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Characterization and management of autochthonous bacterial strains from semiarid soils of Spain and their interactions with fermented agrowastes to improve drought tolerance in native shrub species

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ABSTRACT

Three bacterial autochthonous strains, namely *Enterobacter* sp.; *Bacillus thuringiensis* and *Bacillus* sp. were isolated from the rhizosphere of Mediterranean shrub species growing in a semiarid environment and analyzed alongside with the allochthonous *Bacillus megaterium*, used as reference drought tolerant strain, for their drought tolerance and plant growth promoting rhizobacteria (PGPR) capacities. The preliminary studies, done in axenic culture under non-stress and stress conditions show that *Enterobacter* sp. resulted in the most tolerant bacteria to osmotic stress factors. In contrast, *Bacillus* sp. was the most sensitive bacteria to osmotic stress factors and concomitantly, under these conditions, produced the highest amounts of ACC deaminase, poly-β-hydroxybutyrate and proline, to compensate its lack of stress tolerance. The PGPR activities of the tested bacterial strains under non-osmotic and osmotic stress conditions were determined by evaluating hormone (SA, ABA, JA and IAA) and ACC-deaminase production and phosphate solubilization. To analyze the bacterial efficiency as inoculants four shrubs species (*Thymus vulgaris, Santolina chamaecyparissus, Lavandula dentata* and *Salvia officinalis*), adapted to aridity, were selected. All the tested bacteria improved nutrition and physiological variables related to drought tolerance of the test plant. In addition, in *S. chamaecyparissus* and *S. officinalis* also increased mycorrhizal colonization. The application of fermented agrowaste resulted in effectively improving nutrient uptake and also interacted positively with most of the bacteria increasing plant nutrients content and drought tolerance but their effectiveness depended on the plant species and bacteria involved. In fact, in *B. megaterium* and the fermented agrowaste increased P and K uptake in *S. chamaecyparissus* (by 109% P and by 66% K), in *L. dentata* (by 75% P and 33% K) and in *S. officinalis* (by 63% P and 52% K). However, without amendment, the native *B. thuringiensis* was the most efficient strain in increasing P content in *T. vulgaris* (by 51%) and in *S. chamaecyparissus* (by 11%), and K content in *L. dentata* (by 63%), which decreased the stomatal conductance. Results show that under axenic conditions the stress applied did not suppress the PGPR abilities of assayed bacteria which indicated their potential to be tested as inoculants under detrimental conditions. The applied treatments resulted fundamental for these shrubs to reach their optimal nutritional and physiological traits suggesting their possible applicability under the natural semiarid drought conditions. The multiplicity and complexity of bacterial activities and the intrinsic characteristics of plant reactions to drought could explain the unpredictable results obtained by using these bacteria as plant inoculants. These and other factors are controlling the PGPR effects therefore it made difficult to generalize and to explain the cause/effect of the variable responses to be obtained. The results suggest the potentiality of the target bacteria and fermented agrowaste to be used as a biotechnological tool to help plants and reforestation in semiarid lands.

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1. Introduction

The re-establishment of a plant cover based on autochthonous plant species, adapted to the local environmental conditions, constitutes the most effective strategy for reclaiming degraded areas in semiarid Mediterranean environments (Vallejo et al., 1999). The success of re-vegetation programs in semiarid areas is based on the use of technologies which benefit plant establishment and improve plant drought tolerance. As plants depend on their natural protection systems, including the help from microbial activities involved in stress adaptation, managing of plant associated microbial communities may be one strategy for attenuating the negative effect of detrimental factors such as drought (Azcón et al., 2013; Dimkpa et al., 2009; Pozo et al., 2015).

Plant growth promoting rhizobacteria (PGPR) play important roles in aiding to solve environmental problems and thus can help plant establishment and growth by several direct and indirect mechanisms (Kasim et al., 2013), this leads to increase in tolerance of plants to stress situations as those caused by water shortage (Naveed et al., 2014). In fact, PGPR have been shown to affect the water balance of both well-watered and stressed plants (Kohler et al., 2008). Indeed, physiological variables such as stomatal conductance, transpiration rate and leaf water potential are generally affected by bacterial inoculation under water limited conditions (Benabdellah et al., 2011). Environmental stress factors affecting semiarid ecosystem decreased the diversity and density of microbial populations but microbial propagules do not completely disappear which is an indication of stress adaptation (Azcón et al., 2013; Barea et al., 2011). Drought adapted and tolerant microbial ecotypes are the best candidates to be used as inoculants in reforestation programs under semiarid, water limited conditions (Alguacil et al., 2003; Caravaca et al., 2002).

The application of PGPR for the ecological restoration under natural soil conditions has been little explored. In this respect, the application of organic amendments to the soil, prior to the inoculation of beneficial microorganisms, as PGPR, might be recommended. As previously reported organic amendments are able to increase soil microbiota activity, particularly in degraded soils under semiarid conditions (Medina et al., 2004). The beneficial effects of organic amendments include provision of plant nutrients, increased humus content and thereby increased water-holding capacity, improved soil structure, and increased microbial activity (Caravaca et al., 2002). The extractions of sugar from the sugar beet produced agrowastes, but these products can only be used as organic amendment after biological transformation processes. In this context, the application of fermented agrowaste with microbiologically-solubilized rock-phosphate has been assayed for improving plant performance under stress conditions (Medina et al., 2004). Fermented agrowaste can be used as energy sources for heterotrophic microorganisms such as PGPR as suggested by Bashan and Holguin (1998).

Accordingly, this investigation aims for the isolation, identification and characterization of autochthonous bacteria from semiarid soil (Murcia, province of Spain) for their drought tolerant capacity and to assess their potential to act as PGPR on autochthonous shrubs. The use of drought-tolerant shrubs in semi-arid regions is one of the ways to conserve soil. As previously indicated PGPR can promote plant growth through different mechanisms in which biostimulation and/or biofertilization are involved (Azcón et al., 2013). As biofertilizer, PGPR increase the uptake of nutrients (P from phosphate solubilization and N from N_2-fixation) and as biostimulator by the production and/or modulation of phytohormones (indole acetic acid (IAA), abcisic acid (ABA), salicilic acid (SA), jasmonic acid (JA) and others) affecting plant physiology, root architecture and plant resistance to stress factors (Zahir et al., 2004). The bacterial production of hormones-like compounds has been shown to play an important role in ameliorating effects of drought and other stress factors (Glick, 2012; Groppa et al., 2012).

To evaluate PGPR abilities and the drought resistance capacity of these autochthonous bacterial isolates we determined variables related with plant biostimulation and also with cellular drought tolerance as production of proline, poly-ß-hydroxybutyrate (PHB), antioxidant ascorbate peroxidase (APX) and catalase (CAT) enzymes, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The potential growth of bacterial cells under non-stress and drought stress conditions was also assessed. Drought is connected with the accumulation of ROS causing severe cell oxidative damage. Thus, a decrease in the oxidative stress in cells suggests lower stress symptoms and resulted important for cells survival under drought. Antioxidant enzymes superoxide dismutase (SODs), CATs and APXs are widely distributed in aerobic bacteria but there are few studies in relation to the ability of bacteria to resist drought stress. Proline could contribute to the scavenging of free radicals produced by stress conditions in addition to its main role as an osmoprotectant under water-deficit (Azcón et al., 2010). The PHB is produced by bacteria when they are subjected to stress as a mechanism that favors their establishment and survival (Okon and Itzigsohn, 1992). In this context, Ayub et al. (2004) suggested the relationship between PHB accumulation and high stress resistance. Bacteria are able of ACC deaminase production, the immediate precursor of ethylene in higher plants, and its regulation has been described as the major mechanism by which bacteria exert beneficial effects on plants under abiotic stress conditions (Saleem et al., 2007). Particularly, we hypothesized that drought resistance capacity of bacteria can be ascribed, at least partially, to the proline and antioxidative enzyme metabolism. Thus, we expected that the level of drought adaptation capacity of bacteria ought to be related with the oxidative stress attenuation.

