Native bacteria promote plant growth under drought stress condition without impacting the rhizomicrobiome

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Abstract

Inoculation of plants with beneficial plant growth-promoting bacteria (PGPB) emerges a valuable strategy for ecosystem recovery. However, drought conditions might compromise plant-microbe interactions especially in semiarid regions. This study highlights the effect of native PGPB after one-year inoculation on autochthonous shrubs growth and rhizosphere microbial community composition and activity under drought stress conditions. We inoculated three plant species of semiarid Mediterranean zones, *Thymus vulgaris*, *Santolina chamaecyparissus* and *Lavandula dentata* with a *Bacillus thuringiensis* strain IAM 12077 and, evaluated the impact on plant biomass, plant nutrient contents, arbuscular mycorrhiza fungi (AMF) colonization, soil rhizosphere microbial activity, and both the bacterial and fungal communities. Inoculation with strain IAM 12077 improved the ability of all three plants species to uptake nutrients from the soil, promoted *L. dentata* shoot growth (>65.8%), and doubled the AMF root colonization of *S. chamaecyparissus*. Inoculation did not change the rhizosphere microbial community. Moreover, changes in rhizosphere microbial activity were mainly plant species-specific and strongly associated with plant nutrients. In conclusion, the strain IAM 12077 induced positive effects on plant growth and nutrient acquisition with no impact on the rhizosphere microbiome, indicating a rhizosphere microbial community resilient to native bacteria inoculation.

Introduction

Knowledge of plant-microbe interaction led to the application of microbes as plant growth promoters due to their capability to improve plant development, commonly used to improve crop yields, in ecosystem recovering strategies (Bashan *et al.* 2012). Plant growth-promoting bacteria (PGPB) may play a decisive role in facilitating plant growth in soil and might be especially relevant to vegetation recovery strategies. However, modification in plant microbial community structure caused by inoculation of specific microbes might be buffered by ecosystem resilience (Shade *et al.* 2012), which is driven by the level of diversity of soil biota (Kennedy 1999; Nannipieri *et al.* 2003) and therefore, may compromise the efficiency of applied PGPB. On the other hand, the inoculated microbes may change the microbial community, thus impacting the soil ecosystem functioning and the environment. Hence, it is crucial to determine if effects due to inoculation with PGPB are long-lasting concerning the beneficial impacts on plant growth and effects on soil biota. The effect of PGPB in plants is mainly studied in crops (Chowdhury *et al.* 2013; Scherwinski *et al.* 2008; Lottmann *et al.* 2000), however studies on the application of PGPB as a strategy for ecosystem recovery (Bashan *et al.* 2008; Bashan *et al.* 2012) lack an understanding on the impact of the applied microbes in soil microflora on the long term. Plant-microbe interactions might differ in degraded soil or soil under stress conditions such as drought (Jackson *et al.* 2003; Aboim *et al.* 2008; Peixoto *et al.* 2010).

Alternatively, changes in soil microbial structure may result in undesirable effects if native species critical to plant growth are lost or subsequently have reduced roles. This is particularly relevant as plants interact with multiple soil microorganisms in addition to the inoculated bacteria. For example, native arbuscular mycorrhizal fungi (AMF) perform critical ecological ecosystem functions, especially in semiarid Mediterranean regions (Requena *et al.* 1996). AMF affect plant diversity and productivity and contribute to determining the stability and sustainability of the ecosystem (van der Heijden *et al.* 1998; van der Heijden *et al.* 2006). Therefore, the impact of PGPB inoculation on other native soil microorganisms may be even more important when we consider a degraded environment with reduced biodiversity, what might be more vulnerable to changes in soil microbial composition.

In the best-case scenario, re-vegetation programs should combine the beneficial effects of PGPB with AMF and other microorganisms associated with plant roots as microbial sources for growth and nutrient uptake (Armada *et al.* 2014; Mengual *et al.* 2014; Armada *et al.* 2015a; Armada *et al.* 2016). The selection of autochthonous plant species capable of hosting a high diversity of AMF in combination with PGPB in their rhizospheres may increase plant establishment in stressful environments such as degraded areas in the semiarid Mediterranean.

