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PLANT MICROBE INTERACTIONS

Differential Activity of Autochthonous Bacteria in Controlling Drought Stress in Native *Lavandula* and *Salvia* Plants Species Under Drought Conditions in Natural Arid Soil

Elisabeth Armada · Antonio Roldán · Rosario Azcon

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Abstract The effectiveness of autochthonous plant growth-promoting rhizobacteria was studied in *Lavandula dentata* and *Salvia officinalis* growing in a natural arid Mediterranean soil under drought conditions. These bacteria identified as *Bacillus megaterium* (Bm), *Enterobacter* sp. (E), *Bacillus thuringiensis* (Bt), and *Bacillus* sp. (Bsp). Each bacteria has different potential to meliorate water limitation and alleviating drought stress in these two plant species. *B. thuringiensis* promoted growth and drought avoidance in *Lavandula* by increasing K content, by depressing stomatal conductance, and it controlled shoot proline accumulation. This bacterial effect on increasing drought tolerance was related to the decrease of glutathione reductase (GR) and ascorbate peroxidase (APX) that resulted sensitive indexes of lower cellular oxidative damage involved in the adaptative drought response in *B. thuringiensis*-inoculated *Lavandula* plants. In contrast, in *Salvia*, having intrinsic lower shoot/root ratio, higher stomatal conductance and lower APX and GR activities than *Lavandula*, the bacterial effects on nutritional, physiological and antioxidant enzymatic systems were lower. The benefit of bacteria depended on intrinsic stress tolerance of plant involved. *Lavadula* demonstrated a greater benefit than *Salvia* to control drought stress when inoculated with *B. thuringiensis*. The bacterial drought tolerance assessed as survival, proline, and indolacetic acid production showed the potential of this bacteria to help plants to grow under drought conditions. *B. thuringiensis* may be used for *Lavandula* plant establishment in arid environments. Particular characteristic of the plant species as low shoot/root ratio and high stomatal conductance are important factors controlling the bacterial effectiveness improving nutritional, physiological, and metabolic plant activities.

Abbreviations

PGPR	Plant growth promoting rhizobacteria
GR	Glutathione reductase
APX	Ascorbate peroxidase
SOD	Superoxide dismutase
CAT	Catalase
IAA	Indolacetic acid
Bm	*Bacillus megaterium*
E	*Enterobacter* sp.
Bt	*Bacillus thuringiensis*
Bsp	*Bacillus* sp.

Introduction

Rhizosphere bacteria are ubiquitous soil inhabitants able to establish relationships with plants. Bacteria assist the associated plants in the uptake of mineral nutrients and water and also they increase tolerance to environmental stresses [1]. Bacterial are usually the most numerous organisms which could be cultivable in soil with 10^6–10^9 viable cell by per cubic centimeter [2]. Nevertheless, much more research on the bacteria drought resistance is required to know mechanisms related to grow effect and adaptation to dry soils. Under such drought conditions, the development of indigenous microbial community is limited or even inhibited. Thus, the application of plant growth-promoting microorganisms has been suggested

E. Armada · R. Azcon (✉)
Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Prof. Albareda 1, 18008 Granada, Spain
e-mail: rosario.azcon@eez.csic.es

A. Roldán
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

[3]. The ability of certain bacteria to attenuate detrimental stress effect in plants is previously reported [1, 4, 5].

The physiological benefits of rhizosphere bacteria for the host plants are well known and their effectiveness is ecologically relevant particularly under detrimental conditions. The establishment of a plant cover based on autochthonous plant species is an effective strategy for restoring the Mediterranean semiarid degraded lands. In such areas, having low soil fertility and water deficiency, the establishment of plants is difficult and it requires to apply methods for improving the ability of these plant species to resist the drought environmental conditions [6]. Thus, to carry out successful reforestation programs, it is necessary to apply inoculation technologies which reinforce the limited microbial potential in these degraded areas [4, 7, 8]. Regarding the competitiveness of autochthonous rhizosphere bacteria, one efficient strategy contributing to the establishment of pre-selected beneficial microorganisms in these poor-infertile semiarid soils is through early bacterial establishment in the rhizosphere by inoculation at the seedling stage. Bacterial inoculation, selecting adapted and efficient specific microorganisms, has long been recognized as an interesting possibility to increase plant growth [9]. Nevertheless, the plant growth responses to bacterial inoculation involve from bacterial strain to plant species and even ecotype and site specificity [4]. Authors reported that variable effects were determined depending on plant species, cultivar, and environmental conditions [10].

Lavandula dentata and *Salvia officinalis* constitute important plants for revegetation programs in a semiarid Mediterranean area and to improve the plant establishment by the direct application of bacterial inocula may be a recommended practice. Previous results evidenced that selected bacteria help plants to grow under arid conditions by increasing nutrients supply and water stress tolerance [3].

The role of bacteria in growth, nutrition, and drought tolerance under nutritional limited conditions is based on a range of physiological and cellular mechanisms [1]. In this regard, microorganisms are also able to reduce water stress by alleviating cellular oxidative damage produced in plants under drought conditions. In fact, the view nowadays is to consider ROS as an integrative part of cell signaling metabolism modulated by the cellular redox state loading to different responses related to programmed cell death, plant development or defense, and gene expression [11]. The establishment of inocula in dry soils includes the activation of antioxidant metabolic pathways [12, 13].