Considering these premises, we postulate that the inoculation of autochthonous shrub plants (*Thymus vulgaris*, *Santolina chamaecyparissus, Lavandula dentata* and *Salvia officinalis*) with autochthonous drought resistant bacteria, having PGPR traits, can confer drought tolerance to these plants improving nutrition and altering physiological parameters. PGPR abilities and related processes are regulated in general by activities which confer resistance and intrinsic stress tolerance of both bacteria and plants. Accordingly, the objective of the present study was to isolate and characterize drought tolerant autochthonous bacterial strains, afterthought analyze their effects, in comparison with a reference strain (also drought tolerant) from our culture collection, on growth, nutrition and drought tolerance markers of four autochthonous shrubs and their modulation by the application of a fermented agrowastes (compost). Additionally, autochthonous bacteria can positively interact with native arbuscular mycorrhizal (AM) fungi, existing in the natural soil, thus AM development was also evaluated since such microbial interactions may affect plant drought tolerance. Some bacteria have been named mycorrhiza helper bacteria for their ability to promote mycelia growth and mycorrhiza formation (Frey-Klett et al., 2007).

2. Material and methods

Section 2.1 consists in the isolation of autochthonous bacteria from the rhizosphere of autochthonous shrubs (four) from the semiarid environment. The soil used was located in the Natural Ecological Park “Vicente Blanes” in the Province of Murcia (Southeast Spain). This area suffers drought and low nutrient availability and desertification processes. The soil in the experimental area is a Typic Torriorthent (SSS, 2006) very little developed with a silty–clay texture that facilitates the degradation of soil structure, and low organic matter content. The vegetation in

the zone was predominated by *T. vulgaris*, *S. chamaecyparissus*, *L. dentata* and *S. officinalis* growing with a patchy distribution. The climate in this semiarid Mediterranean zone is a mean annual temperature of 20 °C and rainfall of 250 mm. The main soil characteristics are: organic carbon 0.94%, total N 0.22%, P 1.36 mg kg^{-1} (Olsen test), pH 8.9 and an electric conductivity of 1.55 dS m^{-1}.

Supposedly, isolated bacteria were adapted to drought and only were selected the most abundant and representative bacteria. Later they were identified by molecular techniques. We tested whether these autochthonous isolates actually were drought tolerant bacteria along with a drought-tolerant *Bacillus megaterium* strain (Accession CECRIbio 04 similarity 98%) from a culture collection selected in semiarid zone in previous experiments (Marulanda et al., 2006, 2009). The autochthonous bacterial abilities to cope with drought and their functional traits under osmotic stress conditions were analyzed in the experiment. In a subsequent bioassay (Section 2.2), we evaluated the effect of these selected bacteria on the four most representative autochthonous shrub species growing in a soil under drought conditions. The treatments used in Section 2.2 were: three autochthonous bacteria and one from collection were inoculated in presence or absence of fermented agrowaste in each one of selected shrub. Plants without fermented agrowaste or bacteria were also assayed as controls. Each treatment was replicated five times a total of 50 pots per plant. The experiment consisted of a factorial block design (5×2) for each plant with five inoculations each with and without fermented agrowaste (total 10 treatments).

2.1. Experiment I

2.1.1. Isolation of autochthonous bacteria from a semiarid environment

The soil samples (three repetitions) for bacterial isolation were taken from the rhizosphere of (four) shrubs naturally growing in a Mediterranean semiarid soil from Murcia province (Spain). This soil was used as test soil for the greenhouse inoculation experiment (Section 2.2).

Bacterial isolation was carried out following a conventional procedure: 1 g of homogenized rhizosphere soil was suspended in 9 mL of sterile water, to perform dilutions (10^{-2}–10^{-4}), which were spread on yeast mannitol agar (YMA), potato dextrose agar (PDA), Luria–Bertani (LB) agar and incubated at 28 °C for 48 h, to isolate bacteria. The most representative (abundant) colonies of different morphological appearances (the three most abundant cultivable types) were selected. Morphology and mobility of bacteria were examined by microscopy. In addition, *B. megaterium* from our collection was assayed as reference strain. It was previously selected as PGPR and drought tolerant strain from a similar semiarid soil. These three representative bacterial strains and *B. megaterium* were grown individually in 250-mL flasks containing 50 mL of nutrients broth medium in shake culture for 48 h at 28 °C for inocula preparation.

2.1.2. Molecular identification of the bacterial strains

Identification of isolated bacteria was done by sequencing the 16S rDNA gene. Bacterial cells were collected, diluted, lysed and their DNA used as a template in the PCR reactions. All reactions were conducted in 25 μL volume containing PCR buffer 10×, 50 mM $MgCl_2$, 10 μM each primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (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. PCR products were analyzed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (PerkinElmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program (Altschul et al., 1990). We used others methods as the analysis of proteins "MALDI biotyper" (Bruker Daltonik) to confirm the bacterial identifications.

2.1.3. Bacterial growth under increasing polyethylene glycol (PEG) levels in the growing medium

Autochthonous bacterial isolates and the *B. megaterium* used as a reference strain were grown at 28 °C in an axenic medium (nutrient broth, 8 g L^{-1}) supplemented or not with increasing PEG concentrations (0%, 15%, 30% and 40%) to generate osmotic stress (equivalent to −1.02; −1.50; −3.60 and −3.99 MPa). This allows to test bacterial osmotic stress tolerance along the time, by estimating the number of viable cells, as centrifuged per milliliter. Number of viable cells was estimated after 4 and 6 days of growth following a conventional procedure: 1 mL of suspension was plated in agar nutrient broth medium. The bacterial growth was monitored by measuring optical density at 600 nm. The four PEG treatments were replicated 3 times in the culture of each bacterial strain giving a total of 48 tubes.

2.1.4. Plant growth promoting bacterial activities growing without stress and with stress caused by application 40% polyethylene glycol (PEG) in the growing medium

The four bacterial isolates were cultivated (three replicates) at 28 °C in 100 mL of liquid nutrient medium for 48 h on a rotary shaker at 120 rpm supplemented or not with 40% of PEG (−3.99 MPa) in order to induce drought stress conditions. This level of PEG was selected in preliminary studies as the maximum PEG concentration supportable by bacterial strains.

The accumulation of proline was estimated by spectrophotometric analysis at 530 nm (Bates et al., 1973). The bacterial extracts react 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, shaking vigorously for 20 s. A standard curve was prepared with known concentrations of proline.

Measurement of lipid peroxidation was done by the method based on the reaction of thiobarbituric acid (TBA) with reactive species derived from lipid peroxidation, particularly malondialdehyde (MDA). Detection of thiobarbituric acid reactive species (TBARS) was carried out by a colorimetric assay described by Buege and Aust (1978) with some modifications (Espindola et al., 2003). 50 mg of cells were resuspended in 500 μL of 50 mM phosphate buffer (pH 6.0) containing 10% trichloroacetic acid (TCA), and 0.3 g glass beads were added. The samples were broken by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. After centrifugation, supernatants were mixed with 0.1 mL of 0.1 M EDTA and 0.6 mL of 1% (w/v) TBA in 0.05 M NaOH. The reaction mixture was incubated at 100 °C for 15 min and then cooled on ice for 5 min. The absorbance was measured at 532 nm. Lipid peroxidation was expressed as μmoles of malondialdehyde per gram of dry cell weight.

The method for the extraction of antioxidant enzymes in the microbial cells was described by Azcón et al. (2010). Bacterial cells were homogenized in a cold mortar with 4 mL 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 8 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 6000 rpm for 15 min at 4 °C, and the supernatant was used for enzyme activity determination. Catalase (CAT) activity was measured as described by Aebi (1984), conducted in 2 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 μL of enzyme extract. It was determined the consumption of H_2O_2 and followed by decrease in absorbance at 240 nm for 1 min

(extinction coefficient (ε_{240}) of 39.6 $mM^{-1}\,cm^{-1}$). Ascorbate peroxidase (APX) activity was measured in a 1 mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 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). Total soluble protein amount was determined using Bradford method (1976), and bovine serum albumin as standard.

The poly-ß-hydroxybutyrate (PHB) production of the four bacterial strains on different osmotic concentrations (0% and 40% PEG) in N_2 deficient medium (pH 7) and incubated at 28 °C for 72 h at 120 rpm was measured. PHB produced were extracted as described in the method of Ramsay et al. (1994). The amount of PHB in the extracts was determined spectrophotometrically at 235 nm (Law and Slepecky, 1961; Lee et al., 1995). A standard curve was prepared to determine PHB in mg mL^{-1}.

The production of indole-3-acetic acid (IAA) by these bacteria was determined 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 room temperature, and the optical density at 535 nm was measured in each treatment (Wöhler, 1997). A standard curve was also prepared for IAA determination.

The activity of ACC deaminase enzyme in isolates was measured as described by Penrose and Glick (2003). The enzyme activity was assayed according to a modification of the method of Honma and Shimomura (1978) which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase hydrolyses ACC. The quantity of μmol of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 1.0 mmol and 1.0 μmol. Protein concentration of cellular suspension in the toluenized cells was determined by the method of Bradford (1976).

