaminated with heavy metals. Chemosphere 2009;75:327–34.

Azcón R, Perálvarez MC, Roldán A, Barea JM. Arbuscular mycorrhizal fungi, *Bacillus cereus*, and *Candida parapsilosis* from a multicontaminated soil alleviate metal toxicity in plants. Microb Ecol 2010;59:668–77.

Barea JM, Azcón R, Azcón-Aguilar C. Mycorrhizal fungi and plant growth promoting rhizobacteria. In: Varma A, Abbott LK, Werner D, Hampp R, editors. Plant surface microbiology. Heidelberg, Germany: Springer-Verlag; 2004. p. 351–71.

Barea JM, Pozo MJ, Azcón R, Azcón-Aguilar C. Microbial co-operation in the rhizosphere. J Exp Bot 2005;56:1761–78.

Bashan Y, Holguin G, de-Bashan LE. Azospirillum–plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). Can J Microbiol 2004;50:521–77.

Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil 1973;39:205–7.

Benabdellah K, Abbas Y, Abourouh M, Aroca R, Azcon R. Influence of two bacterial isolates from degraded and non-degraded soils and arbuscular mycorrhizae fungi isolated from semi-arid zone on the growth of *Trifolium repens* under drought conditions: mechanisms related to bacterial effectiveness. Eur J Soil Biol 2011;47:303–9.

Berglund AH, Larsson KE, Liljenberg CS. Permeability behaviour of lipid vesicles prepared from plant plasma membranes – impact of compositional changes. Biochim Biophy Acta Mol Cell Biol Lipids 2004;1682:11–7.

Beyer WF, Fridovich I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 1987;161:559–66.

Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 2003;91:179–94.

Bohnert HJ, Gong QQ, Li PH, Ma SS. Unraveling abiotic stress tolerance mechanisms – getting genomics going. Curr Opin Plant Biol 2006;9:180–8.

Bradford MM. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 1976;72:248–54.

Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol 1985;113:484–9.

Dimkpa CO, Merten D, Svatos A, Buechel G, Kothe E. Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. Soil Biol Biochem 2009;41:154–62.

Dong DF, Liu TN, Yuan JZ, Xie AH, Tu TU. Artificial neural networks method for oil systems identification and its applications. In: Proceedings of the 4th World Congress on Intelligent Control and Automation; 2002. p. 2952–5.

Duncan DB. Multiple range and multiple *F* tests. Biometrics 1955;11:1–42.

Evelin H, Kapoor R, Giri B. Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. Ann Bot 2009;104:1263–80.

Giovannetti M, Mosse B. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 1980;84:489–500.

Hamayun M, Khan SA, Shinwari ZK, Khan AL, Ahmad N, Lee I-J. Effect of polyethylene glycol induced drought stress on physio-hormonal attributes of soybean. Pak J Bot 2010;42:977–86.

Hoque MA, Banu MNA, Okuma E, Murata Y. Exogenous proline and glycinebetaine increase NaCl-induced a scorbate-glutathione cycle enzyme activities, and proline improves salt tolerance more than glycinebetaine in tobacco bright yellow-2 suspension-cultured cells. J Plant Physiol 2007a;164:1457–68.

Hoque MA, Okuma E, Banu MNA, Nakamura Y, Shimoishi Y, Murata Y. Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. J Plant Physiol 2007b;164:553–61.

Kasim WA, Osman ME, Omar MN, Abd El-Daim IA, Bejai S, Meijer J. Control of drought stress in wheat using plant-growth-promoting bacteria. J Plant Growth Regul 2013;32:122–30.

Kohler J, Hernández JA, Caravaca F, Roldán A. Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. Environ Exp Bot 2009;65:245–52.

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, et al. Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. J Biol Chem 2008;283:34197–203.

Liddycoat SM, Greenberg BM, Wolyn DJ. The effect of plant growth-promoting rhizobacteria on asparagus seedlings and germinating seeds subjected to water stress under greenhouse conditions. Can J Microbiol 2009;55:388–94.

Loreto F, Centritto M. Leaf carbon assimilation in a water-limited world. Plant Biosyst 2008;142:154–61.

Lucy M, Reed E, Glick BR. Applications of free living plant growth-promoting rhizobacteria. Antonie Van Leeuwenhoek 2004;86:1–25.

Maheshwari R, Dubey R. Nickel-induced oxidative stress and the role of antioxidant defence in rice seedlings. Plant Growth Regul 2009;59:37–49.

Maillet F, Poinsot V, Andre O, Puech-Pages V, Haouy A, Gueunier M, et al. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 2011;469:58–64.

Marschner H. Mineral nutrition of higher plants. 2nd edn. London, UK: Academic Press; 1995.

Marulanda-Aguirre A, Azcón R, Ruíz-Lozano JM, Aroca R. 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 2008;27:10–8.

Marulanda A, Barea JM, Azcón R. An indigenous drought-tolerant strain of *Glomus intraradices* associated with a native bacterium improves water transport and root development in *Retama sphaerocarpa*. Microb Ecol 2006;52:670–8.