To our knowledge, there is still a lack of understanding how the inoculation of native microbes affects rhizosphere soil microbes, especially mycorrhiza. Several studies have evaluated the impact of inoculated PGPB, however, they usually apply non-native microbes (Schreiter *et al.* 2014) in short-term experiments (Scherwinski *et al.* 2008) and in agricultural fields aiming to increase crop productivity (Lottmann *et al.* 2000). Research should focus on impacts of native PGPB in facilitating plant growth under stress conditions of both soil degradation and drought. Such an evaluation would best combine molecular biological (*i.e.*, next-generation sequencing) and biochemical (assessment of biomarkers and fatty acids) approaches, since an understanding that embraces soil community structure and activity is required. Next generation sequencing of phylogenetic markers such as ribosomal RNA genes provides information on the overall community structure, whereas the analysis of phospholipid fatty acids (PLFA) and enzyme activities provide information on microbial activities. The sequence of ribosomal RNA gene provides the identification of microbial groups that comprise the microbial community through environmental DNA present in the sample, which result in information more closely related to the microbial seedbank concept (Philippot *et al.* 2013). Accordingly, a more comprehensive characterization of microbial activity could also rely on the identification of biomarkers based on the composition of PLFA, on dehydrogenase activity (estimator of basal respiration), urease activity (microbial hydrolyzation of urea) (Lloyd and Sheaffe 1973), and phosphatase activity (microbial mediated phosphorus availability). Altogether, the combined analysis provides information about community structure and potentially viable microbes and their functional activity and helps to highlight the potential impacts of bacterial inoculation in plant rhizosphere.

The aim of this study was to examine the long-lasting impact of a beneficial native bacterial strain IAM 12077 of *Bacillus thuringiensis* inoculated in three plant species (*Thymus vulgaris, Santolina chamaecyparissus* and *Lavandula dentata*), cultured under drought stress conditions on (1) plant growth and nutrition, and (2) the root AMF colonization, the rhizosphere bacterial and fungal community structure (assessed by next-generation sequencing) and the rhizosphere microbial enzyme activities. We hypothesize that after one year, the inoculation of PGPB strain IAM 12077 on plants affects the rhizosphere microbial community assembly and mycorrhiza colonization.

Materials and Methods

Soil bacteria isolation and molecular identification

The bacterial strain used in this study was isolated from the same natural soil used in the microcosm experiment (see description below). The bacterium was isolated from a mixture of rhizosphere soils from several autochthonous shrub species. A homogenate of 1 g soil in 9 mL sterile water was diluted (10^{-2} to 10^{-4}), plated on three different media [Yeast Mannitol Agar, Potato Dextrose Agar, Luria-Bertani Agar (Bertani 1951)] and incubated at 28 ºC for 48 h to isolate bacteria from different taxonomic groups.

Molecular characterization of the selected bacterium was by sequencing the 16S rRNA gene. Bacterial cells were collected, diluted, lysed, and the DNA extracted, which was used as PCR template. The amplification reactions were performed in 25 µL volume containing 10X PCR buffer, 50 mM $MgCl_2$ 10 µL of each primer [27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT)] and 1 µL of 5 U/µl *Taq* polymerase (Platinum, Invitrogen). PCR was carried out in a thermal cycler using following conditions: 5 min at 95 ºC, followed by 30 cycles of 45 s at 95 ºC, 45 s at 44 ºC and 2 min at 72 ºC, and finally one cycle of 10 min at 72 ºC. PCR products were analyzed by 1% agarose gel electrophoresis and purified with the QIAquick Gel extraction kit (Qiagen) for subsequent sequencings using an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to databases (EMBL and GenBank) using BLAST program. Similarity searches at NCBI using BLAST program unambiguously identified the bacterial species similar to *Bacillus thuringiensis* strains. The genetic similarity was also confirmed by phylogenetic analysis of the 16S rRNA gene sequence (Figure S1). The strain is *Bacillus thuringiensis* IAM 12077 (Accession NR 043403.1 or accession D16281).

In previous studies (Armada *et al.* 2015b; Armada *et al.* 2016), the same strain was characterized for growth under non-stress and osmotic stress conditions, ability to synthesize proline or poly-β-hydroxybutyrate, lipid peroxidation, and PGPB characteristics that

included α-ketobutyrate [ACC (1-aminocyclopropane-1-carboxylate) deaminase] and indole acetic acid (IAA) production and phosphate solubilization.

Microcosm experimental design and soil characteristics

The microcosm experimental design was based on two factors: (1) three different autochthonous shrub species: *Thymus vulgaris* (T), *Santolina chamaecyparissus* (S), *Lavandula dentata* (L) and (2) inoculation or not of the autochthonous species with the selected *Bacillus thuringiensis* strain IAM 12077 (non-inoculated, -; inoculated with strain IAM 12077, Bt). Each of six treatments included five biological replicates resulting in 30 pots.