Arid environments determine the ability of organisms to proliferate is such habitat. The microbial ability to adapt to environmental changes is fundamental to the survival of these organisms and several mechanisms are responsible for the required adaptation. Remarkable similarities exist between plants and bacteria in their cellular responses to an osmotic stress [14]. Several organisms (microorganisms and plants) from different kingdoms are able to accumulate the same set of cellular compounds upon exposure to stress conditions. There are close parallelism in the mechanisms that plants and microorganisms use to regulate responses to environmental stresses. In fact, there are processes that enable organisms to cope with environmental changes or stress conditions and they determine the ability of organisms to live in particular environments.

This study reports information on the relevance of cells metabolic processes conducting to proline and indolacetic acid (IAA) microbial production in the growing medium along the time when this medium was added of increasing polyethylene glycol (PEG) to create an osmotic stress. The bacterial IAA productions are related to plant improvement effect and proline is accumulated in the cell under stress condition to protect cells against adverse effect of ROS and stabilizing proteins. This compound increases resistance to water deficiency by that it can be considered a good stress indicator. As well, previous studies [15] report mechanisms commonly involved in the plant growth-promoting activity of bacteria as is the production of phytohormones and particularly IAA plays the most important role in plant growth promotion. Thus, it was selected as representative index of bacterial efficiency.

Plants and microorganisms living in semiarid soils are often adapted to such stress conditions and the applications of such organisms to establish vegetation cover in these areas is an attractive possibility to recover these soils. But plants and microorganisms are affected by these detrimental conditions which alter cells and metabolism reducing growth. Under drought conditions, the relative plant benefit from the microorganisms may be different according to the photosynthetic activity of the associated plant which affects microbial performance. However, adapted/tolerant bacteria can enhance plant growth and nutrition under drought conditions and several physiological mechanisms may enhance the plant resistance to water stress. In general, plants may increase drought tolerance by reducing stomatal conductance and evapotranspiration, by increasing the cellular osmolyte accumulations and by enhancing drought tolerance and/or avoidance strategies.

The aim of this study was to determine the effect of some autochthonous drought-adapted bacteria and one selected as drought tolerant from our collection on the growth, nutrition, and physiological values of *L. dentata* and *S. officinalis*. Both are plants that naturally grown in semiarid soils and are drought resistant. To reach these objectives, we test the mechanisms of bacteria and plant drought resistance and their interactions in drought tolerance.

The specific objectives of this study were the following: (1) isolation and characterization of autochthonous bacteria from rhizospheres of autochthonous plants; (2) to assess in native *Lavandula* and *Salvia* plant species, under drought conditions, the growth promotion, nutrition, and physiological and

biochemical traits related to drought tolerance in both non-inoculated and inoculated plants; and (3) to determine the bacterial characteristics as growth, proline, and IAA production under stress conditions. One reference strain of *Bacillus megaterium* drought-tolerant was used as reference to compare the particular activity among species of bacteria under drought stress conditions.

Values related to bacterial tolerance to osmotic stress as growth was determined along time with increasing levels of PEG in the culture medium. As well, proline and IAA produced were also evaluated in axenic culture under stress (15 % PEG) conditions.

Materials and Methods

Independent experiments were carried out in the present study. One microcosm experiment (experiment I) analyzed the effectiveness of three autochthonous or one of reference drought-adapted bacteria in improving plant growth, physiology, antioxidant activities, and nutrition as indexes of drought tolerance. In a second assay, we determine changes on maintenance of growth of the bacterial cells in axenic culture medium under increasing osmotic stress conditions (by PEG application) and their abilities for proline and IAA production under such stress conditions. These autochthonous bacteria were also identified using molecular methods.

Pot Experiment for Plant Growth

The plants used in the microcosm experiments under greenhouse conditions were *L. dentata* and *S. officinalis*. Both are low-growing shrubs widely distributed in the Mediterranean area selected. They are well adapted to the water stress conditions of this zone and, therefore, potentially could be used in the reforestation of semiarid disturbed lands. In this bioassay, we tested the effect of three autochthonous drought-tolerant bacteria and *B. megaterium* (used as reference, drought-adapted, strain) on these two native shrubs. The plant biomass, nutrition, stomatal conductance, antioxidant (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX)) activities, proline accumulation, and mycorrhizal intra- and extraradical colonization were evaluated. These values were determined after 1 year of plant growth in a natural soil under drought. Five replicated by the Mediterranean test soil used in this greenhouse experiment was air-dried, sieved to less than 2 mm, and mixed with quartz sand (<1 mm) at a soil/sand proportion (5:2, *v*/*v*). The test soil came from Molina de Segura (province of Murcia, Spain). Pots were filled with 500 g of the soil/sand (5:2, *v*/*v*) mixture. The main soil characteristics were pH 8.90, *P* value 1.36 μg/g by Olsen test, organic carbon 0.94 %, total N 0.22 %, and an electric conductivity of 1.55.

Bacteria Isolation and Identification

The autochthonous bacteria, identified as *Enterobacter* sp., *Bacillus thuringiensis*, and *Bacillus* sp. were isolated from the semiarid experimental soil from the Murcia Province (Spain). This area suffers from drought and low nutrients availability and as a result desertification. They were the most abundant bacterial types in such arid soil exhibiting different colony morphology and were isolated from the above-mentioned soil (a mixture of rhizospheres from several autochthonous plant species). For that following serial soil dilutions, 1 g of homogeneized soil was suspended in 100 mL of sterile water (dilution 10^2) and this suspension was further diluted to reach dilution 10^4 to 10^6. These suspensions (10^4 to 10^6 sown in agar nutrient broth medium, 8 g L^{-1}) were cultivated for 48 h at 28 °C. The abundance of those dominant colony forms, preliminarily referred as strains A, B, or C, were (as colony-forming units per milliliter counts) 120.10^4 (A), 85.10^4 (B), and 145.10^4 (C). They were independently grown in 250 mL flasks containing 50 mL of nutrients broth (8 g L^{-1}) medium in shake culture for 48 h at 28 °C. These bacteria isolates were cleaned and maintained suitable for the further in vitro and microcosm applications.

