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Native plant growth promoting bacteria *Bacillus thuringiensis* and mixed or individual mycorrhizal species improved drought tolerance and oxidative metabolism in *Lavandula dentata* plants

E. Armada[a], A. Probanza[b], A. Roldán[c], R. Azcón[a,*]

[a] *Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Prof. Albareda 1, 18008 Granada, Spain*
[b] *Departamento de Ciencias Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad San Pablo CEU, 28668 Boadilla del Monte, Madrid, Spain*
[c] *CSIC-Centro de Edafología y Biología Aplicada del Segura, Department of Soil and Water Conservation, P.O. Box 164, Campus de Espinardo, 30100 Murcia, Spain*

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ABSTRACT

This study evaluates the responses of *Lavandula dentata* under drought conditions to the inoculation with single autochthonous arbuscular mycorrhizal (AM) fungus (five fungal strains) or with their mixture and the effects of these inocula with a native *Bacillus thuringiensis* (endophytic bacteria). These microorganisms were drought tolerant and in general, increased plant growth and nutrition. Particularly, the AM fungal mixture and B. thuringiensis maximized plant biomass and compensated drought stress as values of antioxidant activities [superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase APX)] shown. The AMF-bacteria interactions highly reduced the plant oxidative damage of lipids [malondialdehyde (MDA)] and increased the mycorrhizal development (mainly arbuscular formation representative of symbiotic functionality). These microbial interactions explain the highest potential of dually inoculated plants to tolerate drought stress. *B. thuringiensis* "in vitro" under osmotic stress does not reduce its PGPB (plant growth promoting bacteria) abilities as indole acetic acid (IAA) and ACC deaminase production and phosphate solubilization indicating its capacity to improve plant growth under stress conditions. Each one of the autochthonous fungal strains maintained their particular interaction with *B. thuringiensis* reflecting the diversity, intrinsic abilities and inherent compatibility of these microorganisms. In general, autochthonous AM fungal species and particularly their mixture with *B. thuringiensis* demonstrated their potential for protecting plants against drought and helping plants to thrive in semiarid ecosystems.

1. Introduction

Plants inhabiting arid or semiarid areas suffer from many abiotic stresses such as water deficiency, limitation in essential macronutrients and low organic matter, the latter due mainly to the plants' limited establishment and production. Native plant species establishment are used as the most effective strategy in arid ecosystems and in semiarid Mediterranean areas for reclaiming these degraded soils (Bashan et al., 2012). Mycorrhizae may help plants to thrive in semiarid ecosystems (Azcón et al., 2013). This symbiosis is widespread under natural stress conditions and it occurs in nearly all environments. The arbuscular mycorrhizal (AM) fungi are able to colonize and function in poor degraded ecosystems such as mine soil (Vivas et al., 2003a) or under arid/saline conditions (Ruíz-Lozano et al., 2012), but such detrimental environmental factors have a negative effect on the development of AM symbiosis. The AM fungi have the ability to colonize the roots of most vascular plants and AM colonized plants cope more effectively with water deficit. The mycorrhizal effect is based on direct and indirect mechanisms, for example, mycorrhizal myceliums have access to soil pores therefore being more efficient than roots for nutrient and water extraction (Azcón and Barea, 2010). It is well known that mycorrhizal plants enhanced the uptake of nutrients, especially these with low mobility such as P, Zn, Cu and others. Physiological and biochemical changes related to mycorrhizal plant drought tolerance have been described (Marulanda et al., 2003, 2007). Thus, the plants' ability to cope with environmental stresses is enhanced by AM fungal colonization and AM fungi have been considered an important functional component of the soil/plant system in disturbed soils. There is a lot of evidence that AM fungi are adapted to

* Corresponding author.
E-mail addresses: elisabeth.armada@eez.csic.es (E. Armada), a.probanza@ceu.es (A. Probanza), aroldan@cebas.csic.es (A. Roldán), rosario.azcon@eez.csic.es (R. Azcón).

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edaphic conditions but differences in fungal behaviour, efficiency on plant growth and stress tolerance can be, at least partly, due to the fungus involved. Nevertheless, the whole extent to which the plant benefits from particular AM species is still unknown.

The value to AM-derived nutrients, in terms of its C cost, is therefore likely to vary between particular plant-fungus associations. Carbon demand by each AM fungus is considered a cost of the symbiotic association and this need to be compensated (Wright and Upadhyaya, 1998). Drought highly reduced C assimilation processes by the host plant but plants respond differently to particular AM colonization according to how each fungus affects the process of C assimilation in the host-plant and the C requirements of each fungus. These results highlight the diversity in the way, function and reaction of AM colonization according to partners involved and the environmental conditions. The existence of species' specific interactions between the host-plant and the fungal species underlines the importance of screening of fungal species to maximize the benefits of the symbiosis (Alguacil et al., 2003). Authors reported that the inoculation with a mix of native AM fungi was a more effective treatment for the development of *Retama sphaerocarpa* than an allochthonous fungus, *Glomus claroideum* in a semiarid ecosystem. Other studies have focused on the importance of the origin of the AM fungi to be used as inocula in dry soil when plants were colonized by drought-sensitive or drought-tolerant *Glomus* species (Marulanda et al., 2007).

The assimilation of nutrients by AM colonized plants reflects the amount of direct plant uptake plus the indirect contribution from the AM fungus. However, both ways became weaker when water availability decreased, that even AM colonization decreased. Drought may induce changes in the metabolic capacity reducing the infective fungal capacity but these characteristics have not yet been studied.

Adverse environmental conditions can negatively affect the diversity and number of spores in soil and also the infectivity of AM propagules (Gosling et al., 2013). The negative effect of drought stress can be compensated by rhizosphere bacteria that are able to improve the growth of AM fungi (Vivas et al., 2003a).

Plant growth promoting bacteria (PGPB), as component of soil microbiota, have the potential role of improving the establishment of plant species under arid soil conditions (Hayat et al., 2010). They can colonize root surface and/or intercellular spaces in plant tissues.

Many mechanisms lead to plant growth promotion as phytohormones production, nutrients and water acquisition and others have been described (Armada et al., 2014). But unpredictable results of PGPB inoculation can be found mainly caused by the quality and resistance/tolerance of inoculants to the severe stress conditions. Thus, the bacterial ability to produce compounds that play important role in the process of osmotic adjustment decreasing the cell osmotic potential allowing greater water retention during drought were evaluated under axenic conditions using polyethylene glycol (PEG) as an osmotic stress agent.

Regarding plant biochemical parameters affected by water stress, reactive oxygen species (ROS) have been used as an indicator of drought tolerance. Water stress generates ROS production that may cause lipid peroxidation, protein degradation, membrane injury and cell death (Apel and Hirt, 2004). Major ROS scavenging enzymes include antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) to control the cellular redox status under stress conditions such as drought (Apel and Hirt, 2004; Ferrol and Pérez-Tienda, 2009). These antioxidant enzymes increase the ability of plants to resist environmental stresses. Nevertheless, the effectiveness of autochthonous microorganisms in plant drought tolerance has been scarcely reported (Marulanda et al., 2003, 2007).

The diversity of AM fungi is low compared to that of host plants, although evidence from molecular methods suggests that the diversity of AM fungi is higher than expected (Lumini et al., 2010). However, the relatively low AM diversity shows differences according to fungus, habitants and host species involved (Hazard et al., 2013). It seems attributable to functional differences between AM fungi (Maherali and Klironomos, 2007).

For this paper, the plant *Lavandula dentata* was selected as a representative shrub species from semiarid scrublands in the southeast of Spain. This plant is well-adapted to drought conditions and it was a prevailing plant species growing in the arid zone of study.