The soil used in this experiment is natural soil from the "Vicente Blanes" natural park in Molina de Segura, Murcia, Spain, (coordinates: 38° 12' N, 1º 13' W, 393 m altitude). The soil is a Typic Torriorthent (SSS 2006) containing: organic C 0.94%, total N 0.22%, P $1.36 \cdot 10^{-3}$ g kg^{-1} (Olsen test), pH 8.9 and an electric conductivity of 1.55 $dS \cdot m^{-1}$. The substrate used in this assay consisted of a 5 cm layer of the natural park soil mixed with sterile sand [5/2 (v/v)]. The substrate was added to 0.5 kg capacity pots. One milliliter of pure strain IAM 12077 culture (10^8 $CFU \cdot mL^{-1}$), grown in LB medium broth (Bertani 1951) for 48 h at 28 ºC was applied to the potted seeds at sowing time. The bacterial inoculum (10^8 $CFU \cdot mL^{-1}$) was again applied to the seedlings 15 days later. The control consisted of inoculations with sterilized LB medium.

The three plant species were grown for one year in pots under greenhouse conditions (temperature 19-25 ºC, 16 h/8 h light/dark photoperiod). The photosynthetic photon flux density (PPFD) measured with a light-meter (LICOR, model LI-188B) was 400-700 µmol m^{-2} s^{-1}. During the experiment, plants were subject to drought conditions by limiting the soil water-holding capacity to 50% each day after water application and allowing the water-holding capacity to decrease to ~30% before the next water application.

Plant biomass and nutrient analysis

Plants were harvested one year after planting (five replicates per treatment, n=5), shoots were excised from the roots, and fresh weights of both shoots and roots determined. Subsequently, samples were dried for 48 h at 75 ºC and dry masses determined. Shoot P, K, Ca and Mg (mg $plant^{-1}$), as well as Zn, Fe, Mn and Cu (µg $plant^{-1}$) contents, were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) at the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

Arbuscular Mycorrhizal Fungi (AMF) root colonization, spores isolation and identification

The roots of the three plant species were carefully washed and stained (Phillips and Hayman 1970). The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples (Phillips and Hayman 1970) using the gridline intersect method of Giovannetti and Mosse (1980).

The AMF spores were isolated from the soil samples by a wet sieving process (Sieverding 1991). 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 1994), a mixture of lactic acid to water at 1:1, Melzer's reagent, and water (Spain 1990). For identification of the AMF species, the spores were examined by microscopy at up to 400-fold magnification as described for glomeromycotean classification (Oehl *et al.* 2011).

Rhizospheric soil enzymatic activity

Dehydrogenase activity was determined following Skujins' method (1976) as modified by García *et al.* (1997). One gram of rhizosphere soil was exposed to 0.2 mL of 0.4% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) in distilled water for 20 h at 22 ºC in the dark. The formed iodo-nitrotetrazolium formazan (INTF) was extracted with 10 mL of methanol by shaking vigorously for 1 min and filtering through a Whatman no. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

β-glucosidase was determined using 0.05 M *p*-nitrophenyl-β-D-glucopyranoside (PNG), (Masciandaro *et al.* 1994) as substrate. This assay is also based on the release and detection of *p*-nitrophenol (PNP). Two milliliters of 0.1 M maleate buffer (pH 6.5) and 0.5 mL of the substrate were added to 0.5 g of sample and incubated at 37 ºC for 90 min. The reaction was stopped by addition of tris-hydroxymethyl aminomethane (THAM) according to Tabatabai (1982). The amount of generated PNP was determined by absorbance at 398 nm (Tabatabai and Bremner 1969).

Urease activity was determined by the method of Nannipieri *et al.* (1980) and expressed as µmol $N\text{-}NH_3 \cdot g^{-1}$ $soil \cdot h^{-1}$.

Alkaline phosphatase activity was determined using 0.115 M *p*-nitrophenyl phosphate disodium (PNPP) as substrate. Two milliliters of 0.5 M sodium acetate buffer adjusted to pH 5.5 using acetic acid (Naseby and Lynch 1997) and 0.5 mL of substrate was added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling to 2 °C for 15 min. Then, 0.5 mL of 0.5 M $CaCl_2$ and 2 mL of

0.5 M NaOH were added, and the mixture was centrifuged at 1649 x*g* for 5 min. The *p*-nitrophenol (PNP) formed was determined by absorbance at 398 nm (Tabatabai and Bremner 1969). For controls, the substrate was added before the additions of $CaCl_2$ and NaOH.