Marulanda A, Barea JM, Azcón R. Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments. Mechanisms related to bacterial effectiveness. J Plant Growth Regul 2009;28:115–24.

Marulanda A, Porcel R, Barea JM, Azcón R. Drought tolerance and antioxidant activities in lavender plants colonized by native drought-tolerant or drought-sensitive *Glomus* species. Microb Ecol 2007;54:543–52.

Matilla MA, Pizarro-Tobías P, Roca A, Fernández M, Duque E, Molina L, et al. Complete genome of the plant growth-promoting rhizobacterium *Pseudomonas putida* BIRD-1. J Bacteriol 2011;193:1290–390.

Medina A, Azcón R. Effectiveness of the application of arbuscular mycorrhiza fungi and organic amendments to improve soil quality and plant performance under stress conditions. J Soil Sci Plant Nutr 2010;10:354–72.

Medina A, Azcón R. Reclamation strategies of semiarid mediterranean soil: improvement of the efficiency of arbuscular mycorrhizal fungi by inoculation of plant growth promoting microorganisms and organic amendments. In: Hafidi M, Duponnois R, editors. The mycorrhizal symbiosis in mediterranean environment: importance in ecosystem stability and in soil rehabilitation strategies. New York: Nova Science Publishers; 2012. p. 87–106.

Menconi M, Sgherri CLM, Pinzino C, Navariizzo F. Activated oxygen production and detoxification in wheat plants subjected to a water-deficit program. J Exp Bot 1995;46:1123–30.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. Reactive oxygen gene network of plants. Trends Plant Sci 2004;9:490–8.

Olsen SR, Dean LA. Phosphorus. In: Black CA, Evans DD, White JL, Ensminger LE, Clark FE, Dinauer RC, editors. Methods of soil chemical analysis. Madison, WI: American Society of Agronomy; 1965. p. 1035–49.

Phillips JM, Hayman DS. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 1970;55:159–61.

Porcel R, Barea JM, Ruíz-Lozano JM. Antioxidant activities in mycorrhizal soybean plants under drought stress and their possible relationship to the process of nodule senescence. New Phytol 2003;157:135–43.

Porcel R, Ruíz-Lozano JM. Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. J Exp Bot 2004;55:1743–50.

Porras-Soriano A, Soriano-Martin ML, Porras-Piedra A, Azcón R. Arbuscular mycorrhizal fungi increased growth, nutrient uptake and tolerance to salinity in olive trees under nursery conditions. J Plant Physiol 2009;166:1350–9.

Querejeta JI, Allen MF, Alguacil MM, Roldán A. Plant isotopic composition provides insight into mechanisms underlying growth stimulation by AM fungi in a semiarid environment. Funct Plant Biol 2007;34:683–91.

Ramoliya PJ, Patel HM, Pandey AN. Effect of salinization of soil on growth and macro- and micro-nutrient accumulation in seedlings of *Salvadora persica* (Salvadoraceae). For Ecol Manage 2004;202:181–93.

Romheld V, Kirkby EA. Research on potassium in agriculture: needs and prospects. Plant Soil 2010;335:155–80.

Roth CH, Malicki MA, Plagge R. Empirical evaluation of the relationship between soil dielectric constant and volumetric water content as the basis for calibrating soil moisture measurements. J Soil Sci 1992;43:1–13.

Ruíz-Lozano JM, Porcel R, Aroca R. Evaluation of the possible participation of drought-induced genes in the enhanced tolerance of arbuscular mycorrhizal plants to water deficit. In: Varma A, editor. Mycorrhiza: state of the art, genetics and molecular biology, eco-function, biotechnology, eco-physiology, structure and systematics. Berlin, Heidelberg, Germany: Springer-Verlag; 2008. p. 185–207.

Ruíz-Lozano JM, Porcel R, Azcón C, Aroca R. Regulation by arbuscular mycorrhizae of the integrated physiological response to salinity in plants: new challenges in physiological and molecular studies. J Exp Bot 2012;63:4033–44.

Turkan I, Bor M, Ozdemir F, Koca H. Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Sci 2005;168:223–31.

Vyas P, Gulati A. Organic acid production *in vitro* and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. BMC Microbiol 2009;9:174.

White I, Knight JH, Zegelin SJ, Topp GC. Comments to 'considerations on the use of time-domain reflectometry (TDR) for masuring soil water content' by WR Whalley. J Soil Sci 1994;45:503–8.

Wilde P, Manal A, Stodden M, Sieverding E, Hildebrandt U, Bothe H. Biodiversity of arbuscular mycorrhizal fungi in roots and soils of two salt marshes. Environ Microbiol 2009;11:1548–61.

Zgallai H, Steppe K, Lemeur R. Photosynthetic, physiological and biochemical responses of tomato plants to polyethylene glycol-induced water deficit. J Integr Plant Biol 2005;47:1470–8.