Combined microbial inoculations resulted more effective to induce resistance to drought conditions and in the protection of plants against a drought stress enhance revegation process. We conducted a pot experiment in a semiarid Mediterranean soil under drought conditions and we assayed if *L. dentata* was more benefited from the inoculation with a whole autochthonous AM fungal consortium or from each one of the single native fungal isolates (selecting the five most abundant and representative ecotypes). In addition, the autochthonous beneficial bacteria *Bacillus thuringiensis* was assayed in interaction with native AM fungi (single or mixture) stimulating plant growth, nutrition and drought tolerance. Thus, here we hypothesised that the combined inoculation involving autochthonous microorganism (single or mixed AM fungi and *B. thuringiensis*) could be beneficious to enhance *L. dentata* growth under water stress conditions. The drought tolerance, PGPB characteristics and endophytic conditions of *B. thuringiensis* here used were also evaluated. The aim is to verify the potential of plant coinoculation to increase drought tolerance and to alleviate the impact of water stress. Selected soil microorganism may help an important role in the establishment of autochthonous plant cover under arid environmental conditions.

2. Material and methods

Independent experiments were carried out in the present study. Firstly, an autochthonous bacteria, isolated from the semiarid experimental soil from the province of Murcia (Spain), was identified using molecular methods and in an *in vitro* assay, we determined changes on maintenance of growth of the bacterial cells in axenic culture medium under non stress and stress osmotic conditions [by 40% polyethylene glycol (PEG) application] and their abilities to produce proline, lipid peroxidation (MDA) or poly-ß-hydroxybutyrate (PHB) and PGPB characteristics tested as α-ketobutyrate (ACC deaminase), indole acetic acid (IAA) production and phosphate solubilization under such non-stress and stress conditions. Secondary, a microcosm experiment under drought conditions analyzed the effectiveness of five autochthonous AM fungal species single or in consortium inoculated and the impact of autochthonous bacteria in improving plant growth, physiology, nutrition and antioxidant activities as indexes of drought tolerance.

2.1. Isolation and molecular identification of the bacterial strain

The autochthonous bacteria strain used throughout this study were isolated from the same natural soil used in the bioassay (see description below). The bacterium was isolated from a mixture of rhizosphere soils from several autochthonous shrub species.

A homogenate of 1 g of soil in 9 mL of sterilized water was diluted (10^{-2} to 10^{-4}), plated on three different media [Yeast Mannitol Agar, Potato Dextrose Agar, Luria-Bertani (LB) Agar] and then incubated at 28 °C for 48 h, to isolate bacteria from different taxonomic groups. The selected bacterium was the most abundant bacterial type in such arid soil.

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), (Rees et al., 2004) 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, 45s at 44 °C and 2 min at 72 °C, and finally one cycle of 10 min at 72 °C. The products of PCR were analyzed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QIAGEN) for subsequent sequencing in an automated DNA sequencer (PerkinElmer ABI Prism 373). Sequence data were compared to database (NCBI) using BLAST program.

2.2. Isolation and identification of the arbuscular mycorrhizal (AM) fungi

The method used in the isolation of spores of the arbuscular mycorrhizal fungi from rhizosphere soil samples, called "method wet sieving and decanting" (Gerdemann and Nicolson, 1962) optimizes the separation of the spores from other mineral and organic soil particles.

A suspension of soil in water was filtered through a chain of different diameter mesh strainers (500, 250 and 50 μm). The contents of each sieve were then collected and they were counted using a stereo-microscope (30–40X). The population of arbuscular mycorrhizal was increased through the establishment of plants 'trap' (Sieverding, 1991). This method involves growing plants with a strong dependence on mycorrhizal, in the soil of study. Thus fungal species can complete their life cycles and sporulate mass, resulting in a diverse population of species of AM fungi at different stages of ontogenic.

The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG) (Koske and Tessier, 1983); a mixture of PVLG and Melzer's reagent (Brundrett et al., 1994) a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain, 1990). For identification of the AM fungi species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification (Oehl et al., 2011).

2.3. Evaluation in axenic culture of B. thuringiensis growth, stress tolerance abilities and PGPB characteristics under non-stress and stress (40% of PEG) conditions

2.3.1. B. thuringiensis *growth*

Bacterial strain were cultivated at 28 °C in nutrient broth (Luria-Bertani (LB)) medium supplemented with PEG (40%) to generate osmotic stress (equivalent to −3.99 MPa). This level of PEG was selected in preliminary studies as the maximum PEG concentration supportable by bacterial strain. The number of viable cells was estimated after 4 days of growth following the conventional procedure: 1 mL of suspension was plated in nutrient broth medium. The bacterial growth was monitored by measuring optical density at 600 nm (Armada et al., 2014).

2.3.2. B. thuringiensis *stress tolerance abilities*

The bacterial isolates were cultivated at 28 °C at 120 rpm in 100 mL of liquid nutrient (LB) medium supplemented or not with 40% of PEG (−3.99 MPa) in order to induce drought stress.

The accumulation of proline was estimated by spectrophotometric analysis at 530 nm (Bates et al., 1973). The bacterial extract reacts with ninhydrin and glacial acetic acid for 1 h at 100 °C. The reaction stops by introducing the tubes in an ice bath. The reaction mixture is extracted with 2 mL of toluene, shaking vigorously for 20 s. A standard curve which was prepared with known concentrations of proline.

Measurement of lipid peroxidation was done by the method based on the reaction of 2-thiobarbituric acid (TBA) with reactive species derived from lipid peroxidation, particularly malondialdehyde (MDA). Detection of 2-thiobarbituric acid reactive substances (TBARS) was carried out by a colorimetric assay described by Buege and Aust (1978) with some modifications (Espindola et al., 2003). The absorbance was measured at 532 nm. Lipid peroxidation was expressed as μmoles of malondialdehyde g^{-1} of dry cell weight.

The poly-β-hydroxybutyrate (PHB) production of the bacterial strain 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}.

2.3.3. B. thuringiensis *PGPB characteristics*

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).

The production of indole-3- acetic acid (IAA) by these bacteria was determined using 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.

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 resuspended in 0.9% NaCl to produce equal cell densities among. Solutions were inoculated on the agar plates and incubated at 30 °C, and observed daily for 7 days for the appearance of transparent "halos" (Katznelson and Bose, 1959). Experiments were performed in triplicate. Phosphorus solubilization index was measured using the following formula (Edi-Premono et al., 1996).

$$\mathrm{PSI} = \frac{\text{Colonydiameter} + \text{Halozonediameter}}{\text{Colonydiameter}}$$

2.4. Microbial inoculation in Lavandula dentata *plants under greenhouse conditions*

2.4.1. Experimental design

The experimental work was based on a design with two factors: isolates of arbuscular mycorrhizal fungi species predominant in the study area (see results, five different AM fungi species: *Septoglomus constrictum* EEZ 198; *Diversispora aunantia* EEZ 199; *Archaeospora trappei* EEZ 200; *Glomus versiforme* EEZ 201; *Paraglomus ocultum* EEZ 202 and a mixture or consortium of these AM fungi) and bacte-

rial inoculation treatments [bacteria native isolated of study zone: control (−); *B. thuringiensis* (B.t)].

2.4.2. Test soil and inoculation of microorganisms

The soil used in this experiment is located in the National Park of "Vicente Blanes" in the town of Molina de Segura, Murcia (Spain), (coordinates: 38° 12' N, 1° 13' W; altitude 393 m). The main features of this soil was that it was a soil with low organic matter content and a silty-clay texture which are both causes for very easy ground degradation. The main characteristics of the soil were pH 8.90, P 1.36×10^{-3} g kg^{-1} (Olsen test), organic carbon 0.94%, total nitrogen 0.22%, electrical conductivity of 1.55 dS m^{-1}. The substrate used in this assay consisted in using the previously mentioned Mediterranean soil (sterilized and sieved by 5 mm), and mixed with sterile sand to the ratio of (5:2, (v/v)). Substrate was put into pots with a capacity of 0.5 kg. The plant used in this study was *Lavandula dentata* and was grown under drought conditions for six months in greenhouse.