Rhizospheric soil microbial lipid extraction and PLFA analysis

Lipid extraction, fractionation, mild alkaline methanolysis and GC analysis were according to Frostegard *et al.* (1993a,b). PLFA analysis was carried out in freeze-dried frozen samples (−80 ºC). Lipids were extracted from 3 g lyophilized soil samples using a one-phase mixture (1:2:0.8 v/v/v) of chloroform/methanol/0.15 M, pH 4.0 citrate buffer. After extraction, the lipids were separated into neutral lipids, glycolipids and polar lipids (phospholipids) on silicic acid columns (Merck Kieselgel 60 63-200µm) followed by a mild alkaline methanolysis to form the corresponding fatty acid methyl esters for GC analysis. The fatty acids were identified by their retention times in relation to that of the internal standard (fatty acid methyl esters 19:0 and 12:0).

The following fatty acids were assessed as biomarkers for bacterial biomass: i14:0, i15:0, a15:0, i16:0, 16:1w7t, 17:1w7, a17:1w7, i17:0, cy17:0, 18:1w7c and cy19:0 (Mauclaire *et al.* 2003). PLFA 16:1w5 was used as an indicator of arbuscular mycorrhizal fungi (Olsson *et al.* 1995, Drigo *et al.* 2010). C18:2w6.9 was used as a measure of fungal biomass (Bååth 2003). Methylated fatty acid (10Me16:0) was used as a specific biomarker for *Actinomycetes* (Frostegard *et al.* 1993a,b; Welc *et al.* 2010). The ratios of Gram-positive to Gram-negative bacteria were calculated by taking the sum of the PLFAs i-C14:0, i-C15:0, a-C15:0, i-C16:0, i-C17:0 and a-C17:0 reflected the amount of Gram-positive bacteria, whereas C16:1w7, C17:0 cy and C18:1w7 reflected the amount of Gram-negative bacteria (Frostegård and Bååth 1996; Zelles 1997).

Rhizospheric soil DNA extraction, PCR conditions for fungal and bacterial tag-encoded amplification and sequencing

DNA was extracted from 0.5 g of soil using the fast DNA Spin Kit for soil (MO BIO Laboratories Inc., Carlsbad CA, USA) and quantified by 260 nm absorbance (Nanodrop Technology, Wilmington, DE, USA). The integrity of the DNA was verified by 1% agarose gel electrophoresis using TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). For fungal 18S rRNA partial gene amplification, primers described by Verbruggen *et al.* (2012) were used. The 5′ terminus of primers contained an adaptor sequence and a multiplex identifier tag (MID, 12 different 10-bp-long tags), which resulted in the following primer constructs (adaptor in boldface): Forward (FF390.1), 5′-CTATGCGCCTTGCCAGCCCGCTCAG-(MID)-CGWTAACGAACGAGACCT-3′, Reverse (FR1), 5′-CGTATCGCCTCCCTCGCGCCATCAG-(MID)-AICCATTCAATCGGTAIT-3′. PCR reactions contained 2.0 µL of 10 µM each forward and reverse primer, 5.0 µL 10x PCR-buffer, 5.0 µL of 2 mM dNTP's, 0.5 µL BSA, 33.10 µL Milli-Q water and 0.40 µL FastStar Expand TAQ DNA polymerase (5 U/µL). The PCR conditions were 95 ºC for 5 min followed by 25 cycles at 95 ºC for 30 s, 57 ºC for 1 min and 72 ºC for 1 min, a final elongation step at 72 ºC for 10 min. Products were purified using QIAquick PCR Purification Kit (Qiagen).

For bacteria, the V4 region of the 16S rRNA gene was amplified by PCR using 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT -3') primers. The 515F primer included the Roche 454-B pyrosequencing adapter (a 10-bp barcode) unique to each sample, and a GT linker, while 806R included the Roche 454-A sequencing adapter (a 10-bp barcode), unique to each sample, and a GG linker. PCRs contained 1.0 µL of 5 µM each forward and reverse primers, 2.5 µL 10x PCR-buffer, 2.5 µL of 2 mM dNTPs, 16.80 µL Milli-Q water and 0.20 µL of 5 U/µL FastStar Expand TAQ DNA polymerase under the following conditions: 95 ºC for 5 min followed by 30 cycles of 95 ºC for 30 s, 53 ºC for 1 min and 72 ºC for 1 min, a final elongation step at 72 ºC for 10 min. Products were purified using QIAquick PCR Purification Kit (Qiagen). Amplicons were quantified and equimolar pooled. The samples were sequenced (Macrogen Company Inc., South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA).