To determine phosphate solubilization index (PSI), each bacterial culture was assayed on Pikovskaya agar plates (Pikovskaya, 1948) containing tricalcium phosphate ($Ca_3(PO_4)_2$) as insoluble phosphate source. Cells were grown overnight in LB medium, next they were washed twice with 0.9% NaCl and re-suspended in 0.9% NaCl to produce equal cell densities among all the isolates. Solutions were inoculated on the agar plates and incubated at 30 °C, and observed daily for 7 days for appearance of transparent "halos" (Katznelson and Bose, 1959). Experiments were performed in triplicate. Phosphorus solubilization index was measured using following formula (Edi-Premono et al., 1996):

$$\mathrm{PSI} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

2.1.5. Hormones production by the bacterial strains growing without and with 15% of polyethylene glycol (PEG) in the growing medium

Bacterial strains were grown in LB medium with and without 15% PEG (−1.50 MPa) for four days to determine the production of these phytohormones. Treatments were replicated three times. The PEG concentration here used (15%) was selected because bacterial growth was quite considerable and it avoid problems in the detection of these hormones.

Bacterial culture medium (0.2 g) was homogenized in 5 mL ultrapure water and added with 20 μL of a mixture of internal standards containing, 50 ng [$^2H_6$]-ABA, 50 ng [$^2H_4$]-SA, 50 ng [$^2H_6$]-JA, and 50 ng [$^2H_5$]-OPDA (12-oxo phytodienoic acid). Centrifugation was performed at 5000 × *g* for 15 min, the pellet was discarded, the pH of the supernatant was adjusted to 2.8 with acetic acid, and the supernatant was partitioned twice against an equal volume of diethyl ether (Durgbanshi et al., 2005). The aqueous phase was discarded, and the organic fraction was evaporated. The solid residue was resuspended in 1.5 mL methanol (MeOH) and filtered through a 0.22 μm cellulose acetate filter. The organic fraction was evaporated at 35 °C in a Speed Vac model SC110 (Savant Instruments Inc., New York, NY, USA) and resuspended in 50 μL 100% MeOH. A 5 μL aliquot of this solution was injected into the HPLC system.

HPLC analysis was performed using an Alliance 2695 (Separation Module, Waters, Milford, MA, USA) quaternary pump equipped with an auto-sampler. A Restek C18 (Restek, Bellefonte, PA, USA) column (2.1 × 100 mm, 5 μm) was used at 28 °C with injected volume 5 μL. The binary solvent system used for the elution gradient consisted of 0.2% acetic acid in H_2O (solvent B) and MeOH (solvent A) at a constant flow rate of 200 μL min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied (*t* (min), % A): (0, 40), (25, 80), with 7 min for re-equilibration. MS/MS was performed using a Micromass Quatro Ultima™ "*Pt*" double quadrupole mass spectrometer (Micromass, Manchester City, UK). All of the analyses were performed using a turbo ion spray source in negative ion mode with the following settings for SA, JA, ABA, and OPDA: capillary voltage −3000 V, energy cone 35 V, RF Lens1 (20), RF Lens2 (0.3), source temp 100 °C, de-solvation temp 380 °C, gas cone 100 L h^{-1}, gas de-solvation 70 L h^{-1}, collision (50), and multiplier (650). The MS/MS parameters were optimized in infusion experiments using individual standard solutions of SA, JA, ABA and OPDA at a concentration of 10 ng μL^{-1} diluted in mobile phase A/B (40:60, v/v). MS/MS product ions were produced by collision-activated dissociation of selected precursor ions in the collision cell of the mass spectrometer, and mass was analyzed using the second analyzer of the instrument. Quantification was performed in the multiple reaction monitoring (MRM) mode.

2.2. Experiment II

2.2.1. Fermentation agrowaste process

Aspergillus niger NB2 strain was used in this study. It was shown to produced organic acids, mainly citric acid when growing on complex substrates and to mineralize lignocellulosic materials (Vassilev et al., 1998) and solubilized the rock phosphate (RP) (Medina et al., 2006). Sugar beet waste a lignocellulosic material (cellulose (29%), hemicellulose (23%) and lignin (5%)), was ground in an electrical grinder to 1 mm fragments. It was mixed at a concentration of 10% with 50 mL Czapek's solution containing (grams per liter of distilled water): $FeSO_4$, 0.01; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; $NaNO_3$, 3.0; sucrose, 30; K_2HPO_4, 1.0 and a final pH of 7.3 ± 0.2 for static fermentation in 250 mL Erlenmeyer flasks. Rock-phosphate at a concentration of 1.5 g L^{-1} was added. This medium was inoculated with 3 mL of *A. niger* spore suspension (1.2 × 10^6 spores). Static fermentation was performed at 28 °C for 20 days resulting in a product that can be used as organic amendment in the soil/plant system.

2.2.2. Bacterial inoculation and plant growth conditions

The substrate used in this assay consisted in the target soil, previously described. It was screened (5 mm), and mixed with sterile sand (5:2 (v/v)). The capacity of pots was of 0.5 kg.

The fermented agrowaste was mixed at 2% (v/v) with the soil in half of the pots. Pots filled with 0.5 kg of the soil/sand mixture added or not with fermented agrowaste were stabilized for two weeks before to start the experiment. One milliliter of pure bacterial culture (10^8 cfu mL^{-1}) of each bacteria (*B. megaterium*, *Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) grown in LB medium for 48 h at 28 °C, was applied to the appropriate pots. These treatments were replicated five times with a total of 200 pots placed in a random complete block designs.

Shrub seedlings were grown in 0.5 kg pots in a greenhouse under controlled conditions (18–24 °C, with a 18/6 light/dark period and 50% of relative humidity). A photoperiod of 16 h at a photosynthetic photon flux density (PPFD) of 400–700 μmol m^{-2} s^{-1} as measured with a light meter (model LI-188B; Licor Inc., Lincoln, NE, USA) was maintained during the experiment by supplementary light to compensate natural illumination. Water was supplied daily to maintain constant soil water close to field capacity (17% volumetric soil moisture) during 2 weeks after transplanting. After this time, and during a period of 1 year, these plants were allowed to dry until soil water content was 50% of field capacity. However, during the 24-h period comprised between each re-watering the soil water content was progressively decreasing until a minimum value of 30% of field capacity. Soil moisture was measured with an ML2 X ThetaProbe (AT Delta-T Devides Ltd., Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moisture (Roth et al., 1992). This volumetric soil moisture is considered to be a normal environmental condition in dry Mediterranean areas. A completely random experimental design was adopted.

2.2.3. Plant biomass and nutrients content

One year after planting, plants were harvested (five replicates per each treatment). Dry biomass of roots and shoots (data not-shown) and nutrients concentrations were determined.

Shoot content (milligram per plant) of P, K, Ca, Mg as well as of Zn, Fe, Mn and Cu (microgram per plant) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Mineral analysis was carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura (CSIC) Murcia, Spain.

2.2.4. Stomatal conductance, photosynthetic efficiency and proline content in shoot of L. dentata *and* S. officinalis

Before harvest some physiological plants values as stomatal conductance and photosynthetic efficiency was measured, only in *L. dentata* and *S. officinalis*. In the two remaining plants (*T. vulgaris* and *S. chamaecyparissus*) because of its reduced number of leaves and small area was impossible to make the mentioned determinations.

Stomatal conductance was measured by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK).

Photosystem II efficiency was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll a fluorescence. FluorPen quantifies the quantum yield of photosystem II as the ratio between the actual fluorescence yield in the light-adapted state (F_v) and the maximum fluorescence yield in the light-adapted state (F_m), according to Oxborough and Baker (1997). Measurements of stomatal conductance and photosynthetic efficiency were taken in the 2nd youngest leaf of two different plants of each treatment.

The proline was extracted in 100 mM phosphate buffer (pH 7.8) from 0.5 g of fresh leaves, previously immersed in liquid N_2 and stored at −80 °C according to Bates et al. (1973). Proline was estimated by spectrophotometric analysis at 520 nm using the ninhydrin reaction (Bates et al., 1973).