One milliliter of pure bacterial culture (10^7 cfu mL^{-1}) grown in nutrient broth medium for 48 h at 28 °C, was applied to the appropriate pots at sowing time just below plant seedlings, and 15 days later the bacterial culture (1 mL, 10^7 cfu mL^{-1}) was applied around the plant on the soil. Five grams of different isolates of arbuscular mycorrhizal fungi (AMF) species and consortium of AMF per pot were applied to each one of the appropriate pots at sowing time just below the seeds. Five replicates of each treatment were used, making a total of 70 pots.

2.4.3. Plant growth conditions

These plants were grown for six months in pots containing a mixture of sterile soil and sterile quart sand (5:2, (v/v)) under greenhouse conditions (temperatures ranging from 15 °C to 21 °C; 16/8 light/dark photoperiod, and a relative humidity of 50–70%). A photosynthetic photon flux density of 400–700 μmol m^{-2} s^{-1} was applied as supplementary light. Plants were grown during the experiment under drought conditions by keeping soil water capacity to 50% each day after water application but water levels decreased gradually during the day to nearly 20% water capacity until the next water application.

2.4.4. Plant biomass analyses

After six months of growth, plants were harvested (five replicates of each treatment) shoots and roots were weighed and dried for 48 h at 75 °C to obtain dry weights. Shoot/root ratio (g) was also calculated.

Shoot content (mg $plant^{-1}$) of C, N, P, K, Mg and Ca as well as of Mn, Cu, Fe, and Zn (μg $plant^{-1}$) 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.

2.4.5. Root colonization

2.4.5.1. Mycorrhizal colonization. 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) after counting 150 intersections. Mycorrhizal development was evaluated by the method of Trouvelot et al. (1986) using MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The parameters measured according to this method were the frequency of AM colonization in the sample (F%), intensity of AM colonization in the whole root system (M%), and absolute arbusculum richness (A%) referred to the calculated whole root system respectively. The images were performed with a Nikon Eclipse 50i microscope equipped with a Nikon DS-Fi1 camera.

2.4.5.2. B. thuringiensis *endophytic colonization.* Bacterial endophytic colonization was realized only in two treatments (B.t; B.t + MIX) due to lack of root biomass in the remaining treatments and also by our interest in evaluates the bacterial endophytic colonization mainly in these two treatments. Roots containing rhizospheric soil were treated according to Forchetti et al. (2007). One centimeter root section from the two treatments was aseptically excised, and homogenates were serially diluted in 0.1 M $MgSO_4$ to enumerate the bacteria colonizing the root (cfu per cm) (Idris et al., 2007).

Transmission electron microscopy (TEM) in root of two treatments mentioned above (B.t; B.t + MIX) were fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) dehydrated in a graded series of ethanol and embedded in unicryl resin. Ultrathin sections were examined with a transmission electron microscope JEOL 1011.

2.4.6. Oxidative damage to lipids in shoots

The non-mycorrhizal plants [non-inoculated (−) and inoculated with *B. thuringiensis* (B.t)] showed insufficient shoot biomass for the following determinations.

Lipid peroxides were extracted by grinding 0.5 g of shoot with ice-cold mortar and 5 mL of trichloroacetic acid (TCA) 5%. Homogenates were centrifuged at 12,290 × *g* for 10 min. The chromogen was formed by mixing 0.5 mL of supernatant with 1.5 mL of a reaction mixture containing 20% (w/v) TCA, 0.5% (w/v) TBA, and by incubating the mixture at 95 °C for 30 min (Minotti and Aust, 1987). After cooling at room temperature, absorbance of samples was measured at 532 nm. Lipid peroxidation was estimated as the content of TBARS and expressed as equivalents of MDA according to Halliwell and Gutteridge (1989). The calibration curve was made using MDA in the range of 0.1–100 μmol. A blank for all samples was prepared by replacing the sample with extraction medium.

2.4.7. Antioxidant enzymatic activities in shoot (SOD, CAT, APX and GR)

The antioxidant enzymatic activities of the non-mycorrhizal plants [non-inoculated (−) and inoculated with *B. thuringiensis* (B.t)] were not performed due to small amount of shoot biomass, insufficient to proceed for its determination. The method followed for the extraction of enzymes of shoot tissues was that described by Aroca et al. (2003). Total SOD activity (EC 1.15.1.1) (Burd et al., 2000) was measured on the basis of SOD's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C. CAT activity (EC 1.11.1.6) was measured as described 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}). APX activity (EC 1.11.1.11) was measured in a 1 mL reaction volume containing 80 mM potassium phosphate buffer (pH 7.0), 0.5 mM hydrogen peroxide and 0.5 mM sodium ascorbate. The H_2O_2 was added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). GR activity (EC 1.20.4.2.) was estimated by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH (Carlberg and Mannervik, 1985). The reaction mixture (1 mL) contained 50 mM Tris buffer, 3 mM $MgCl_2$ (pH 7.5), 1 mM oxidized glutathione, 100 μL enzyme extract and 0.3 mM NADPH was added and mixed thoroughly to begin the reaction. The results were

expressed in mmol NADPH oxidized mg^{-1} protein, 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 (Bradford, 1976) and bovine serum albumin as standard.

2.5. Statistical analyses

Data from both experiments were analyzed using the SPSS 21 software package for Windows and were subjected to a one-way general linear model ANOVA (analysis of variance) which was used to determine the effect of each treatment. Duncan's multiple-range test (Duncan, 1955) was used for post hoc analysis to determine differences between means. Differences were considered significant at $p \leq 0.05$. Percentage values were arcsine-transformed before statistical analysis.

3. Results

3.1. Identification of bacterial strain and of arbuscular mycorrhizal (AM) fungi

Each sequence obtained was compared with the database of 16S rDNA from the NCBI/BLAST. The similarity unambiguously identified the bacterium as *B. thuringiensis* (Accession NR 43,403.1, identity 98%).

Fungal characterization has been done using morphological techniques (Barea et al., 2011). The more predominant AMF species identified in the study area were: *Septoglomus constrictum*, *Diversispora aunantia*, *Archaespora trappei*, *Glomus versiforme* and *Paraglomus ocultum*, which were cataloged and included in the collection of EEZ (codes 198-202).

3.2. Characterization of bacterial osmotic stress tolerance and PGPB activities

Table 1 shows the *B. thuringiensis* growth, the bacterial stress tolerance and its PGPB characteristics under non-stress and stress (40% PEG) conditions. Osmotic stress decreased more bacterial growth than its PGPB abilities. In fact, the stress highly increased ACC deaminase production, slightly reduced IAA and it does not change phosphate solubilization. The stress tolerance parameters either did not change as PHB production or increased as proline or MDA. Regarding these *in vitro* results, the stress applied in the culture medium did not reduce the bacterial potential to improve plant growth. 3.3. Plant biomass production and nutrients uptake *L. dentata* inoculated plants have higher root and shoot biomass under the drought condition compared to non-inoculated plants. *L. dentata* showed significant growth difference according to the single mycorrhizal species (or mixture) inoculated and the particular interaction of each AM fungus with *B. thuringiensis* (Fig. 1).

The most efficient mycorrhizal fungus in increasing shoot biomass were *A. trappei* and *P. occultum* yielding 0.61 and 0.51 g shoot dry weight respectively while control non-inoculated plants yielded 0.14 g. These fungal inocula promoted increases in plant growth of 336% (A.t) and 264% (P.o). Single Bt increased shoot growth by 21% and *B. thuringiensis* associated with *S. constrictum*, *G. versiforme* or the mixture of native fungi improved the effectiveness of these fungi in enhancing shoot growth by 12.8% (S.c), 27.3% (G.v) and 22.9% the fungal mixture (Fig. 1A). However, the opposite effect was observed when *B. thuringiensis* was associated to *D. aunantia* .