Statistical analysis

Basic and univariate statistics

Kolmogorov–Smirnov and Lilliefors tests were used for assessing the normality of the data. Most variables of plant biomass were normally distributed or could be normalized by data transformation. Homogeneity of variance was checked by using the Brown–Forsythe test for unequal replication numbers. Statistical analysis consisted of two-way ANOVA followed by Tukey-Kramer's test. Correlation and regression analyses were also performed to identify univariate interactions among variables. For the regression analysis, two values of shoot biomass were considered outliers and therefore, excluded from this analysis. To reduce both family-wise error and the false discovery rate, all multiple comparisons passed to a step-down resampling algorithm (Westfall and Young 1993) while the correlation analysis had their p-values corrected by the Benjamini-Hochberg false discovery rate adjustment (Benjamini and Hochberg 1995).

Multivariate statistics and Generalized Linear Models

The sequenced datasets (bacterial and fungal) were analyzed using the "phyloseq" package in R (McMurdie and Holmes 2013). The bacterial and fungal operational taxonomic unit (OTU) abundances were summarized at the Class and Genus taxonomical levels, respectively, and the Hellinger transformation was adopted prior to ordination methods (Legendre and Gallagher 2001) in order to stabilize the mean-variance relationship and to avoid confounding location and dispersion effects (Warton *et al.* 2012).

The "ade4" R package (Dray and Dufour 2007) compared the effect of treatment on the various datasets (plant biomass and nutrients, microbial enzymatic activity, soil microbial community). Between-class analysis (BCA) measured the amount of variance restricted to the grouping factor as a percentage of the total inertia (Dray and Jombart 2011; Thioulouse *et al.* 2012). BCA is an alternative method to linear discriminant analysis for which the number of samples is smaller than the number of variables (Thioulouse *et al.* 2012). Principal Component Analysis (PCA) was applied to each community data set prior to BCA. Monte-Carlo tests of the treatment groups were performed with 999 permutations. We also performed Co-Inertia analysis to investigate the degree of data co-structure (Dray *et al.* 2003) by evaluating the covariance between our four different datasets: (i) plant biomass and nutrients, (ii) microbial activity, (iii) bacterial and (iv) fungal community structure.

Because both microbial enzymatic activity and soil microbial community (fungi and bacteria) datasets presented an overdispersal variance, we applied a generalized linear model (GLM) based on tweedie and negative binomial distribution to investigate the effects of both plant and bacterial inoculum on microbial activity and structure, respectively. To avoid sequencing bias common in next-generation platforms (Lee *et al.* 2012; McMurdie and Holmes 2014), we decided to use the total number of reads per sample as a covariance effect in our generalized linear models. The effect of each treatment in the microbial community was evaluated by the Wald's test (W) and Wilks's lambda test for the enzymatic activity.

The R environment (R Development Core Team 2007) and "ade4" (Chessel *et al.* 2004), "mvabund" (Wang *et al.* 2012) and multcomp (Hothorn *et al.* 2008) packages were used.

Results

Plant growth, nutrition, and symbiotic parameters

Inoculation with strain IAM 12077 increased the shoot biomass of all three plant species compared to the control: 65.8% for *L. dentata*, 31.8% for *S. chamaecyparissus* and 17% for *T. vulgaris* (Figure 1A). However, inoculation weakly influenced root biomass ($p<0.06$) (Figure 1B).

Inoculation with strain IAM 12077 increased both macro and micronutrients in shoots, but all three plant species responded differently: P and K increased by 51% and 47%, respectively, for *T. vulgaris* , while K, Ca and Mg increased by 63%, 27% and 36% for *L. dentata* compared to non-inoculated controls (Table 1). Inoculation with strain IAM 12077 also increased the shoot content of the micronutrients Zn, Fe and Cu for *T. vulgaris* and Zn, Mn and Cu for *S. chamaecyparissus* compared to non-inoculated controls (Table 1). In general, the shoot nutrient content of *S. chamaecyparissus* contained the largest amount of P, while *L. dentata* accumulated more K, Ca and Mg than the other two species in this study (Table 1).

Remarkably, the plant P content was not significantly correlated with the percentage of AMF colonization ($p>0.20$). The predominant AMF species identified in the native consortium in soil of this study were: *Septoglomus constrictum, Diversispora aunantia, Archaespora trappei*, *Glomus versiforme*, and *Paraglomus ocultum*, which were cataloged and included in the collection of EEZ (codes EEZ 198 to EEZ 202). The analysis of AMF root colonization revealed a distinct pattern. Among all non-inoculated species, *T. vulgaris* had the highest percentage of AMF root colonization. Bacteria inoculation on *S. chamaecyparissus* significantly increased the percentage AMF root colonization by 92% and total AMF colonization by 145% with respect to the non-inoculated controls (Table 2). However, we did not detect any statistical differences in AMF root colonization for *L. dentata* with or without inoculation. We also report a significant relationship between the percentage of AMF root colonization and shoot dry mass ($r^2=0.48$, $p<0.01$) (Supplementary Figure S1).