2.2.5. Percentage of arbuscular mycorrhizal (AM) fungal root colonization and glomalin production

Intraradical arbuscular mycorrhizal (AM) fungal colonization was assessed 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). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

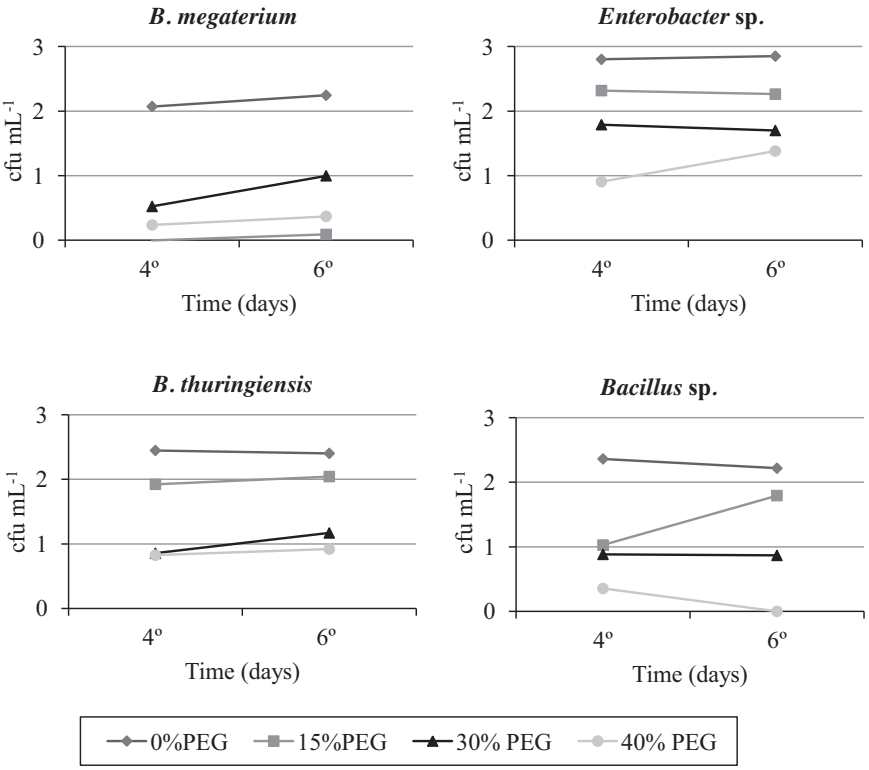

Fig. 1. Viable cells (centrifuged per milliliter) of bacterial strains growing in axenic nutrient medium supplemented with increasing levels of PEG (equivalent to −1.02; −1.50; −3.60 and −3.99 MPa) at different time intervals (from 4 to 6 days).

The extraradical fungal development was determined as glomalin related soil protein (GRSP), operationally measured as Bradford-reactive soil protein (Rillig, 2004). It was recovered from soil according to the method described by Wright and Upadhyaya (1998) with minor modifications. For the easily extractable fraction of GRSP (EE-GRSP) samples of 1 g soil were subjected to extraction with 8 mL of 20 mM citrate pH 7.0, and autoclaving for 30 min at 121 °C.

Glomalin is a stable molecule with a high C content (until 50%) (Rillig, 2004) and acts in the soil aggregation (Wright and Upadhyaya, 1996).

2.3. Statistical analysis

Data from both experiments were analyzed using the SPSS 21 software package from Windows. For Section 2.1, we used a one-way ANOVA, followed by Duncan's multiple-range test (Duncan, 1955) to find out significant differences at $p \leq 0.05$. Section 2.2, was based on a randomized complete factorial block design (5×2) for each plant species, consisting of 5 inoculations treatments with and without fermented agrowaste giving a total of 10 treatments. These treatments were analyzed with a general linear model ANOVA, followed by Duncan's multiple range test to find out significant differences at $p \leq 0.05$. Percentage values were arcsine-transformed before statistical analysis.

For the Pearson correlation analyses significant differences were determined at $p \leq 0.001$ in Section 2.1 and $p \leq 0.01$ in Section 2.2.

3. Results

3.1. Experiment I

Autochthonous bacterial strains were identified as *Enterobacter* sp. (Accession NR 044977.1 similarity 99%), *B. thuringiensis* (Accession NR 043403.1 similarity 98%) and *Bacillus* sp. (Accession NR 043403.1 similarity 91%). These bacteria were assayed under axenic conditions to evaluate their osmotic stress tolerance and their PGPR characteristics under stress situations.

The increasing levels of PEG in the growing medium specifically affected bacterial growth. *Enterobacter* sp. was the most stress tolerant bacteria showing the greatest growth under −3.6 and −3.9 MPa, whereas *B. megaterium* was the most sensitive to such conditions (Fig. 1). The PEG concentration is correlated with the growth of each bacterial species ($r = -0.75$; $p \leq 0.001$).

Table 1 shows bacterial metabolic characteristics related to stress tolerance and/or their PGPR abilities under stress (40% PEG) and non-stress conditions (0% PEG) in the growing medium. We observed that stressed bacterial cells accumulated more proline, particularly *Bacillus* sp. and *Enterobacter* sp., the two strains which were the lower proline producer in non-stress conditions. The oxidative lipid damage (MDA) increased with the stress particularly in *B. megaterium* and *Enterobacter* sp. and did not change in *Bacillus* sp. The CAT and APX antioxidant activities varied with the bacterial strain involved. *B. thuringiensis* showed the highest levels of antioxidants without stress and *Bacillus* sp. and *Enterobacter* sp. the lowest CAT and APX activities irrespective of stress conditions. With the application of stress factor increased both antioxidant activities in the allochthonous *B. megaterium*. Nevertheless the 40% PEG did not change (*Bacillus* sp. or *Enterobacter* sp.) or reduced (*B. thuringiensis*) these both antioxidant activities. The PHB accumulation was quite similar in the three *Bacillus* strains but under osmotic stress conditions only *B. thuringiensis* and *Bacillus* sp. increased but *B. megaterium* decreased the synthesis of this compound (Table 1). With regard to PGPR activities the highest production of IAA was found in *Enterobacter* sp. non-stressed cells, but with the stress situation (40% PEG) IAA production decreased in this bacterial culture. In contrast, *B. megaterium* significantly increased IAA production under osmotic stress conditions by 4.5 folds, or did not change in *B. thuringiensis* and *Bacillus* sp. cultures (Table 1).

The stress conditions reduced ACC production in *B. megaterium* and *Enterobacter* sp. or increased this compound in *B. thuringiensis* and *Bacillus* sp. All target bacteria were able to produce other phytohormones such as SA, JA, ABA and OPDA under moderate stress (15% PEG) and non-stress conditions (Table 2). Without

Table 2
Salicilic acid (SA), jasmonic acid (JA), abcisic acid (ABA) and 12-oxo phytodienoic (OPDA) produced by the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) growing four days in axenic culture medium supplemented or not with 15% polyethylene glycol (PEG).

	(PEG)	SA (pmol g^{-1})	JA (pmol g^{-1})	ABA (pmol g^{-1})	OPDA (pmol g^{-1})
B. megaterium	0%	1494.5 a	525.9 cd	163.3 ab	1577.7 a
	15%	1351.0 a	186.1 b	152.8 a	3813.5 c
Enterobacter sp.	0%	13,321.2 d	400.7 c	162.6 ab	2700.3 b
	15%	3498.0 b	108.3 a	153.2 a	1891.3 ab
B. thuringiensis	0%	1722.7 a	376.4 c	164.9 b	1283.8 a
	15%	7051.6 c	209.8 b	145.8 a	2949.3 bc
Bacillus sp.	0%	17,220.5 e	428.3 c	148.8 a	3176.8 b
	15%	3091.8 b	145.8 b	150.5 a	8648.9 d

Values within each column, having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 3$).

Table 1
Proline, lipid peroxidation (MDA), antioxidant enzymatic (catalase (CAT) and ascorbate peroxidase (APX)) activities, poly-β-hydroxybutyrate (PHB), indolacetic acid (IAA), α-ketobutyrate (ACC-deaminase) production and phosphorus solubilization index (PSI) by the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) after four days of growth in axenic culture medium supplemented or not with 40% polyethylene glycol (PEG).