Great differences in root development between inoculated and non-inoculated plants were also observed. The bacteria improved root growth by 50% but this effect was always higher for the mycorrhizal inoculated plants. In some cases, *B. thuringiensis* decreased this value in AM colonized plants. The dual inoculation of *G. versiforme* plus *B. thuringiensis* resulted to be the most effective treatments in increasing the root growth over non-inoculated plants by 412% (Fig. 1B).

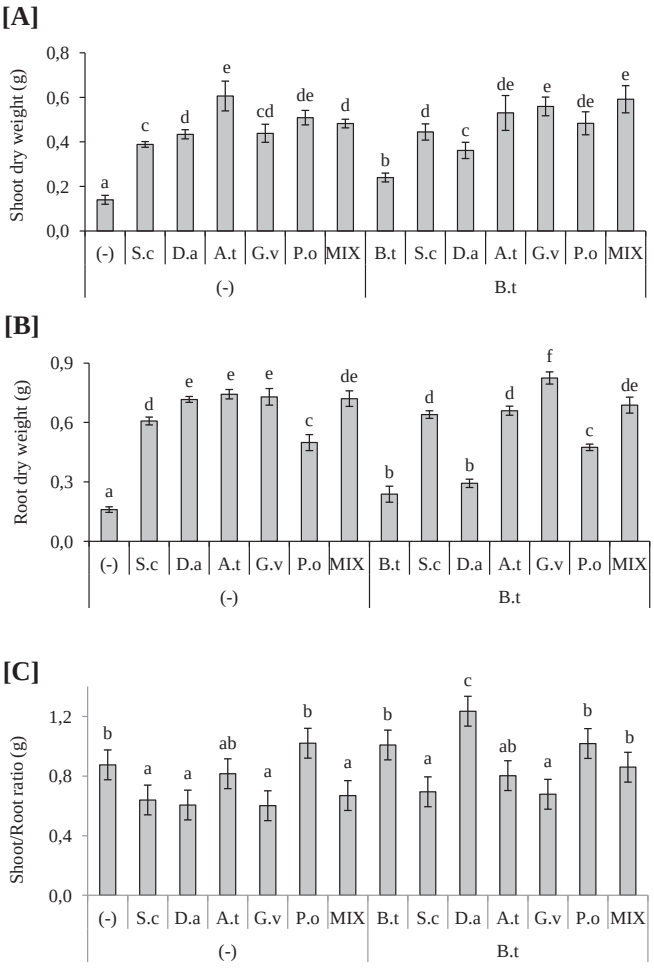

Fig. 1. Shoot dry weight (g) [1A], root dry weight (g) [1B] and shoot/root ratio (g) [1C] in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p < 0.05$) determined by Duncan's multiple-range test ($n = 5$).

AM fungal colonization by *S. constrictum*, *D. aunantia*, *G. versiforme* and mix increased more root than shoot biomass under drought conditions. But when associated to *B. thuringiensis* (*D. aunantia* and mix) increased this ratio (Fig. 1C).

Under these drought conditions, the uptake of whatever nutrient analyzed was enhanced by the mycorrhizal colonization. But this effect was variable according to the colonizing fungal species. The colonization with *A. trappei* enhanced the C, N, P, K, Mg and Ca shoot content in a higher extent than the rest of the colonizing AM fungal species or the mix (Figs. 2 and 3). *B. thuringiensis* maximized the shoot accumulation of some of these nutrients (C, N and Ca) when associated to *G. versiforme* or (C, N and K) when associated to the fungal mixture. As results show micronutrients such as Mn, Cu, Fe and Zn were differently increased by the microorganisms applied and the highest values were observed in dually inoculated plants.

The positive effect of *B. thuringiensis* in increasing micronutrients content was only observed in interaction with *S. constrictum*

Table 1
Bacterial growth (cfu mL^{-1}), drought tolerance abilities [proline, lipid peroxidation (MDA) and poly-β-hydroxybutyrate (PHB) production] and PGPB activities [indole acetic acid (IAA), phosphate solubilization index (PSI) and α-ketobutyrate (ACC-deaminase) accumulations] by *Bacillus thuringiensis* (B.t) grown for four days under non-stress and osmotic stress conditions produced by a concentration of 40% polyethylene glycol (PEG) in the growing medium.

	[PEG]	cfu mL^{-1}	mmol proline mg^{-1} protein	μmol MDAg^{-1} dry cell weight	mg PHB mL^{-1}	μg IAAmg^{-1} protein	PSI	mmol α-ketobutyrate mg^{-1} protein
B.t	0%	2.18b	0.12a	0.7a	0.33a	18.2b	1.56a	0.20a
	40%	0.83a	0.31b	4.4b	0.38ab	13.0a	1.37a	0.41b

Within each parameter values having a common letter are not significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test ($n = 4$).

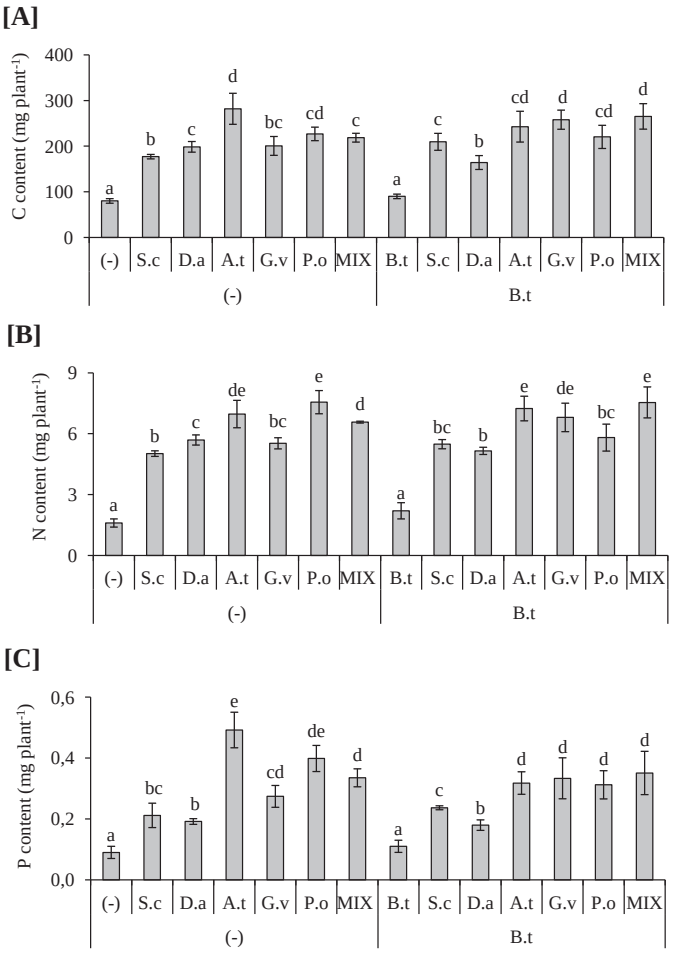

Fig. 2. C [2A], N [2B] and P [2C] content (mg plant^{-1}) in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p < 0.05$) determined by Duncan's multiple-range test ($n = 3$).