Microbial enzymatic activity and PFLA composition in the rhizosphere of the three plant species

β-glucosidase activity was highest in *L. dentata* rhizospheres, alkaline phosphatase activity was highest in *S. chamaecyparissus* rhizospheres, and dehydrogenase activity was highest in both plant species. Inoculation with strain IAM 12077 did not affect any of the enzymatic activities measured for all three plant species, except that the urease activity of *S. chamaecyparissus* increased by 30.5% compared with the non-inoculated control (Table 2).

The lipid abundance of the microbial community including bacteria, fungi, *Actinomycetes*, AMF, Gram-positive (G+) and Gram-negative (G-) bacteria, total PLFA and total neutral lipids fatty acids (NLFA) was lower in *L. dentata* rhizosphere. Bacteria, fungi, AMF, and total PLFA significantly increased in *S. chamaecyparissus*, while *Actinomycetes*, G+ and G- bacteria increased for both *T. vulgaris* and *S. chamaecyparissus.* ANOVA analysis revealed significant differences ($p\leq0.05$) for the microbial biomarkers of bacteria, fungi,

Actinomycetes, G+ and G- bacteria and total PLFA of the rhizosphere of all three autochthonous plants species. Inoculation with strain IAM 12077 for each plant species did not significantly affect the lipid abundance of the rhizosphere microbial community, although inoculated *L. dentata* had a low content of acidic phospholipid biomarkers, but the content of NLFA was significantly increased compared to the non-inoculated control (L(-)) (Table 3).

There was a significant effect for all three plant species with respect to the profile of microbial PLFA in the rhizospheres. Significant differences were found for the bacterial biomarkers (C17:1w8c, C18:1w9t) and fungal biomarkers (C18:1w9c, C18:2w6c) (Supplementary Table S1). Inoculation did not significantly influence the profiles of fatty acids (Wilk's = 0.39, p>0.05).

BCA analysis also pointed to statistically relevant differences for non-inoculated plants and to a non-significant difference for bacterial inoculation. The results showed that 98.5% of the total variance in this dataset relates to the differences between plant species (Figure 2) where we observed that the microbial activity (enzymatic activity) and PFLA composition of *L. dentata* presented a distinct profile compared to those of *T. vulgaris* and *S. chamaecyparissus*.

Bacterial and fungal community structure

The numbers of total sequences were 10,7667 of fungi and 81,135 of bacteria. The sequence reads resulted in 900 operational taxonomic units (OTUs) for fungi and 3,756 OTUs for bacteria. Supplementary Table S2 presents the total abundance medians and interquartile range for both bacterial and fungal communities according to treatments.

Analysis of absolute abundance of both bacterial (W = 119.1, p = 0.82) and fungal (W = 64.7, p = 0.65) species at the family level for the various treatments did not reveal any significant effects after one year of strain IAM 12077 inoculation. Most bacterial abundance (53%) was related to six major taxa: *Rubrobacter* (13.3%), unclassified *Solirubrobacterales* (10.8%), unclassified *Actinobacteria* (9.2%), *Solirubrobacter* (8.2%), *Streptophyta* (7.1%) and unclassified *Geodermatophilaceae* (5.3%). Contrastingly, fungal taxa accounted for 55% of total abundance reported for the experiment, and the fungi belonged to taxa that included *Hypocreales* (22%), *Hypocreomicetidea* unclassified (9.4%), *Dothideomycetes incertae sedis* (8.6%), *Agaricales* (7.8%) and *Glomus* (7.6%).

The population abundance of *Bacillales* order which comprises our strain IAM 12077 inoculum also did not show any difference among treatments, after one year of inoculation (W=5.47, p>0,05). In summary, our analysis of changes in the microbial communities due to inoculation showed no significant effects. We also observed only a small impact due to the total number of reads in our analysis (W=1.72×10^{-12}, p>0.05).

Interactions of microbial community structure and activity, and shoot nutrient acquisition

The co-inertia analysis showed a significant covariance between bacterial and fungal soil rhizosphere communities, and between microbial activity and abundance (enzymatic activity and PFLA composition) and plant biomass and nutrients (Figure 3). We found that 55% of total data variance for plant nutrients and biomass correlated with microbial activity and abundance, while 72% of fungal community variability strongly associated with bacterial community variance.