One milliliter of pure bacterial culture (10^8 cfu mL^{-1}) grown in nutrient broth medium for 24–48 h at 28 °C of temperature was applied to the appropriate pots at sowing time just below to plant seedlings, and 15 days later the bacterial culture (1 mL, 10^8 cfu mL^{-1}) was applied around the plant on the soil.

Identification of bacteria isolates 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 in 25 μL volume containing PCR buffer 10×, 50 mM $MgCl_2$, 10 μM of each primers 27FA (AGAGTTTGATCCTGGCTCAG) and 1492RA (GGTT ACCTTGTTACGACTT), and 5U/μL of *Taq* polymerase (Platinum, Invitrogen).

The PCR was performed in a thermal cycle with 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 1 cycle of 10 min at 72 °C. The products of PCR were analyzed by 1 % agarose gel electrophoresis. Extraction of DNA bacterial used QIAquick Gel extracton kit (QUIAGEN). Each sequence was compared with the database of 16S rRNA, the NCBI/BLAST. Autochthonous bacterial strains were identified as *B. thuringiensis* (98 %), *Bacillus* sp. (91 %), and *Enterobacter* sp. (99 %).

Plant Growth Conditions

These plants were grown for 1 year in pots containing a mixture of natural soil and quartz sand (5v/2v) under greenhouse conditions (temperature ranging from 19 to 25 °C, 16/8

light/dark photoperiod, and a relative humidity of 50–70 %). A photosynthetic photon flux density of 400–700 μmol m^{-1} s^{-1} was applied as supplementary light. Plants were grown along the experiment under drought conditions by keeping soil water capacity to 50 % each day after water application but water level decreased along day to nearly 30 % water capacity to the next water application.

Measurements

One year after planting, plants were harvested (five replicated per each treatment). Dry biomass of roots and shoots, nutrients concentrations, and mycorrhizal infection were determined.

Shoot concentrations (in milligram per gram) of P, K, Ca, and Mg as well as of Zn, Fe, and Cu (in microgram per gram) were determined from five different replicates per treatment after by flame photometry and colorimetry, respectively (Analytical Service of the "Centro de Edafología y Biología Aplicada del Segura" CSIC, Murcia, Spain).

Before harvest, some physiological plants values as stomatal conductance were measured (see below).

Stomatal Conductance

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

Root Colonization

Roots were carefully washed and stained. The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples [16], using the gridline intersect method [17] where the root sample was spread out evenly in dishes that had gridlines marked on the bottom to form 1.27 cm squares. Vertical and horizontal gridlines were scanned under a dissecting microscope at ×40 to ×100 magnification. The absence or presence of AM colonization was recorded at each point where a root intersected a line and at least 100 gridline intersects were tallied as the authors recommended.

The mycorrhizal extraradical mycelium was evaluated following the methodology proposed [18] which measured easily extractable protein.

Antioxidant Enzymatic Activities

Regarding method for the extraction of enzymes, plant cells were homogenized [19] 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) [20] was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50 % at 25 °C. CAT activity (EC 1.11.1.6) was measured as described [21]. Consumption of H_2O_2 (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min was monitored. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 100 μL of enzyme extract in a 2 mL volume. APX activity (EC 1.11.1.11) was measured in a 1-mL reaction volume containing 80 mM potassium phosphate buffer (pH 7.0), 2.5 mM hydrogen peroxide, and 1 M 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 [22]. GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH [23]. The reaction mixture (1 mL) contained 50 mM Tris buffer, 3 mM $MgCl_2$, 1 mM oxidized glutathione, and 50 μL enzyme extract, and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were expressed in micromole NADPH oxidized per gram fresh weight per minute, and the activity was calculated from the initial speed of reaction and the molar extinction coefficient of NADPH (ε_{340}=6.22 mM^{-1} cm^{-1}). Total soluble protein amount was determined using the Bradford method [24] and BSA as standard.

Shoot Proline Content

Free proline was extracted from 0.5 g of fresh leaves [25]. The methanolic phase was used for quantification of proline content. Proline was estimated by spectrophotometric analysis at 530 nm of the ninhydrin reaction [26].

In vitro experiment to determine microbial characteristics

Bacterial Growth Under Increasing PEG Levels in the Culture Medium

The bacterial isolates were checked in an additional in vitro experiment for testing the drought tolerance abilities to a reference strain. For that, the growth of drought-tolerant autochthonous bacteria under increasing PEG levels was assayed in comparison to a reference *B. megaterium* strain from our collection. The bacterial strains were cultivated at 28 °C in nutrient medium supplemented with 0, 15, 30, and 40 % of PEG. These treatments were replicated three times.

The number of viable cells was estimated along the time, from 3 to 6 days.

Production of IAA and Proline by the Bacteria Under 15 % PEG Along Time (5, 6, and 7 days)

The production of IAA by the bacteria was determined using the Salper reagent [27]. Three milliliters of fresh Salper reagent were added to free cell supernatant and kept in complete darkness for 1 h, and the optical density at 535 nm was measured in each treatment. A standard curve was prepared for IAA (Sigma, USA). The proline was estimated by spectrophotometric analysis at 515 nm [26].

Statistical Analyses

The data results of both experiments were subjected to analysis of variance (ANOVA) and Duncan's multiple-range test [28]. Percentage values were arc sine-transformed before statistical analysis.