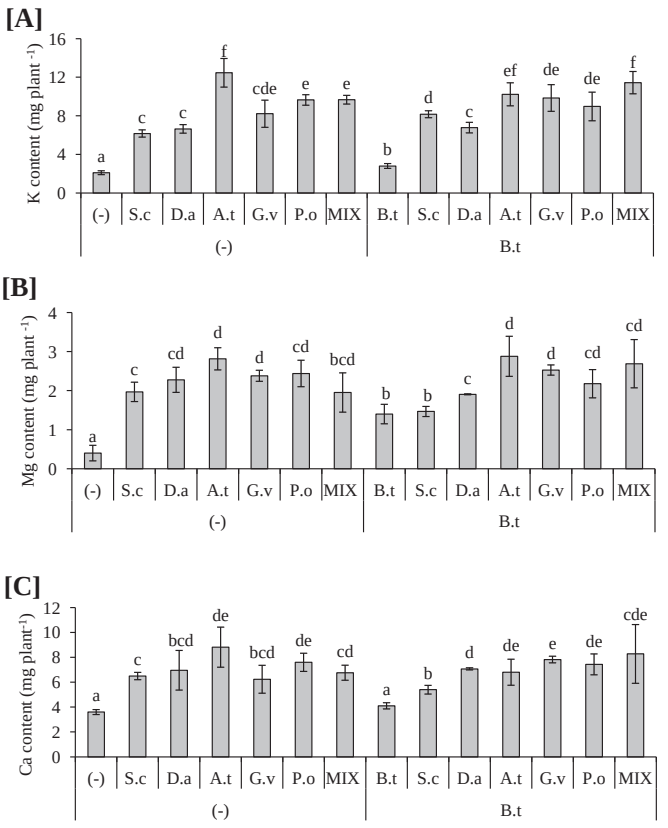

Fig. 3. K [3A], Mg [3B] and Ca [3C] content (mg plant^{-1}) in *Lavanduladentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p < 0.05$) determined by Duncan's multiple-range test ($n = 3$).

(Cu content) or with *D. aunantia* and *G. versiforme* (Fe content) (Fig. 4). Non-mycorrhizal control plants show that micronutrients are present in low concentrations in the soil solution and particularly Zn and Cu have a low mobility which normally causes deficiencies.

3.3. Root colonization

The percentage of AM colonized roots (%AMF) (Fig. 5A) was highly variable depending on the fungal inoculum involved and ranged from 13% (*D. aunantia*) to 52% (MIX + B.t). Regarding whatever colonizing parameters (%AMF, F%, M% or A%) the highest values were observed in roots colonized by the Mix that significantly differed from single fungi. The bacterial inoculation tends to increase %AMF by 18% in single *A. trappei* and by 24% in *S. constrictum* colonized plants. But the highest%AMF colonization was obtained in plants inoculated with the fungal mixture irrespective of bacteria (Figs. 5 A and 6). Nevertheless, the most important effect of *B. thuringiensis* on the symbiotic development was the improvement of the most important mycorrhizal parameters as M% and particularly A% in plants colonized by most of the fungi.

The arbuscular colonization was limited (less than 10%) in single AM-colonized plants but in plants colonized by the fungal mixture it was much higher (24.6%). The interaction with *B. thuringiensis* enhanced the formation of this important propagule by 545% (*A. trappei*), by 294% (*G. versiforme*) and by 561% (*P. occultum*). Thus the arbuscular development was maximized in plants colonized by dual *B. thuringiensis* and the fungal mixture.

The different species of arbuscular mycorrhizal fungi colonize the roots of a more or less intense way as was also observed on microscopic images (Fig. 6). The photos show that the strains *S. constrictum* (A1), *G. versiforme* (A4), *P. ocultum* (A5) and the mixture of AM fungi (A6) developed the highest level of intracellular

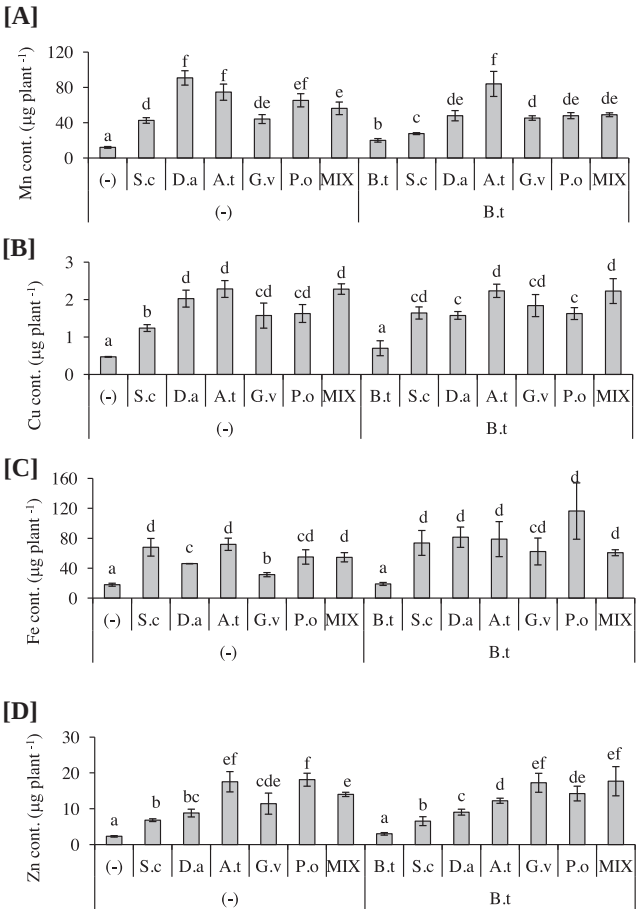

Fig. 4. Mn [4A], Cu [4B], Fe [4C] and Zn [4D] content (µg plant^{-1}) in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p < 0.05$) determined by Duncan's multiple-range test ($n = 3$).

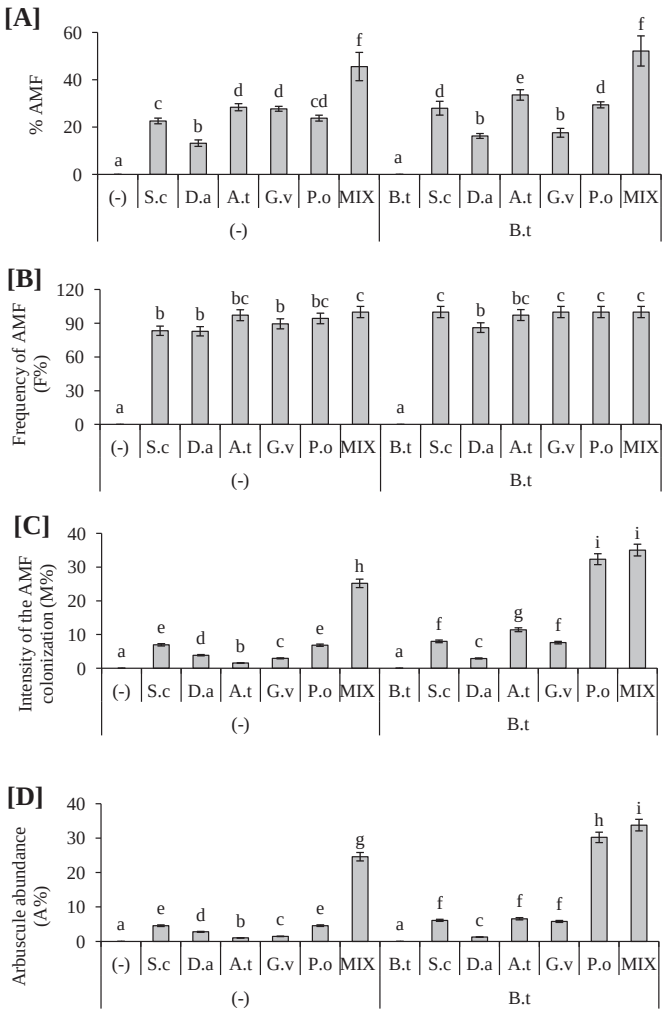

Fig. 5. Percentage of AM colonization [5A], frequency of AMF colonization in the whole root system (F%) [5B], intensity of AMF colonization in the whole root system (M%) [5C] and arbusculum abundance (A%) [5D] in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their interaction with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p \leq 0.05$) determined by Duncan's multiple-range test ($n = 3$).

hyphae. In root colonized by *A. trappei* (A3) and *P. ocultum* (A5) is observed the formation of extracellular hyphae but the dual inoculation of these fungi with *B. thuringiensis* decreases this extracellular development. In general, the co-inoculated roots increased the intraradical growth and development of most of AMF species (except in *D. aunnatia*) as also values of M% and A% showed. Species such as *G. versiforme* (B4), *P. ocultum* (B5) and mixture of AM fungi (B6) co-inoculated with *B. thuringiensis* increased the presence of arbuscules as Fig. 5D also shows but B.t as well promotes the formation of vesicles in the roots colonized with mixture of AM fungi (B6).