Based on the co-inertia analysis results, we investigated how plant biomass and nutrients interacted with microbial activity and abundance. We found 20 positive interactions and 8 negative interactions (Figure 4). Total fungi activity and abundance correlates negatively with root dry weight (r=-0.51, p<0.05) and is negatively associated with plant Mg-content (r=-0.55, p<0.05). β-glucosidase relates positively with plant K (r=0.75), Ca (r=0.67) and Fe contents (r=0.51) and was negatively associated with the percentage of AMF (r=0.53) and Mn (r=0.60) content. We also report a negative relationship between dehydrogenase and the percentage of AMF (r=-0.67, p<0.05) and the total AMF colonization. The same was true for alkaline phosphatase activity, which correlated negatively with the percentage of AMF (r=-0.63, p<0.05) and total AMF colonization (r=-0.55, p<0.05). On the other hand, enzymatic activity, lipid acid abundance, fatty acid composition of total bacteria, total fungi, *Actinomycetes* and mycorrhiza likely contributed to the accumulation of P (r_{bac}=0.58, r_{fun}=0.55, r_{act}=0.58, r_{myc}=0.57), Zn (r_{bac}=0.54, r_{fun}=0.50, r_{act}=0.54, r_{myc}=0.54), Mn (r_{bac}=0.72, r_{fun}=0.73, r_{act}=0.67, r_{myc}=0.71) and Cu (r_{bac}=0.52, r_{fun}=0.52, r_{myc}=0.52). We also report a strong relationship between the presence of Gram-negative bacteria and plant Mn content (r=0.57, p<0.05).

We also evaluated the co-occurrence between bacterial and fungal communities in the rhizosphere of all the three plant species (Figure 5). We found 122 positive associations and 61 negative associations between the rhizosphere bacterial and fungal communities. Presence of the genus *Glomus* correlated negatively with bacteria from the groups *Sphingosinicella* and *Acidobacteria* Gp6, while the presence of *Glomus* correlated positively with *Acidobacteria* Gp16. We observed two positive interactions between the genus *Paraglomus* with *Pseudomonadaceae* (unclassified) and *Microvirga*.

In summary, our analysis showed the interactions between microbial activity and abundance (enzyme activity and PFLA composition) and plant biomass, and between bacterial and fungal communities, under drought conditions are majorly positive, while interactions of two genera of mycorrhiza (*Glomus* and *Paraglomus*) associated both positively and negatively with bacterial groups.

Discussion

Inoculation with strain IAM 12077 on seed and seedlings of three plant species resulted in plant growth and nutrient content acquisition, after one year under drought stress conditions. The inoculation increased shoot growth of all three autochthonous plant species studied. Strain IAM 12077 may enhance plant growth by various mechanisms such as optimizing the supply of nutrients and the solubilization of inorganic phosphorus (Glick 1995; He *et al.* 1997; Leggett *et al.* 2001). Hence, the application of phosphate solubilizing bacteria (PSB) could be a reasonable substitute for chemical phosphate fertilizers (Khan and Zaidi 2006). Previously, Armada *et al*. (2015b) showed that inoculation with strain IAM 12077 enhanced maize nutrient uptake of P by 37% and Fe, Zn, and Cu, indicating the capacity of this strain on solubilization of non-available nutrients and the production of siderophores. Thus, this strain alone strongly impacted the plant nutrient uptake. The enhanced access to soil nutrients likely explains the increase of plant biomass, but strain IAM 12077 also seems to possess mechanism to improve plant tolerance under adverse conditions (Armada *et al*. 2016), and other growth promoting compounds such as indole-3-acetic acid (IAA). Altogether, the bacteria alone promoted plant growth without resulting in long-term changes on AMF colonization and the rhizosphere microbial community.