Results

Differential Bacterial Effects on Plant Growth Responses Mycorrhizal Colonization and Plant Nutrition

As the results show, the inoculations of these bacteria resulted effective for plant growth and nutrition under the drought conditions along the experimented period here used (1 year). Nevertheless, responses of *L. dentata* and *S. officinalis* to the native and reference bacterial strains inoculated resulted different. In *L. dentata*, the autochthonous *B. thuringiensis* clearly caused the highest beneficial effect on shoot and root growth (Table 1). Nevertheless, *Enterobacter* did not affect *L. dentata* biomass. In the case of *S. officinalis*, the plant reaction to the bacteria applied was different and less relevant than in *L. dentata*. In fact, all of the inoculated bacteria enhanced *S. officinalis* growth but nonsignificant differences in *S. officinalis* plants on shoot growth between the inoculated with each one of the four bacteria were observed (Table 1). In *S. officinalis*, the bacterial inoculation increased particularly root development being *B. megaterium* and *B. thuringiensis* the most effective strains in increasing this value by 53 and 43 %, respectively, over controls plants. These bacteria also significantly increase total AM colonization in both plants (Table 1).

The bacterial inoculation of each bacteria increased the mycorrhizal potential of the natural soil particularly in *S. officinalis* (Table 1). Nevertheless, the mycorrhizal frequency, arbuscules production (a % and A %), and the extraradical mycorrhizal mycelium, estimated as glomalin content, in rhizosphere soil were not affected by the bacterial treatments

Table 1 Effect of autochthonous bacterial strains (*Enterobacter*, *B. thuringiensis*, *Bacillus* sp.) and the reference *B. megaterium* on shoot and root growth (in milligram) and total AM colonization in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions

	Shoot dry weight (mg)	Root dry weight (mg)	Shoot/root ratio	Total AM colonization
Lavandula dentata				
Control	650 a	360 a	1.80 ab	97 a
B. megaterium	970 b	460 b	1.50 a	138 b
Enterobacter	680 a	420 ab	1.62 s	168 b
B. thuringiensis	1090 c	510 c	2.14 b	153 b
Bacillus sp.	860 b	410 ab	2.10 b	115 a
Salvia officinalis				
Control	510 a	790 a	0.64 b	324 a
B. megaterium	670 b	1210 b	0.55 a	726 c
Enterobacter	620 b	880 a	0.70 b	466 b
B. thuringiensis	620 b	1130 b	0.55 a	655 c
Bacillus sp.	650 b	970 ab	0.67 b	475 b

Within each plant and value means followed by the same letter are not significantly different ($P \leq 0.05$) after ANOVA and Duncan tests

(data not shown). Shoot/root ratio was greater in *L. dentata* than in *S. officinalis* (Table 1).

In *S. officinalis*, inoculated bacteria did not increase K uptake but in *L. dentata* a big enhancement in K content was found in plants inoculated by bacteria (except *B. megaterium*) particularly by *B. thuringiensis* that increased this nutrient by 63 % (Table 2). Similarly, the highest bacterial effect on Ca and Mg content was determined in this plant associated to *B. thuringiensis*. Nevertheless, in both shrubs plants, nonsignificant differences in P content were found as result of the bacterial inoculation (Table 2). Concerning to the microelements acquisition, different trends were also observed in these both plants as affected by the bacteria inoculated. In *L. dentata*, *B. thuringiensis* enhanced Zn, Mn, and Cu by 23, 54, and 39 %, respectively. This bacterium did not increased any of these micronutrients in *S. officinalis* (Table 3).

Differential Bacterial Effects on Plant Physiological and Antioxidant Responses

As Fig. 1 shows, *B. thuringiensis* highly depressed stomatal conductance in *L. dentata* but such bacterial effect was not observed in *S. officinalis*. In *S. officinalis*, the most active bacteria in decreasing such value was *B. megaterium* (Fig. 1).

Regarding the antioxidant activities (Fig. 2), we can observe that in *S. officinalis*, in different way than in *L. dentata*, the antioxidant APX, GR activities, and proline did not change or were increased as affected by the bacterial

Table 2 Effect of autochthonous bacterial strains (*Enterobacter*, *B. thuringiensis*, *Bacillus* sp.) and the reference *B. megaterium* on P, K, Ca, and Mg shoot acquisition (milligram/plant) inoculated in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions

	P	K	Ca	Mg
Lavandula dentata				
Control	0.624 ab	13.512 a	13.297 b	2.143 ab
B. megaterium	0.542 a	18.353 b	15.270 b	2.360 ab
Enterobacter	0.502 a	15.026 a	9.831 a	1.449 a
B. thuringiensis	0.644 b	21.967 c	16.833 c	2.911 c
Bacillus sp.	0.642 b	19.478 b	15.449 bc	2.563 bc
Salvia officinalis				
Control	0.635 a	9.874 a	8.288 a	2.557 a
B. megaterium	0.757 a	9.953 a	12.191 b	3.228 a
Enterobacter	0.710 a	8.903 a	10.797 b	2.703 a
B. thuringiensis	0.779 a	9.416 a	11.196 b	2.876 a
Bacillus sp.	0.765 a	10.652a	16.918 c	3.304 a

Within each plant and value means followed by the same letter are not significantly different ($P \leq 0.05$) after ANOVA and Duncan tests

inoculations particularly by the native strains. In *L. dentata*, the opposite bacterial effects were found on these values. GR activity and proline were highly decreased in *L. dentata* by whatever bacteria inoculated.