The endophytic colonization of *B. thuringiensis* in roots of *L. dentata* was also determined (Fig. 7(1)). It was of 5.6×10^6 cfu cm^{-1}, being the B.t cells mainly located in the intracellular zone (Fig. 7(2A)). But B.t inoculation with the mixture of AM fungi reached the cells number to 9.0×10^6 cfu cm^{-1}. As consequence, the presence of fungal mixture increased the population of *B. thuringiensis* by 61% (Fig. 7(1)), located in the intracellular and extracellular zones of the plant cortex cells (Fig. 7(2B)). Thus, an interactive relationship was detected between both microorganisms and the competence by roots occupancy by these endophytic organisms is excluded.

3.4. Oxidative stress

Drought stress is accompanied by an increase in oxidative stress indicators (MDA). In non-mycorrhizal plants the limited and insufficient shoot of biomass made impossible to carry out these determinations. MDA content was quite similar among mycorrhizal plants. However, the coinoculation with *B. thuringiensis* highly decreased this oxidative stress. This decreasement ranged from 47% in *S. constrictum* colonized plants until 73% in *P. occultum* inoculated *L. dentata* (Fig. 8).

3.5. Antioxidant enzymes activity

Antioxidant activities were only determined in mycorrhizal plants because of the lack of material in control plants (Fig. 9). *P. occultum* colonized plants showed the lowest SOD, CAT, APX and GR and although differences with others fungi were non-significant in most of the cases. Nevertheless, it is worthy to note that the highest activities were observed in plants inoculated by *D. aunantia*, *A. trappei* or by the fungal mixture with *B. thuringiensis* . These micro-

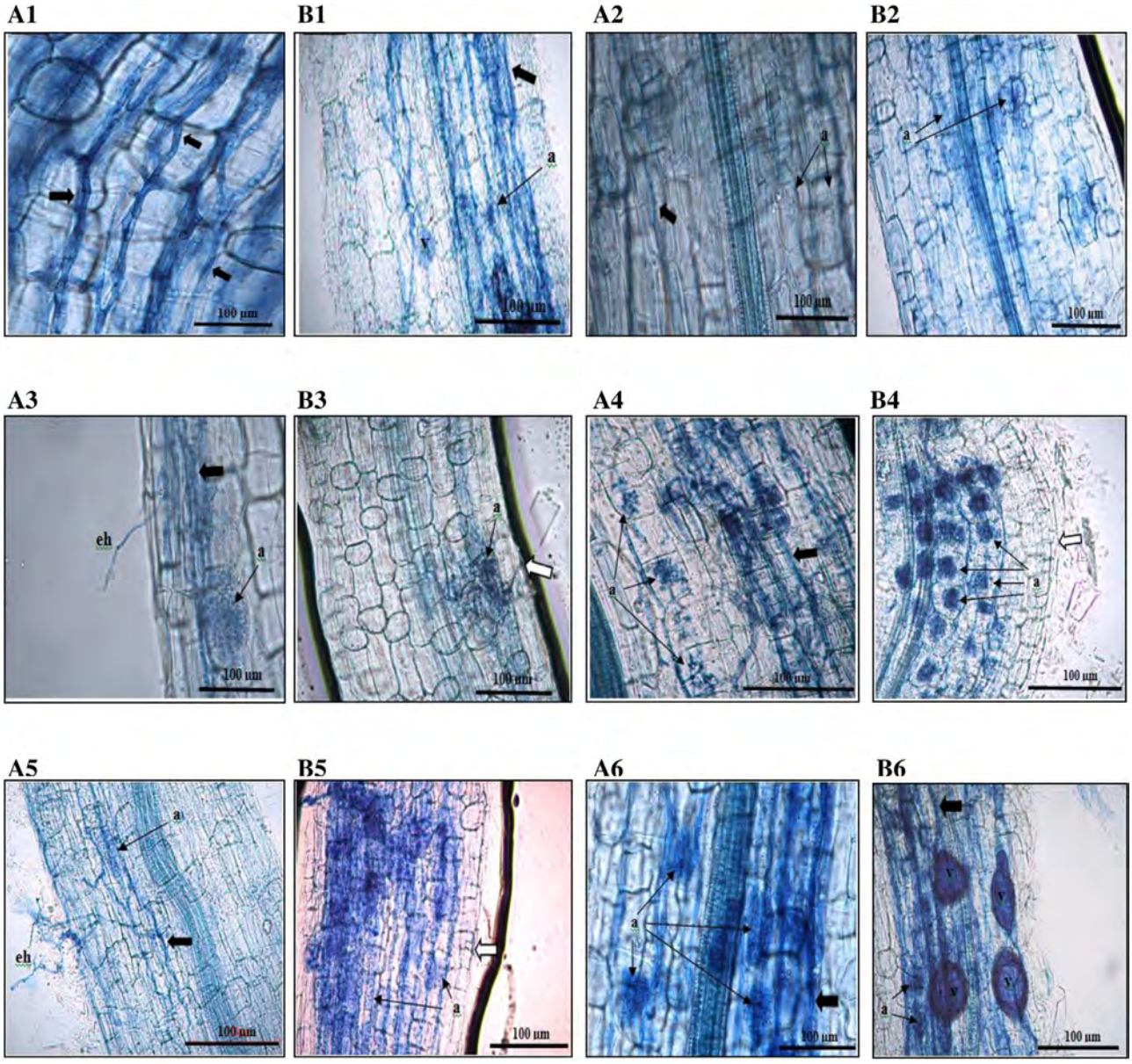

Fig. 6. Single arbuscularmycorrhizal fungal species (A1-5) or mixed (A6) colonizing *Lavandula dentata* roots. (A1: *Septoglomus constrictum* EEZ 198; A2: *Diversispora aunantia* EEZ 199; A3: *Archaeospora trappei* EEZ 200; A4: *Glomus versiforme* EEZ 201; A5: *Paraglomus ocultum* EEZ 202 and A6: a mixture or consortium of these AMF). And the effect of endophytic *B.thuringiensis* (B) on each one of AM fungal species (B1-5) and then mixture (B6). Intracellular hyphae (black arrow); extracellular hyphae (eh) and entry point (white arrow); arbuscule (a); vesicle (v). Bars = 100 µm.

bial treatments were the most active in increasing SOD, CAT and APX activities in *L. dentata* (Fig. 9).

The antioxidant enzymes activities of *L. dentata* varied slightly depending on the activity and the reaction of the bacteria was also different according to the antioxidant activity and the fungus involved.

4. Discussion

In this study as in others previous the applications of microbial inoculants have proved to be a useful strategy for the establishment of native shrubs species in degraded environments (Armada et al., 2014; Mengual et al., 2014).

L. dentata was selected as a representative shrubs species in Mediterranean zones and inoculated plants improved defense mechanisms to cope with drought in the nutrient deficient semiarid experimental soil here used. The inocula effectiveness were based on to enhance nutrients uptake and physiological/biochemical values (Armada et al., 2014; Marulanda et al., 2007). The excellent efficiency of nutrient acquisition in mycorrhizal plants colonized by whatever inoculated fungal strain could explain the differences in some parameters evaluated in these plants. Particularly, arbuscular-mycorrhizal fungi enhanced the supply of nutrients of low mobility such as P and K, contributing to the best availability of these nutrients from soil to associated plants. The most efficient single autochthonous fungus on nutrients uptake was *A. trappei* or the fungal mixture plus *B. thuringiensis* that respectively increased, over non inoculated control, C by 251% or 231%; N by 335% or 371%; P by 444% or 288% and K 493% or 444%. The highest C content in inoculated plants is one of the main plant strategies for drought stress tolerance (Gale and Zeroni, 1985). These two fungal treatments were also similarly effective in increasing shoot biomass production over control plants.