	(PEG)	mmol proline (mg^{-1} prot)	mmol MDA g^{-1} (dry cell weight)	mmol CAT (mg^{-1} prot)	mmol APX (mg^{-1} prot)	mg PHB (mL^{-1})	mg IAA (mg^{-1} prot)	mmol α-ketobutyrate (mg^{-1} prot)	PSI
B. megaterium	0%	0.14 b	2.5 b	164 e	2340 c	0.32 c	38.2 b	0.65 c	1.90 c
	40%	1.21 e	30.0 g	401 f	4888 d	0.01 a	183.2 d	0.35 b	1.63 bc
Enterobacter sp.	0%	0.05 a	6.7 d	22 b	238 a	0.01 a	110.0 c	0.37 b	2.06 c
	40%	1.10 d	20.5 f	2 a	217 a	0.08 b	53.0 b	0.22 a	1.00 a
B. thuringiensis	0%	0.12 b	0.7 a	606 g	11,760 e	0.33 c	18.2 a	0.20 a	1.56 b
	40%	0.31 c	4.4 c	46 d	586 b	0.38 cd	13.0 a	0.41 b	1.37 b
Bacillus sp.	0%	0.05 a	10.0 c	32 c	277 a	0.31 c	10.0 a	0.41 b	1.00 a
	40%	1.50 f	10.2 c	26 b	261 a	0.45 d	10.3 a	1.09 d	1.00 a

Values within each column, having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 3$).

stress induction *Bacillus* sp. produced the highest amount of SA but stress greatly decreased the ability of this bacterium for the production of these compounds. The stress, in general, reduced JA production (having a significant correlation $r = -0.941$; $p \leq 0.001$), but enhanced OPDA production by most of the tested bacteria (except in the *Enterobacter* sp.). Nevertheless, ABA production was quite similar under non-stress and stress conditions in all bacteria tested. But no generalization can be given on SA production with regards stress effect (stimulating for *B. thuringiensis,* negative for *Bacillus* sp. and *Enterobacter* sp. and neutral for *B. megaterium*). The decreased SA amount under stress was only noted in the greatest SA producer as *Bacillus* sp. and *Enterobacter* sp. strains (Table 2). These results showed the complexity of mechanisms involved in the bacterial drought tolerance.

All the assayed bacteria showed PSI ability being this value highest in *B. megaterium* and *Enterobacter* sp. but the PSI was greatly depressed by the stress induction in *Enterobacter* sp. (Table 1).

In general, these drought tolerant bacteria highly reduced the levels of PGPR metabolites as ACC-deaminase, IAA, and PSI by the osmotic stress, as well the antioxidant CAT activity.

3.2. Experiment II

Plant biomass (shoot and root growth) were not significantly affected by the treatments applied (data not shown). Nevertheless, shoot nutrients content in the four shrub species grown with and without fermented agrowaste were affected, but differently, by the bacterial inocula. In soil without fermented agrowaste addition *B. thuringiensis* increased P content ranging between 11% in *S. chamaecyparissus* to 51% in *T. vulgaris*, and for K content the effect ranged between 28% in *S. chamaecyparissus* to 63% in *L. dentata*. Nutrient uptake was maximized by *B. thuringiensis* inoculation in most of the shrubs grown without fermented agrowaste, with the exception of *S. officinalis* (Table 3).

Nutrient contents were significantly increased by the fermented agrowaste application in three of the four shrubs, excluding *S. officinalis*. Fermented agrowaste application had an important effect in increasing shoot P and K content in *T. vulgaris* by 100% (P) and by 87% (K) in comparison to plants grown without fermented agrowaste application. Nevertheless, shrubs grown with fermented agrowaste (excluding *T. vulgaris*) and inoculated with *B. megaterium* showed maximum P and K uptake. Such effects in the case of P were: 75% (*L. dentata*), 63% (*S. officinalis*) and 109% (*S. chamaecyparissus*). In the case of K were: 33% (*L. dentata*) and 66% (*S. chamaecyparissus*). A similar trend was observed for Ca and Mg contents in these three shrubs when *B. megaterium* was inoculated (Table 3). Micronutrient (Zn, Fe, Mn and Cu) content was also enhanced when these plants were inoculated with *B. megaterium* in fermented agrowaste amended soil (Table 4). Indeed, treatments involving *B. megaterium* inoculation and fermented agrowaste, dually applied, significantly enhanced nutrient uptake in most of the target plant species (Tables 3 and 4).

Physiological parameters related to drought tolerance as proline content, stomatal conductance and photosynthetic efficiency were measured only in *L. dentata* and *S. officinalis*, as in these species shoot biomass manipulation was easier. *S. officinalis* exhibited higher accumulation of proline in fresh leaves than *L. dentata* in absence of fermented agrowaste. In *L. dentata*, bacterial inoculation decreased proline accumulation, but *B. thuringiensis* plus fermented agrowaste caused a significant increase of proline in this plant species (Fig. 2A). Nevertheless, in *S. officinalis* (without fermented agrowaste) the opposite trends were found since whatever bacterial treatment did not either change or increase proline accumulation. In general, the fermented agrowaste reduced proline production in both plants but most of bacteria increased proline level in amended soil (Fig. 2A).

Table 3
P, K, Ca and Mg content (miligram per plant) in four autochthonous plants (*Thymus vulgaris*,*Santolina chamaecyparissus*, *Lavandula dentata* and *Salvia officinalis*) non-inoculated (control) or inoculated with the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) grown in an arid Mediterranean soil amended or not with fermented agrowaste (CO), under drought conditions.

	P		K		Ca		Mg	
	(−)	CO	(−)	CO	(−)	CO	(−)	CO
Thymus vulgaris								
Control	0.6 a	1.2 d	7.1 a	13.3 d	6.0 a	11.3 c	1.7 a	2.9 d
B. megaterium	0.7 b	0.9 c	9.3 b	11.2 c	7.0 a	7.7 ab	2.0 ab	2.2 b
Enterobacter sp.	0.7 b	0.9 c	10.0 b	10.9 bc	8.7 b	10.3 b	2.2 b	2.6 c
B. thuringiensis	0.9 c	1.1 cd	10.4 b	12.9 d	7.9 b	9.1 b	2.2 b	2.5 c
Bacillus sp.	0.8 c	1.2 d	10.3 b	10.5 b	8.4 b	9.9 b	2.0 b	2.8 cd
Santolina chamaecyparissus								
Control	0.9 a	1.1 b	10.1 a	11.4 b	8.4 b	9.3 b	1.0 a	1.2 b
B. megaterium	0.8 a	2.3 c	8.6 a	18.9 c	6.8 b	15.2 d	0.9 a	2.4 d
Enterobacter sp.	1.0 b	1.0 b	11.9 b	8.4 a	11.2 c	5.2 a	1.4 b	0.8 a
B. thuringiensis	1.0 b	1.0 b	12.9 b	11.2 b	11.9 c	9.0 b	1.4 c	1.1 b
Bacillus sp.	0.9 a	0.9 a	11.3 b	11.0 b	8.8 b	8.9 b	1.1 a	1.1 a
Lavandula dentata								
Control	0.6 ab	0.8 b	13.5 a	19.6 bc	13.3 b	10.9 a	2.1 b	1.9 a
B. megaterium	0.5 a	1.4 c	18.4 b	26.0 d	15.3 b	16.9 c	2.4 ab	2.7 b
Enterobacter sp.	0.5 a	1.3 c	15.0 a	26.1 d	9.8 a	16.3 c	1.4 a	2.9 c
B. thuringiensis	0.6 ab	0.9 b	21.9 c	19.1 bc	16.8 c	11.1 a	2.9 bc	1.6 a
Bacillus sp.	0.6 ab	0.9 b	19.5 b	20.4 bc	15.4 bc	13.0 ab	2.6 b	2.1 ab
Salvia officinalis								
Control	0.6 a	0.8 a	9.9 a	8.7 a	8.3 a	7.3 a	2.6 a	2.7 a
B. megaterium	0.8 a	1.3 b	9.9 a	13.2 b	12.2 b	12.7 b	3.2 ab	3.5 bc
Enterobacter sp.	0.7 a	1.1 ab	8.9 a	12.9 ab	10.8 b	11.2 b	2.7 a	3.3 ab
B. thuringiensis	0.8 a	1.0 ab	9.4 a	10.3 a	11.2 b	12.0 b	2.9 ab	2.7 a
Bacillus sp.	0.8 a	1.1 ab	10.6 a	10.9 a	16.9 c	11.7 b	3.3 ab	3.2 ab

Within each shrub species and each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

Table 4
Zn, Fe, Mn and Cu content (microgram per plant) in four autochthonous plants (*Thymus vulgaris*, *Santolina chamaecyparissus*, *Lavandula dentata* and *Salvia officinalis*) non-inoculated (control) or inoculated with the reference *Bacillus megaterium* or autochthonous bacterial strains (*Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* sp.) grown in an arid Mediterranean soil amended or not with fermented agrowaste (CO), under drought conditions.