The higher APX and GR activities were determined in non-inoculated *L. dentata* while the highest proline accumulation was determined in *S. officinalis* plants. As well, proline as GR and APX activities highly decreased in *L. dentata* by the bacteria applied. In *S. officinalis*, the bacterial inocula did not downregulated any of these activities as in *L. dentata* did. The similar SOD activity here observed in these plants and the lack of change as results of whatever bacterial inoculation indicates the lower value of this enzymatic activity as drought stress index in these plants (Fig. 2).

Bacterial Growth and Response Under Drought Conditions

With regard to the bacterial growth under increasing PEG levels in the axenic medium, we tested that *Enterobacter* exhibited the highest growth (cfu) which is indication of tolerance to the stress caused by the highest levels of PEG. In contrast, the reference strain *B. megaterium* resulted the most sensitive to whatever PEG level in the growing medium since all of the PEG concentrations used highly reduced the bacterial growth (Fig. 3).

Results of proline accumulation in the bacterial cells growing under 15 % PEG indicated that after 5 days of culture autochthonous bacteria show the greatest values and the reference *B. megaterium* the lowest. Nevertheless, the maximum proline production was determined in *Bacillus* sp. in a later growth periods (after 6 and 7 days of growth) (Fig. 4).

In the same way than proline, IAA production (under 15 % PEG) by the reference *B. megaterium* had the lowest amount at whatever culture time. The greatest IAA production was reached in *B. thuringiensis* culture irrespective of time of determination (Fig. 4).

Discussion

In this study, we evaluated the effectiveness on plant growth, antioxidant defense, and nutrition of three autochthonous drought-adapted rhizosphere bacteria and one allochthonous (also drought-tolerant) of reference under drought conditions in a natural semiarid soil. No previous information reports the results on bacteria inoculation on shrub development in a natural soil under stressful conditions and the results show that the inoculated drought-resistant bacteria were able to enhance growth and to improve plant performance under such stressed drought conditions.

The relationship between plant nutrition and drought stress is important due to nutritional unbalances caused by drought. Results show that particularly *B. thuringiensis* increased K^+, Ca^{++}, and Mg^{++} content in shoot of *L. dentata* plants. As it is well-known, K^+ content is an inorganic important osmolyte during drought. K^+ as inorganic osmolyte is important in water homeostasis under water deficit and it is able to regulate the stomatal opening, osmotic balance, maintenance of turgor pressure, and reduction of transpiration under drought stress [29]. Ca^{++} is also an important element controlling several

Table 3 Effect of autochthonous bacterial strains (*Enterobacter* sp., *B. thuringiensis*, and *Bacillus* sp.) and the reference *B. megaterium* on Zn, Fe, Mn, and Cu content (microgram/plant) inoculated in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions

	Zn	Fe	Mn	Cu
Lavandula dentata				
Control	38.504 b	104.247 b	13.409 a	5.189 b
B. megaterium	37.806 b	67.026 a	17.192 b	5.628 b
Enterobacter sp.	31.227 a	58.727 a	13.481 a	4.413 a
B. thuringiensis	47.391 c	100.209 b	20.599 c	7.226 c
Bacillus sp.	41.621 b	122.200 b	17.753 b	5.678 b
Salvia officinalis				
Control	29.495 b	56.409 b	17.300 b	3.981 a
B. megaterium	26.385 a	80.935 c	17.810 b	4.492 a
Enterobacter sp.	28.464 b	53.168 b	16.894 b	4.381 a
B. thuringiensis	24.600 a	50.378 b	19.657 bc	4.531 a
Bacillus sp.	30.573 b	57.186 b	23.974 c	6.080 b

Within each plant and value means followed by the same letter are not significantly different ($P \leq 0.05$) after ANOVA and Duncan tests

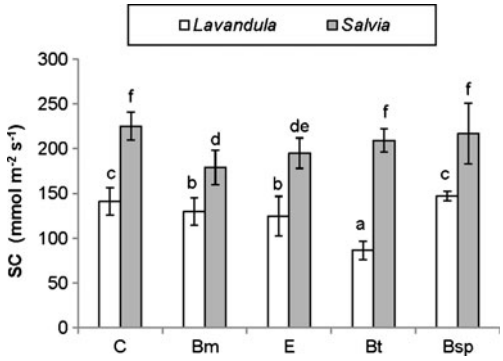

Fig. 1 Effect of autochthonous bacterial strains *Enterobacter* sp. (*E*), *B. thuringiensis* (*Bt*) and *Bacillus* sp. (*Bsp*) and the reference *B. megaterium* (*Bm)* on stomatal conductance (*SC*) in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions. Means followed by the same letter are not significantly different ($P \leq 0.05$) after ANOVA and Duncan tests

physiological processes under water stress conditions such as transpiration, cell wall synthesis, and cell division. Moreover, Ca^{++} is able to stabilize the membrane systems acting as an important cell protectant and Mg^{++} modulates the ion balance in cell, chloroplast, vacuolar membranes, and stomatal opening highly related to drought stress [30]. *B. thuringiensis* induced increase in all of these nutrients which indicate that the photosynthetic functioning is affected in a lower extent by drought in inoculated plants. In addition, micronutrients as Zn^{++}, Mn^{++}, and Cu^{++} also increased in *B. thuringiensis*-inoculated *L. officinalis* plants. It is known that drought stress may affect not only the availability of micronutrients particularly of those slow diffusing but also the competitive uptake and transport is affected. All these changes were considered adaptative responses to the water deficiency. The lack of change or depressing effect in Fe^{++} content after the inoculation may be due to the lack of disturbance of this element by the drought.

In these stressed drought soils, plants are more dependent on microbial activity which is able to increase nutrients and water uptake [4]. The persistence and survival of bacterial community in the rhizosphere soil is very important in stressed environment for the establishment of plants in such environments [31]. But the endophytic condition of these bacteria, as here was tested, is an important mechanism of inocula survival along time.