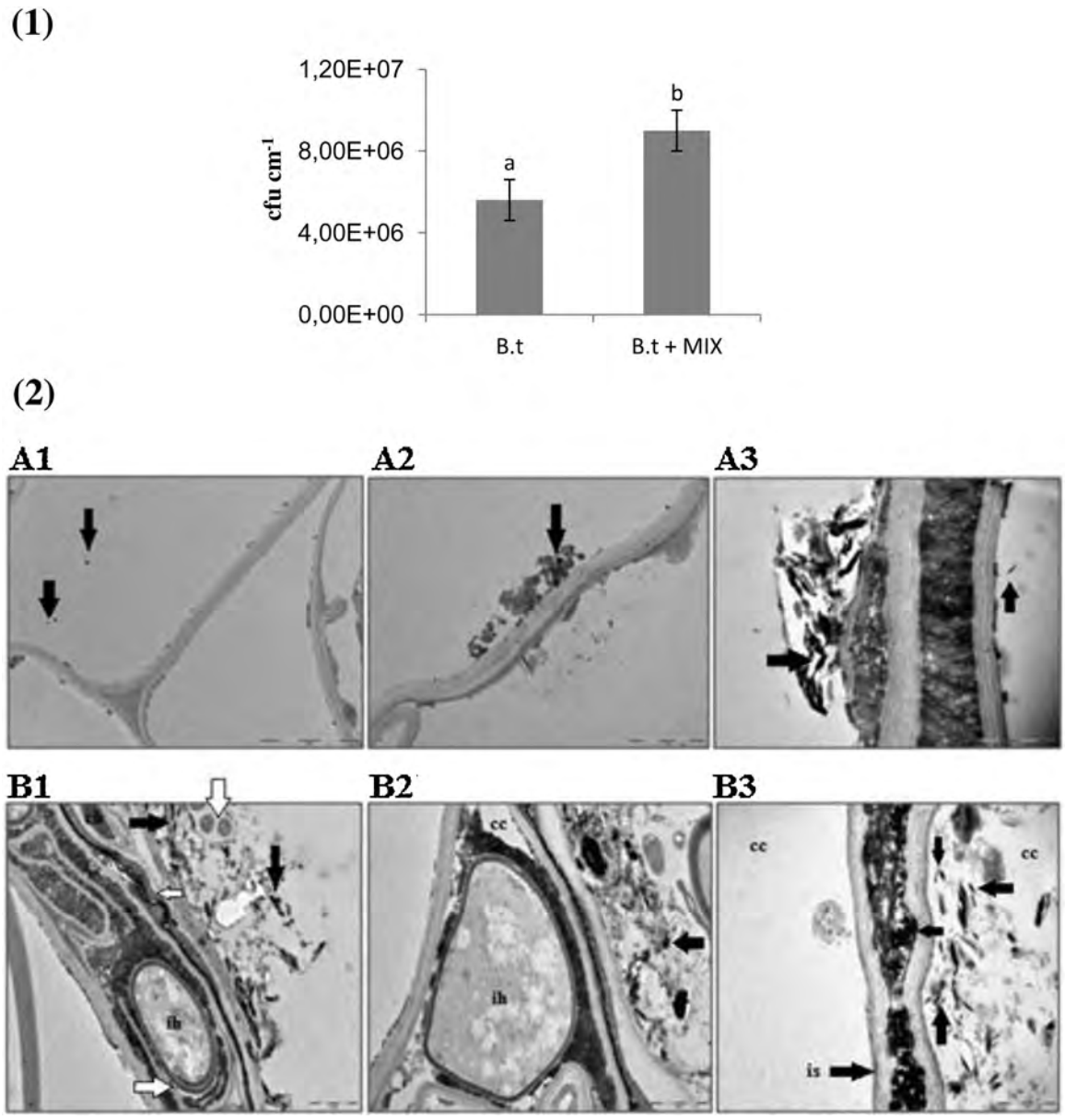

Fig. 7. (1) Bacterial endophytic colonization in roots of *Lavandula dentata* inoculated with autochthonous (B.t) dually inoculates with mixture autochthonous arbuscular mycorrhizal fungi (MIX). Different letters indicate significant differences ($p \leq 0.005$) determined by Duncan's multiple-range test ($n = 5$). (2) Transmission electron micrographs (TEM) showing intraradical hyphae (ih) penetrating cortical root cell of *Lavandula dentata* colonized by endophytic bacteria (*Bacillus thuringiensis*). (A) Presence of *B. thuringiensis* in intracellular cell (black arrow). (B) Bacteria endophytic colonization in intra and extracellular zones of cortex cells harbouring a functional arbuscule with well-separated hyphal structures and details of arbuscular branches (white arrow). The organelle-rich plant cytoplasm surrounding each arbuscule branch, which is enclosed by the periarbuscular membrane (white arrow). Chloroplats (ch); cell cytoplasm (cc); intercellular space (is). Bars = 5 μm (A1); 2 μm (A2; A3; B1; B2); 1 μm (B3).

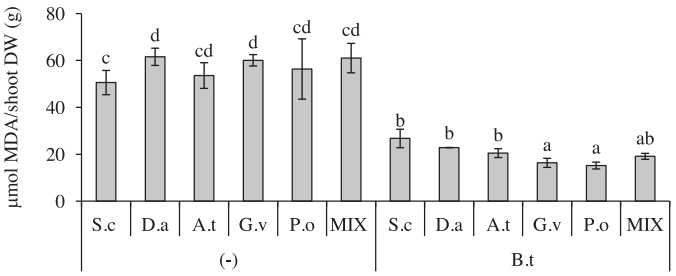

Fig. 8. Oxidative damage to lipids in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p < 0.05$) determined by Duncan's multiple-range test ($n = 3$).

The highest AM colonization and mycorrhizal activity was found in dually inoculated plants as the arbuscular production shows. In fact, the impact of bacteria in increasing drought tolerance processes seem more associated to the proportion of intraradical structures as arbuscules than to the percentage of root colonized as previously reported (Marulanda et al., 2003; Vivas et al., 2005). The effectiveness of *B. thuringiensis* in enhancing the mycorrhizal functional and metabolic status of whatever autochthonous fungal ecotype here used had a greater relevance on plant biochemical status than on growth and nutrition (Vivas et al., 2003b). With regard mycorrhizal intensity (M%) less than 10% was observed in plants inoculated with any of the fungal isolates while the mixture of them increased this value to 25%. Nevertheless, this bacteria highly increased this M% value in plants colonized by *A. trappei* (641%), by *G. versiforme* (160%), by *P. occultum* (374%) and AMF mixture