	Zn		Fe		Mn		Cu	
	(−)	CO	(−)	CO	(−)	CO	(−)	CO
Thymus vulgaris								
Control	33.3 a	54.2 b	42.8 a	118.5 c	40.4 a	69.7 c	4.2 a	6.1 d
B. megaterium	46.7 b	45.4 b	60.9 ab	98.5 bc	50.3 ab	54.8 b	5.5 c	6.0 cd
Enterobacter sp.	53.8 b	57.1 b	80.1 b	120.5 c	62.8 b	61.3 b	4.9 b	6.6 d
B. thuringiensis	54.1 b	55.1 b	115.2 c	112.7 c	46.5 a	62.0 b	6.4 d	6.1 d
Bacillus sp.	45.8 b	50.8 b	119.2 c	147.1 d	50.7 a	61.8 b	5.5 c	6.9 d
Santolina chamaecyparissus								
Control	60.9 c	62.3 cd	87.2 c	99.5 d	100.2 b	97.5 c	11.1 c	8.7 b
B. megaterium	53.6 b	87.1 de	70.0 b	123.7 e	74.0 a	198.8 d	9.7 b	12.3 d
Enterobacter sp.	72.8 d	40.9 a	77.8 b	27.9 a	127.7 c	79.0 a	12.5 d	5.2 a
B. thuringiensis	89.7 e	61.0 c	78.4 b	89.8 c	121.9 c	98.8 c	13.2 d	9.6 b
Bacillus sp.	78.0 d	60.4 c	77.7 b	89.4 c	98.9 c	97.6 c	12.4 d	9.4 b
Lavandula dentata								
Control	38.5 b	36.6 b	104.2 b	85.2 b	13.4 a	17.9 b	5.2 b	6.3 c
B. megaterium	37.8 b	43.7 b	67.0 a	103.3 b	17.2 b	27.1 d	5.6 b	6.9 c
Enterobacter sp.	31.2 a	45.2 c	58.7 a	108.9 b	13.5 a	19.2 b	4.4 a	7.3 c
B. thuringiensis	47.4 c	46.0 c	100.2 b	79.9 b	20.6 c	16.0 b	7.2 c	5.8 b
Bacillus sp.	41.6 b	46.5 c	122.2 b	93.3 b	17.8 b	20.3 b	5.7 b	5.3 b
Salvia officinalis								
Control	29.5 b	23.9 a	56.4 b	39.1 a	17.3 b	15.0 a	4.0 a	4.0 a
B. megaterium	26.4 a	29.4 b	80.9 c	108.3 cd	17.8 b	23.8 c	4.5 a	5.8 b
Enterobacter sp.	28.5 b	28.7 b	53.2 b	92.6 c	16.9 b	20.4 bc	4.4 a	4.8 a
B. thuringiensis	24.6 a	31.1 b	50.4 b	74.6 c	19.7 bc	20.9 bc	4.5 a	4.9 a
Bacillus sp.	30.6 b	25.8 a	57.2 b	54.8 b	24.0 c	19.8 bc	6.1 b	5.4 b

Within each shrub species and each parameter, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

The stomatal conductance mainly depends on the plant used. In general, *S. officinalis* showed a higher stomatal conductance than *L. dentata*, this value was in *L. dentata* (without fermented agrowaste) by *B. thuringiensis* reduced and in both plants with by *B. megaterium* (with fermented agrowaste). Nevertheless, the bacterial inoculants could also influence this parameter (Fig. 2B).

The photosynthetic efficiency of photosystem II (F_v/F_m) was less modified in *S. officinalis* than in *L. dentata* by the bacterial treatments and/or fermented agrowaste (Fig. 2C). This value dropped by the treatments applied (bacteria or fermented agrowaste) only in *L. dentata*. However, in fermented agrowaste amended soil it was increased by the bacteria inoculation. Again, *B. thuringiensis* decreased photosynthetic efficiency in *L. dentata* without fermented agrowaste in concordance with the reduction of stomatal conductance (Fig. 2B). However, the reduction of stomatal conductance in *L. dentata* caused by *B. megaterium* + fermented agrowaste (Fig. 2B) was not reflected in the photosynthetic efficiency (Fig. 2C).

A wide range of natural AM colonization levels (as a percentage of root length colonized), was found in the four shrub species (Fig. 3). In control plants grown without fermented agrowaste the greatest AM colonization was observed for *T. vulgaris* and the lowest in *S. chamaecyparissus*. All bacterial inoculation treatment increased percentage of the mycorrhization in *S. chamaecyparissus* and *S. officinalis*, while this effect was only observed in *L. dentata* when inoculated with *Enterobacter* sp. The highest percentage of natural AM root colonization in each plant growing in soil without fermented agrowaste was reached in the bacteria-inoculated plants, with the exception of *B. thuringiensis* and *Bacillus* sp. when inoculated in *T. vulgaris* (Fig. 3).

In control plants grown with fermented agrowaste the greatest AM colonization was observed in *S. chamaecyparissus*. In general, bacterial inoculation resulted less effective in increasing AM colonization in presence of fermented agrowaste in the medium. Nevertheless, inoculation of *S. officinalis* with *B. megaterium*, *Enterobacter* sp. and *B. thuringiensis* enhanced AM colonization in presence of fermented agowaste (Fig. 3).

Values of glomalin, as GRSP, content reflect the amount of extraradical mycelium (Fig 3). The fermented agrowaste was effective in increasing GRSP in *L. dentata* and *S. officinalis* and the bacterial treatment did not affect this response variable. Correlations cannot be generalized since they are produced only significantly between fermented agrowaste and extra mycorrhizal (GRSP) development ($r = 0.546$; $p \leq 0.01$) but no in intra mycorrhizal ($r = -0.115$; $p \geq 0.05$) development.

4. Discussion

The three *Bacillus* sp. and the *Enterobacter* sp. bacterial strains here selected were able to produce IAA and to phosphate solubilise (particularly *B. megaterium* and *Enterobacter* sp.) under non-stress and stress conditions in vitro. Minaxi (2011) also reported multiple plant promoting traits in a *Bacillus* sp. isolated from semiarid crops. *Bacillus* was the most abundant genus in the rhizosphere of autochthonous drought-adapted target plants, probably because the ability of these bacteria to form spores allows a better survival under stress conditions (Marulanda et al., 2006). Nevertheless, *Enterobacter* sp. resulted in the most tolerant bacteria able to survive under 40% of PEG in the growing medium. Indeed, under the greatest osmotic stress assayed (40% PEG), shows a high level of proline and PHB but the lowest antioxidants activities (CAT and APX) for osmotic cellular adaptation, as previously found (Marulanda et al., 2009). Such bacterial activities may represent an important protection against water limitation. Nevertheless, the MDA production, as indicator of stress oxidation, resulted in a higher level than expected. These intrinsic metabolic bacterial characteristics in the axenic culture under non-stress and stress conditions support the fitness of these bacteria to facilitate