The axenic culture studies confirmed that the reference allochthonous *B. megaterium* exhibited the lowest tolerance to water deficit caused by osmotic stress (PEG) while autochthonous strains, particularly *Enterobacter*, resulted the most tolerant to the highest stress (30 and 40 % of PEG) in the growing medium. Regarding the proline production by these bacteria as compatible solute able to help cells in the

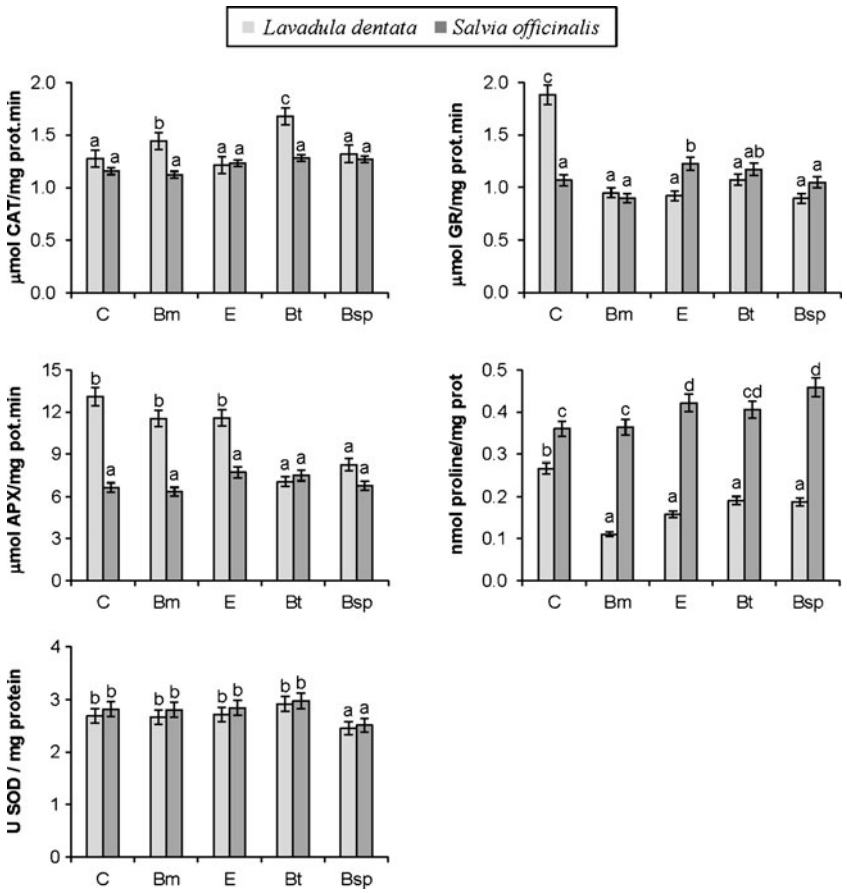

Fig. 2 Effect of autochthonous bacterial strains *Enterobacter* sp. (*E*), *B. thuringiensis* (*Bt*) and *Bacillus* sp. (*Bsp*) and the reference *B. megaterium* (*Bm*) on CAT, APX, GR, and SOD antioxidant activities in shoot and proline accumulation in two autochthonous plants (*L. dentata* and *S. officinalis*) growing in a natural arid Mediterranean soil under drought conditions. Means followed by the same letter are not significantly different ($P \leq 0.05$) after ANOVA and Duncan tests

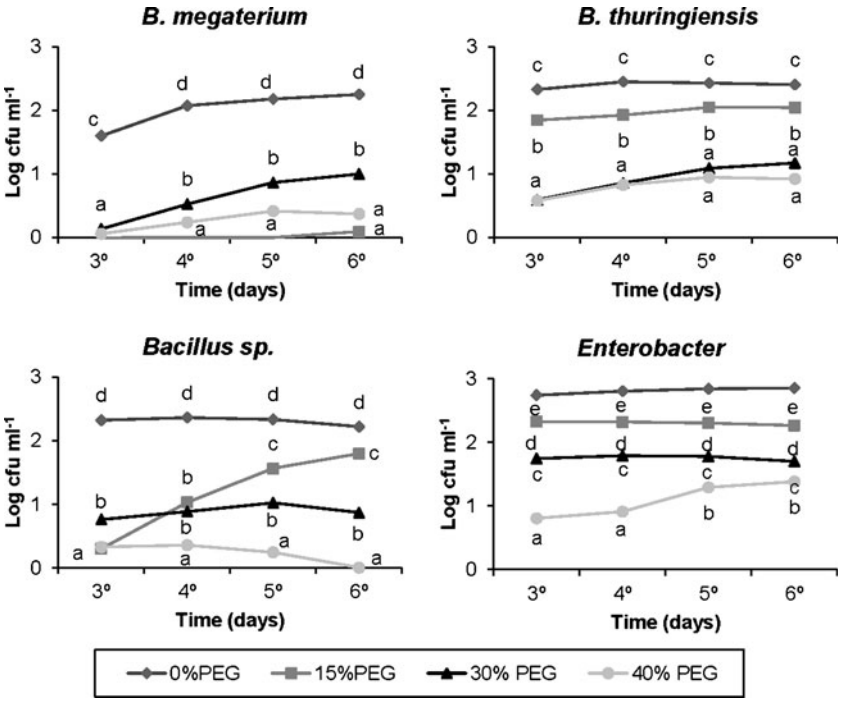

Fig. 3 Viable cells (log cfu per milliliter) of bacterial strains growing in axenic nutrient medium supplemented with increasing levels of PEG at different time intervals (from 3 to 6 days)