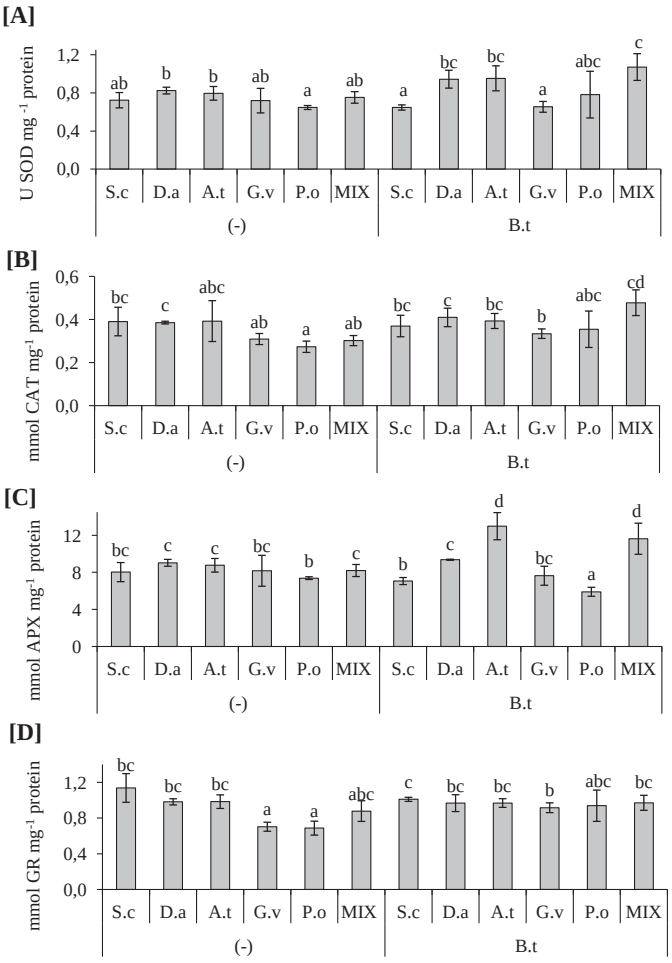

Fig. 9. Antioxidant enzymatic activities (Superoxide dismutase [9A], Catalase [9B], Ascorbate peroxidase [9C] and Glutathione reductase [9D]) in *Lavandula dentata* non-inoculated or inoculated with autochthonous arbuscular mycorrhizal fungus *Septoglomus constrictum* (S.c), *Diversispora aunantia* (D.a), *Archaespora trappei* (A.t), *Glomus versiforme* (G.v), *Paraglomus ocultum* (P.o) (single or a mixture of them) and their inoculation with autochthonous *Bacillus thuringiensis* (B.t). Different letters indicate significant differences ($p < 0.05$) determined by Duncan's multiple-range test ($n = 3$).

(by 39%). Nevertheless, the most important mycorrhizal value is the presence of arbuscules within root cells (A%) since they are the fungal structures involved in the bidirectional soil/plant nutrient exchange. The arbuscular production (measured as A%) was the greatest in plants colonized by mixture of fungal strains particularly in interactions with the bacteria. Marulanda et al. (2009) and Vivas et al. (2005) reported that the beneficial effect of bacterial inoculation was less relevant on AM-colonization than stimulating the active structures in the AM colonization affecting the metabolic and physiological fungal activities that are considered the most important indexes of effective AM symbiosis (Guillemin et al., 1995) which may mitigate the depressive effects of drought.

According to results the plant roots also provided an accurate environment for this endophytic bacterium as microscopic analysis show the bacterial population was enhanced in AM colonized roots. Both microorganisms showing the root niche are also protected from the negative effect of drought allowing a better growth and functioning as results show.

The *B. thuringiensis* ability for IAA production and other beneficial compounds may play important roles in root and microbial growth under stress conditions (Dobra et al., 2010). In addition, ACC is a precursor of ethylene synthesis and ACC deaminase changes the ACC into ammonia and α-ketobutyrate. Therefore, the lowering of ethylene levels in plants exposed to drought is essential for drought tolerance (Glick, 2004). This bacteria can be considered a plant-stress homeostasis-regulating rhizobacteria by the biosynthesis of these phytohormones (Cassán et al., 2014). As well, PHB as carbon storage polymers can support the survival and reproduction of microorganisms under adverse conditions and to improve their tolerance to osmotic stress.

And important result is that *B. thuringiensis* associated to whatever AM fungus alleviates oxidative stress generated in *L. dentata* plants with water deficiency as evidenced by the decreasement of MDA levels. This indicated that the microbial interaction of autochthonous microorganisms (bacteria and AM fungi) may restore the damage in membrane integrity and functionality caused in response of water limitation. Apparently, the dual inoculation of these microorganisms particularly contributed to induce plant drought tolerance.

The modulation of plant antioxidant responses by the microbial treatments applied could be considered one of the most important beneficial effects on the performance of plants grown in arid/semiarid environments. A decreasement of oxidative stress could be considered an indication of induced plant resistance to drought under semiarid conditions. These microorganisms may significantly increase growth and nutritional status under stressed conditions by affecting the antioxidant enzymatic pool and therefore lowering oxidative stress markers.

During periods of drought some metabolic pathways are uncompleted and the electrons, with a high-energy state, are transferred to molecular oxygen to form reactive oxygen species (ROS) that are toxic to molecules and causes oxidative damage to proteins, DNA and lipids (Miller et al., 2010). The oxidative stress caused by drought leads to uncontrolled oxidation and radical chain reactions if the scavenging system of a plant does not cope well with the accumulation of ROS (Gunes et al., 2009). ROS is an early event in stress responses (Singh et al., 2011). The efficient destruction of O_2^- and H_2O_2 requires to be modulated by the action of antioxidant enzymes acting in synchrony. In order to understand the antioxidant defense mechanisms, the analyzed results show that plants colonized by the fungus *D. aunantia*, *A. trappei* or the fungal mixture were more capable in combating drought induced oxidative damage, with more effective antioxidant machinery particularly when associated with *B. thuringiensis* . When cytotoxic ROS are produced in excess by drought stress they can destroy the normal cell metabolism through oxidative damage of lipids and antioxidant activities are the form to repair and restore cellular damage. The reduced oxidative damage in inoculated plants indicates a superior capacity to adapt drought stress by developing a highly efficient defense system. These results suggest that inoculated plants have compensatory/adaptive mechanisms of defense against the oxidative stress caused by water deficiency. But, an activation of antioxidant plant apparatus may not be attributed to the regulation of only one particular enzymatic activity but rather to the complex up-regulation of several ROS-scavenging enzymes as SOD, CAT, APX and GR. The antioxidant defense of SOD is made by eliminating ROS that generated H_2O_2 that is removed by CAT, APX and GR. Thus, the role of any of these enzymes is important in protecting plants from drought-induced oxidative stress.

Responses from the individual antioxidant enzymes and their variation with respect to the colonizing fungal species may depend on the availability of plant micronutrients content. Enzymes such as CAT, APX and SOD are metalloenzymes whose activities are determined partly by the availability of the metals that they utilize. The enhancement of Mn, Cu, Fe and Zn in inoculated plants could be involved in the SOD activity observed in these plants since SOD isoenzymes (being Cu-SOD and Zn-SOD the most abundant) were determined in mycorrhizal plants (Arines et al., 1994). The effi-

ciency of *B. thuringiensis* and the fungal mixture on plant mineral nutrition may be related with the improvement of biochemical metabolism observed.

The effectiveness of inoculated microorganisms is not always recorded here as an stimulation of plant biomass or nutrition but in general, it was evidenced as an improvement of biochemical values related to water status (Kohler et al., 2009).

Results show that each autochthonous AM fungus and the mixture of them showed different levels of effectiveness in enhancing plant nutrition and tolerance to drought.

Differences between the inoculation with single AM fungus *versus* the complex fungal mixture of this community were expected. Under the severe drought conditions here used, single *A. trappei* resulted to be the most efficient fungus in increasing *L. dentata* growth and in promoting C, N, P, K, Mg and Ca uptake. These results contrasts with the initial hypothesis that the inoculation with a mixture of AM fungal isolated would be better under stress conditions than a single isolate as was previously suggested by Caravaca et al. (2005). Variations among fungal isolates in their ability to produce biochemical changes related with plant drought stress tolerance (antioxidants enzymes, and MDA) were also observed. But the main effects attributed to the microbial ability to alleviate plant drought stress were maximized when the fungal mixture was associated with *B. thuringiensis* .

In conclusion, each autochthonous AM strain has a different inherent potential for improving plant performance under drought conditions. The general results emphasize that the autochthonous bacterial strain (*B. thuringiensis)* increased the mycorrhizal performance of autochthonous mycorrhizal fungi. The bacterium was more efficient at increasing the potential of the mixed fungal community assayed.

Based on results and regarding practical sight the applications of combined microbial treatments involving autochthonous PGPB and AMF seem to be the most suitable procedure to the establishment and restoration of plant cover in degraded arid soils. Nevertheless, the adequate selection of these microorganisms and their combination must be considered as crucial for developing practical methodologies in the restoration programs.

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