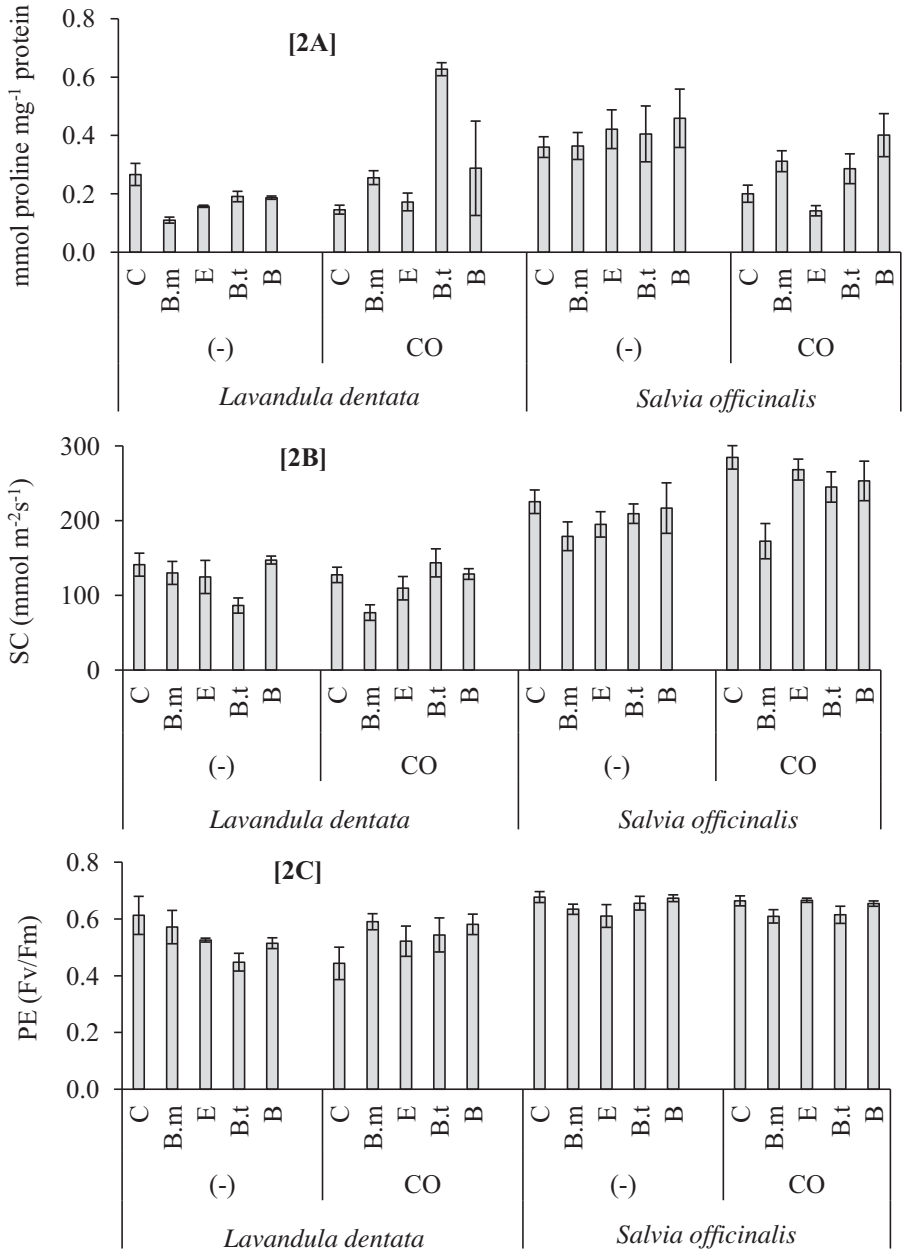

Fig. 2. Proline accumulation [2A], stomatal conductance (SC) [2B] and photosynthetic efficiency (PE) [2C] in *Lavandula dentata* and *Salvia officinalis* non-inoculated (control) or inoculated with the reference *Bacillus megaterium* (Bm) or autochthonous bacterial strains (*Enterobacter* sp. (E); *Bacillus thuringiensis* (Bt) and *Bacillus* sp. (B)) grown in an arid Mediterranean soil amended or not with fermented agrowaste (CO), under drought conditions. Errors bars represent standard errors ($n = 3$).

revegetation of semi-arid areas. However, many factors may affect the performance of inoculated bacteria under natural conditions (Bais et al., 2006).

It has been shown that the exposure of bacterial cells to osmotic stress induce the production of reactive oxygen species (ROS) that disturb the metabolic balance of the cells and cause oxidative stress (Maksimovic et al., 2013). Here the antioxidants activities in the bacterial cultures did not correlated with the osmotic tolerance capacity since the two most tolerant bacteria, *B. thuringiensis* and *Enterobacter* sp., showed the highest CAT and APX (*B. thuringiensis*) and the lowest (*Enterobacter* sp.) activities. Antioxidant activities in stressed cells are highly variable depending of bacteria involved but these enzymes reflects the modified redox status of the stressed cells. Proline accumulation in cells not only has an osmolyte function but also maintains the redox balance and radical scavenging (Szabados and Savoure, 2010).

The production of ACC by the target bacteria was also evaluated because it is the precursor for ethylene synthesis in plant. Bacterial ACC deaminase converts the ACC to ammonia and α-ketobutyrate, thereby lowering ethylene levels in inoculated plants (Glick et al., 1998). The lowering of ethylene levels is essential when plants are exposed to environmental stressors as drought (Glick, 2004). *Bacillus* sp. was the most drought sensitive bacteria and it produced the highest ACC-deaminase and proline accumulation under stress conditions. Both compounds would account for the compensation of the bacterial lack of stress tolerance (40% of PEG addition). However, this bacterial strain changed very little the APX and CAT activities and lipid peroxidation (MDA) under the stress conditions tested. These antioxidant bacterial activities play an important role facilitating the removal of free radicals (Wang et al., 2007). Perhaps in this bacterial strain the low reaction of these antioxidant activities were compensated by the contribution of high PHB and/

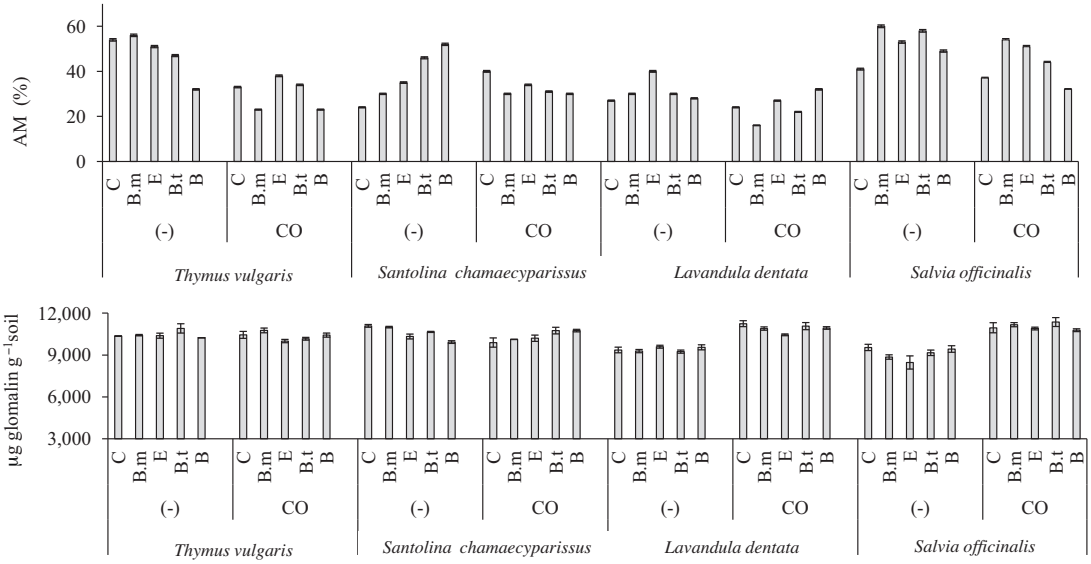

Fig. 3. Percentage of root AM colonization and glomalin content in rhizosphere soil of four autochthonous shrubs (*Thymus vulgaris, Santolina chamaecyparissus, Lavandula dentata* and *Salvia officinalis*) non-inoculated (control) or inoculated with the reference *Bacillus megaterium* (Bm) or autochthonous bacterial strains (*Enterobacter* sp. (E); *Bacillus thuringiensis* (Bt) and *Bacillus* sp. (B)) grown in an arid Mediterranean soil amended or not with fermented agrowaste (CO), under drought conditions. Errors bars represent standard errors ($n = 3$).

or ACC-deaminase production in alleviating cell osmotic stress. Nevertheless, the low survival of this bacterial strain under 40% PEG is contrasting with its abilities to synthesize these compounds.

Production of IAA-like compounds is common in *Bacillus* strains and this bacterial trait may improve root growth during the early plant growth stage. In addition, stomatal closure and transpiration reduction in response to water deficiency in plants may be induced by auxins. The participation of bacterial auxins in the responses to water stress was also observed by Havlova et al. (2008). Drought significantly increased IAA production in *B. megaterium*, as previously reported by Dobra et al. (2010), corroborating that IAA also plays an important role in the stress responses. In fact, this bacterium decreases stomatal conductance in *L. dentata* (with fermented agrowaste) and in *S. officinalis* (with and without fermented agrowaste).

The production of hormones, such as ABA, SA, OPDA and JA, was also tested for the target bacteria, because these signal molecules are the basis for important mechanisms to cope with osmotic stress and be considered as PGPR (Pozo et al., 2015). Particularly ABA is described as the primary chemical involved in acting as signal of osmotic stress (Schurr et al., 1992). In plants, ABA has been proposed to play a role in water transport via activation of aquaporins such as plasma membrane intrinsic proteins type 1 (PIP1) (Parent et al., 2009). Actually, ABA plays an important role in the stress signal transductions (Aroca et al., 2008). The bacterial strains here tested produced quite similar levels of ABA under moderate osmotic stress conditions. In addition to ABA, the hormone JA has been shown to protect cells from osmotic stressors (Pedranzani et al., 2003). The JA is considered as a growth regulator able to induce tolerance to stress conditions. Here, the highest producers of JA under non-stress conditions was *B. megaterium*, the most PEG-sensitive bacteria, while the lowest JA producers was *Enterobacter* sp. under stress conditions, the most PEG-tolerant bacteria. In this context, significant differences between *B. megaterium* and *Enterobacter* sp. in JA production were observed with and without PEG application. Nevertheless, the coordinated action of ABA and JA protected cells from the effects of stress. Observations by Brossa et al. (2011) indicate a relationship between JA and lipid peroxidation but in the here tested bacteria such relation was not observed. The SA synthesized by bacteria also plays an important role in osmotic stress tolerance (Gautam and Singh, 2009). This phenolic hormone is associated to abiotic stress responses and thus, it was determined in the target bacteria because of it signaling activity on the antioxidant defence system (Zhou, 1999). *Bacillus* sp. synthesized the highest amount of SA under whatever tested condition. All those hormones (ABA, SA and JA) synthesized by these bacteria may play also an important role in mediating plant reactions to drought (Groppa et al., 2012).