osmoregulation processes and to facilitate water uptake in response to the stress [32], we determine that the reference *B. megaterium* also resulted the lowest proline producers under stress conditions. Proline induces the adjustment of cell osmotic potential and this is indicative of osmotic adaptation by the bacterial cells. In fact, the cells of *Bacillus* sp. required a greater proline accumulation than the other bacteria assayed as strategy to cope with drought (applied as PEG). Proline may be used for compensating the bacterial lack of drought tolerance. Similarly, the IAA production by these bacteria under stress conditions evidences their particular ability to promote plant growth under such environmental stress [33–36]. The reference *B. megaterium* also showed the lowest capacity for IAA production under stress conditions. IAA prevents the sensitivity to ethylene suppressing ethylene-initiated abscission signaling [37]. Microorganisms, depending on the environmental damage, can increase the activity preceding the final loss of function of a certain threshold value.

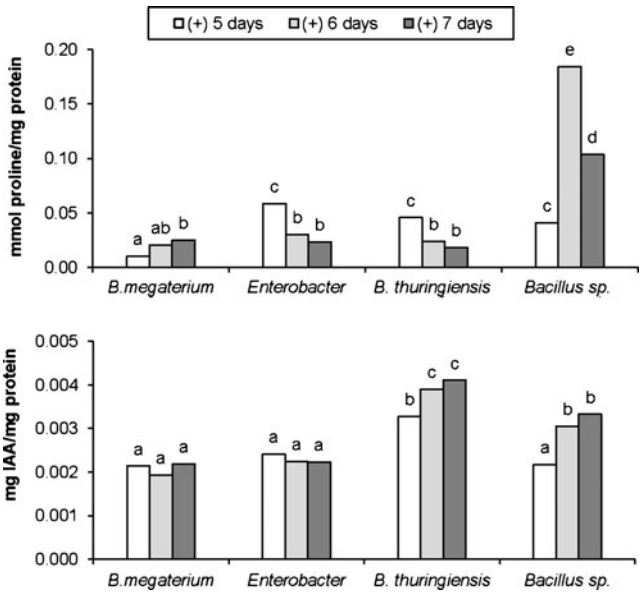

Fig. 4 Cell proline accumulation and indoleacetic acid (IAA) production by native and reference bacteria growing in axenic nutrient medium supplemented with PEG (15 %) at different time intervals (from 5 to 7 days)

In general, in the past, plant growth-promoting rhizobacteria (PGPR) have been used mainly for plant growth promotion by producing plant growth regulators. The ability of autochthonous bacteria to produce auxin-indole derivatives (as here was measured, under osmotic stress, in the axenic culture medium) can cause part of the stimulating effects tested under these stress conditions. But recent studies show additional beneficial effects on different plant species through the bacterial ability to improve tolerance toward abiotic stresses [1, 38]. Several stress markers analyzed by molecular and biochemical methodologies studied the role of priming on different stress tolerance mechanisms by PGPR [5, 39]. Studies [4] show that plants colonized by the *B. megaterium* strain here used increased water content in *Trifolium repens* under water stress. This effect is particularly important in drought environments for preventing damage and enhancing plant survival under arid conditions. Nevertheless, it seems that various mechanisms were functioning in the stimulation of plant drought tolerance by the inoculated bacteria. In this study, K uptake was increased for the bacterial inocula more in

L. dentata than in *S. officinalis* being *B. thuringiensis* the most active bacterial strain which resulted in very efficient enhancing K particularly in *L. dentata* (63 % over control). Here, in *L. dentata*, the K content correlated positively with the enhancement of plant biomass and a decrease of stomatal conductance as affected by the bacterial inoculation. Zhang et al. [40] reported that the salt tolerance in *Arabidopsis thaliana* was mediated through regulation of the HKT1 potassium transporter when inoculated with a *Bacillus* strain. The bacterial activity increasing K in *L. dentata* can be recognized as a very important mechanism to support drought conditions. Concomitantly, stomatal conductance was highly decreased in *L. dentata* inoculated with *B. thuringiensis*. This reduced evapotranspiration by the bacterial inoculation avoided water deficits.

One important mechanism related to stress tolerance is to alter oxidative stress that is necessary for plant survival under drought stress. Few data are available about the mechanism involved in bacterial-mediated plant antioxidant protection and the relevance of such processes in plants survival and adaptation to drought under arid conditions. Plants have no immune system but they have alternative defense strategies as tools to overcome stress constraints, adapt to the changing environments, and survive. The accumulation of ROS in plant cells under stress are removed by enzymatic systems and the increase in antioxidant enzymatic activities is correlated with the stress severity [41, 42]. Here, in *L. dentata*, APX activity was highly decreased (by 85 %) when inoculated with *B. thuringiensis* and it is considered the key antioxidant enzyme in the ascorbate–glutathione redox cycle and APX plays an important role in scavenging ROS [43]. In parallel, in *B. thuringiensis*-inoculated *L. dentata*, the GR activity also was highly depressed (by 57 %) and it has a central role in maintaining the reduced glutathione pool during the drought stress [44].

Antioxidant activities, particularly APX and GR, decreased in *L. dentata* colonized by the most effective bacteria (*B. thuringiensis*), indicating an important relationship among the level of antioxidant responses and this plant's adaptation to the drought stress but this effect varied according the plant species involved. The reduction of these antioxidant production in bacterial-inoculated plants means an energy save in favor of vital processes [45]. This is one procedure to decrease the detrimental effects caused by drought. As well, the decrease observed in such antioxidant activities in inoculated plant responses to drought represents the better adaptation to the stress conditions, showing that lower antioxidant activities indicate a reduction of ROS level in stressed plants [5].

Regarding values of these antioxidant activities in *S. officinalis* inoculated by this bacteria (*B. thuringiensis*), different results than in *L. dentata* were found. In fact, both plants differ in antioxidant activities in response to stress. In general, *S. officinalis* shows lower intrinsic GR and APX activities than *L. dentata* which supports the hypothesis about the ability of this plant to have reduced these antioxidants levels under water stress conditions. The low CAT activity of *L. dentata* in response to drought may be caused by the use of GR and APX that have a much higher affinity for H_2O_2 than CAT [46]. *S. officinalis* has lower APX and GR but occasionally higher CAT than *L. dentata* and such antioxidant differences reflected intrinsic osmotic stress tolerance under drought. Such plant diversity in stress tolerance implies that inoculated bacteria may play multifaceted role to sustain drought avoidance in these plants.

In contrast, both *L. dentata* and *S. officinalis* maintained similar SOD activity and without any change in inoculated plants. This is an indication about the nonsignificant role of this activity in the defense against the oxidative stress induced by drought. Nevertheless, changes in plant CAT, APX, and GR activities as result of inocula applied would be useful markers for the bacterial effect on strategies of drought tolerance in these plants.

Results show that stress mechanisms are different between these plant species. Thus, according to these results, the plant responses to bacterial inoculation on drought tolerance was different probably due to the relative effect of the bacterial colonization changing nutrition and physiology in each host plant.

The variation found between the protective enzymatic systems as affected by bacterial inocula in these two plants suggests that the bacterial effectiveness in drought tolerance act through particular and more or less specific mechanisms depending on the host plant. There is limited information on the varied growth-promoting effect of particular bacteria on host plant under different environmental natural conditions. Thus, it is important to identify the relevant factors involved in the plant responses under drought stress conditions to ascertain the bacterial effectiveness in arid environments. Previous studies reported that microbial groups as mycorrhizal fungi and/or PGPR also change antioxidant activities [47, 48].

In *L. dentata*, whatever inoculated bacteria increased K content, in particular the most efficient is *B. thuringiensis*, and in contrast, in *S. officinalis* any of them increase this nutrient. The osmotic stress tolerance can be modulated by the accumulation of this cation. Potassium is considered the most important inorganic osmolyte.

The bacteria applied also stimulate root growth being such effects strongest in *S. officinalis* that particularly has the greatest root development. An important mechanisms related to the enhancement of plant tolerance to drought may be the change in shoot/root ratio in inoculated plants and it could improve the ability of these plants for increasing their water content. Plants as *S. officinalis* having a well-developed root system, particularly when inoculates with particular bacteria have the highest possibility for taking up water from the medium. Inoculated bacteria were able to produce IAA under

drought conditions (as in axenic conditions was observed) and this phytohormone can be responsible for the root enhancement in inoculated plants. IAA production may also improved water use efficiency regulating plants physiological status as here was evidenced. Thus, the bacterial inocula may also affect the adjustment of water partitioning into apoplastic or symplastic space improving the drought tolerance [49].

S. officinalis shows a higher adaptation and/or tolerance and suffer less than *L. dentata* under the stress and results suggest that inoculated *L. dentata* plants have an increased possibility of water acquisition under water limitation.

S. officinalis shows the highest amount of proline and the lowest GR and APX activities. It seems that in this plant proline correlated with a negative regulation of GR and APX activities. In contrast, low proline in *L. dentata* and high GR and APX activities suggest a more direct role of these enzymes in the *L. dentata* protection against oxidative injury. Cells with a greater proline accumulation have a lower lipid peroxidation by drought stress. The efficient and active role of proline in depressing ROS damage has been suggested [50].

Curiously, these nutritional, physiological, and biochemical differences between *S. officinalis* and *L. dentata* significantly affected their particular response to the bacterial inocula applied. Differences in whatever parameter here evaluated reflected the diversity and particular stress tolerance of these plants. These and previous results indicate that each plant may play a multifaceted role to maintain health and a multiplicity of factors may be involved in reaching the optimum growth under drought conditions [4, 47, 48, 51].

Inoculated autochthonous bacteria have a varied and strong impact in improving plant stress tolerance mechanisms [1]. Bacteria can help plants in the osmoregulation processes and in improving homeostatic mechanisms upon stress challenge [49]. As results show, a combination of nutritional, metabolic, physiological, and morphological changes on the inoculated plants are carried out by the bacteria able to control drought stress in plants. But plant characteristics are important factors affecting the bacterial role in plant adaptation to drought.

According to the results, *B. thuringiensis* produced the highest amount of IAA and proline (at 15 % PEG) in axenic culture and this is correlated with the greatest *L. dentata* growth and K nutrition and the lowest stomatal conductance and antioxidant activities. In fact, these measurements resulted an useful marker of bacterial effectiveness in this plant under water stress conditions. As well, it is important, from a practical point of view, to know that these bacteria were able to survive and to multiply to reach a sufficient population to express himself activities under stress conditions. This suggest that they can maintain a long time their biochemical traits related to positive effects in inoculated plants under water-limiting conditions. The water limitation and osmotic stress negatively affect plant growth but the bacterial inoculation was able to attenuated these detrimental effects.

The use of bacteria to control drought stress in plants is an important and sustainable strategy. But the related processes seem to be regulated differently according to the natural resistance and intrinsic stress tolerance of the plants. The selection of microorganisms involved is important to reach the maximum plant benefit. However, further research studies are required to establish the main processes by which bacteria improve plant performance.

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