Autochthonous bacteria here selected to be used as inoculants for the target shrubs produced different quantities of IAA, ACC-deaminase and PSI and also differed in their ability to produce antioxidants, proline, PHB, ABA, JA, OPDA and SA under stress and non-stress conditions. These physiological and biochemical bacterial traits did not totally explain the benefits obtained by inoculated plants under drought conditions.

In this study we demonstrated that both survival and nutrition of shrub plants were highly benefited by the autochthonous inoculated bacteria and/or fermented agrowaste application in soil affected by drought. The enhancement of nutrients uptake (mainly P and K) in inoculated plants added of fermented agrowaste may affect plant water relations and drought tolerance (Subramanian et al., 2006). In dry soils, the P availability is highly reduced since the decline in soil moisture results in an important lowering in the rate of nutrients diffusion in the soil solution, particularly those having a low diffusing rate, like P.

Fermented agrowaste amendment improves nutrient uptake in most of shrubs assayed (except in *S. officinalis*), as previously found in the legume shrub *Anthyllis cytosoides* (Medina et al., 2004). Along the fermentation process by *A. niger* can occur simultaneous activities such as the mineralization of the lignocellulosic agrowaste compounds, the biosynthesis of organic acids, as the tricarboxilic citric acid, and consequently rock phosphate solubilization (Vassilev et al., 1998). In addition, the fermented agrowaste added to the soil seems to be used as C source and energy for the inoculated bacteria which could lead to an enhancement of the beneficial bacterial activity resulting in increased functional traits that benefited the shrubs nutrition here tested. Particularly, inoculation of *B. megaterium* in fermented agrowaste soil enhanced nutrient uptake, such as P, K, Ca and Mg, by *S. chamaecyparissus*, *L. dentata* and *S. officinalis*.

In *L. dentata* and *S. officinalis* values of proline, stomatal conductance and photosynthetic efficiency were also analyzed. The improvement of such specific characteristics of these two shrubs could be considered as strategies to facilitate water stress tolerance by bacteria and/or fermented agrowaste applied. The stomatal conductance and nutrients as K and Ca content (higher in *L. dentata* than in *S. officinalis*) are important physiological and nutritional values to adapt plants to drought since stomatal closure preserves water lost.

Both under axenic and natural conditions we found that the stress factors applied did not suppress the PGPR abilities of the autochthonous drought-adapted bacteria which indicated their potential to be used as inoculants under such detrimental conditions. Due to their adaptability to stress the bacterial cells may improve its competitive advantage for coping with the stress situation. Thus, the interest of microbial inoculations and their effectiveness increased under unfavorable environmental situations as it is drought (Belimov et al., 2009). However, since many mechanisms and factors may be involved in the adaptation and response to drought, the prevailing mechanisms for stress tolerance of the target bacteria and/or inoculated plants are difficult to be established. The PGPR ability of *B. megaterium* was related to endogenous ABA content in tomato plants (Porcel et al., 2014).

Nutrient acquisition of the target shrubs was differently affected by the inoculation with each one of the PGPR bacteria applied. This may be due to differences in the specific bacterial characteristics, i.e., ability to produce hormones, to colonize roots (Li et al., 2000), to solubilise P or to hydrolyze ACC. However, it is not clear from these results the main bacterial activity involved in the positive effects and potential to minimize the deleterious effect of drought in these inoculated plants (Glick, 2012). The target bacteria, in general, safeguard the plants from the deleterious effects of drought by producing phytohormones, ACC deaminase, PHB, antioxidant enzymes and by increasing nutrients availability in different amount. But these microbial traits seem not always efficiently functioning under such stress conditions in each one of the shrubs. Thus, inoculation with PGPR induces different range of plant tolerance to abiotic stresses with an osmotic component like drought and in this effect may account the level of improvement of physiological and biochemical parameters related with water status (Kohler et al., 2009) and the characteristics of plant involved (Porcel et al., 2014). In fact, the effect of each bacteria on plant physiological values as leaf transpiration and photosynthetic efficiency (PE) cannot be generalized (Alguacil et al., 2009). Results related to a deficient nutrition caused by osmotic stress in non-inoculated plants could be induced by lower root, nutrients and water uptake capacity.

Since ROS produced by the stress situation are removed by several enzymatic systems, it is clear whether the enhancement of these activities in cells correlated with the stress severity (Koussevitzky et al., 2008). APX is the key antioxidant enzyme in the ascorbate/glutathione cycle (Orvar and Ellis, 1997). Enzymatic systems resulted here sensitive and indicative of the bacterial effectiveness in supporting drought impact in the stressed cells. The lowering in these activities in bacterial cells under water stress may be interpreted as a higher water retention and subsequent increased drought stress tolerance.

Proline is also an important compound involved in turgor maintenance. This osmolite is often synthesized by cells in response to stress factors mediating osmotic adjustment and the accumulation of this compounds increases cell resistance to water deficiency (Kishor et al., 2005). Bacterial inoculation and/or fermented agrowaste application decreased proline accumulation in *L. dentata,* which may reflect an increased drought tolerance.

In *L. dentata* the bacteria and the fermented agrowaste highly increased K^+ retention and it is considered as one of the key features of osmotic stress tolerance (Shabala and Cuin, 2008). Tolerant varieties are capable to better retain K^+ (Chen et al., 2007).

Nevertheless, it is very difficult to attribute the bacterial effectiveness to specific nutritional or physiological activities. The particular bacterial effectiveness on the performance and drought tolerance ability in the test plants depended on the plant species involved. It is important to assess whether tolerant mechanisms are not only transient but also long-term lasting.

Significant differences in the natural AM colonization level among the test plants were evident. In *T. vulgaris* plants showed the highest AM colonization levels, which were not affected by bacterial inoculation. In contrast, all bacteria increased the mycorrhization degree in *S. chamaecyparissus* and *S. officinalis*, thereby acting as mycorrhiza helper bacteria (Frey-Klett et al., 2007). Fermented agrowastes applications decreased the ratio of AM intra and extra-radical colonization in all plants, which suggest a particular stimulating effect of this amendment on the fungal mycelia developed in soil, in comparison with that developed inside the root. The extraradical mycelium size was quite similar irrespective of plant and the bacterial inoculum involved. Thus, significant increases of macro and micro nutrients uptake by *T. vulgaris*, *S. chamaecyparissus* and *L. dentata* inoculated with *B. thuringiensis* cannot be explained by an enlargement of the extraradical mycelium emerging from the root systems of those naturally AM-colonized plants.

Structural soil stability of the degraded test soil has been shown to be significantly improved by about a 79% by the addition of fermented agrowaste (Alguacil et al., 2003). The glomalin, present in the extraradical mycelia component, is a recalcitrant glycoprotein acting as a binding agent in the aggregation process (Lovelock et al., 2004). An improved soil structure means an increased water retention, nutrient uptake, drainage, aeration and root growth, which consequently determines an improvement of soil quality and fertility (Caravaca et al., 2002).

All the applied treatments resulted fundamental for the target shrubs species to reach their optimal nutritional and physiological traits under conditions which are characteristics of the natural semiarid Mediterranean drought conditions. The diverse bacterial activities and plant characteristics could explain the unpredictable effectiveness of inoculated bacteria. Detailed molecular and physiological studies will be helpful for understanding microbial and plant tolerance and adaptative processes that are yet poorly understood (Cappellari et al., 2013), and these are the subject of current research. In any case, further experiment under natural soil conditions should be conducted for a proper exploitation of stress-adapted PGPR in the restoration of degraded ecosystems. The selection of efficient bacterial strains with well-defined mechanisms, consistent and reproducible activities under field conditions is very important to develop PGPR inocula.

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