Our analysis also showed no significant effect of strain IAM 12077 in both percentages of AMF and total AMF colonization. In general, AMF present host preference or host specificity (Vandenkoornhuyse *et al.* 2003; Öpik *et al.* 2006; Alguacil *et al.* 2009), which might explain the lack of inoculated strain influence on this plant-fungi relationship. Nevertheless, the impact of bacteria in increasing drought tolerance processes seems to be more associated with the proportion of intraradical structures such as arbuscules than to the percentage of root colonized as previously reported (Marulanda *et al.* 2003; Vivas *et al.* 2005; Armada *et al.* 2016). This specificity has ecological importance for revegetation programs in ecosystems that include autochthonous shrubs (Armada *et al.* 2014; Mengual *et al.* 2014; Armada *et al.* 2015a,b; Armada *et al.* 2016), and our analysis suggests that inoculation with strain IAM 12077 may contribute to promoting P uptake without compromising plant-AMF relationships. However, we found that microbial activity of β-glucosidase, dehydrogenase and alkaline phosphatase were negatively correlated with the percentage of AMF colonization in roots. When measuring soil enzyme activities, it should be considered that potential activities are determined (Schloter *et al.* 2003). Notwithstanding, evaluating soil enzymatic activity remains useful as an indicator of biochemical potential, possible resilience, and a sensor of changes in soil key functions (Taylor *et al.* 2002). Therefore, soil microbial activity, especially that of alkaline phosphatase points to a role for non-AMF microbial activity in solubilizing P for uptake in plants (Nakas *et al.* 1987), which may contribute to the lack of significance between plant P-content and percentage of AMF root colonization. We report a positive correlation between bacterial and fungal activities. Therefore, strain IAM 12077 induced plant P uptake and plants interacted with soil-active microbes for continuous acquisition of soil nutrients, without the dependence on AMF for nutrient uptake.

The strain IAM 12077 in the present study presents a great potential for improving nutrient acquisition, especially compared to organic fertilizer sources (Güneş *et al.* 2014). This might explain the increase of phosphorus content in *T. vulgaris* due to strain inoculation (+51%). Inoculation with strain IAM 12077 increased shoot K content for the three plant species studied and had the greatest influence on *L. dentata* (+63%). Potassium is one the most important soluble inorganic nutrients and regulates water uptake capacity by the roots (Wang *et al.*, 2013), likely an essential process during plant growth under water stress conditions. According to Armada *et al.* (2016), the strain IAM 12077 modulates the plant antioxidant responses by decreasing oxidative stress, which contributes to improve the nutrient uptake and plant growth performance under stress conditions. The lack of significant changes in rhizosphere microbial community after one year of inoculation together with the increased nutrient concentration in plant tissue may suggest the nutrient acquisition resulted from a long-lasting effect of strain IAM 12077.

We also found that strain IAM 12077-inoculated seeds and seedlings exhibited increase in both Ca and Mg contents compared to non-inoculated plants. Calcium acts in membrane protection, and magnesium contributes to modulation of ionic currents across chloroplasts and vacuole membranes (thus, regulating the stomatal opening and ion balance in cells), both of which are phenomena of particular relevance under drought conditions (Parida and Jha 2013). The enhancement of Mg content for inoculated plants suggests a reduced impact of drought on the functioning of the photosynthetic apparatus in these three plant species when colonized by strain IAM 12077.

The plants used in our study belong to two different families (*Lamiaceae* and *Asteraceae*) but are all autochthonous drought-tolerant shrub species with deep roots that help to cope with nutrient stress in eroded soils (Francis and Thornes 1990). They belong to the natural succession of the shrubland community of semiarid Mediterranean ecosystems in the southeast of Spain (Alguacil *et al.* 2011). *T. vulgaris* is heliophylous plant that grows well in drained and calcareous soils, usually reaching 15-30 cm height (Dorling 2008). *S. chamaecyparissus* grows well on rocky soils with button-like flower-heads in summer; consists of an aromatic shrub that grows up to 75 cm (Dorling 2008). Finally, *L. dentata* prefers well-drained alkaline soils and sunny conditions (González 2007) and grows to 60 cm with linear or lance-shaped leaves and the whole plant is also strongly aromatic with a widely known fragrance (Bayer 2006). Considering that three plants in this study prefer well drained soil and they are naturally tolerant to drought conditions, the one year of controlled water-holding capacity simulated the aspects of stressful water conditions likely inducing the plants to intensify their interactions with soil

microbiome, as indicated by the significant covariance between the soil microbial activity and plant nutrients and biomass. The microbial abundances, bacterial (C17:1w8c, C18:1w9t) and fungal biomarkers (C18:1w9c, C18:2w6c) and enzymatic activities differed significantly in the rhizosphere of the three plant species studied. However, strain IAM 12077 inoculation did not significantly influence the profile of fatty acids in the rhizosphere of these species. Therefore, after one year of inoculation, there was nearly no effect of the inoculated bacteria with respect to determining rhizosphere microbial activity and abundance (enzyme activity and PLFA composition), despite measurable contributions to plant growth and nutrient uptake. These results suggest a potential application of strain IAM 12077 as part of a revegetation strategy for enhancing plant growth and uptake of nutrients with a minimized impact on rhizosphere microbial activity and abundance.

The PLFA technique provides a rapid and inexpensive method to access microbial biomass and composition (Frostegård *et al.* 2011), which may be even more sensitive in detecting shifts than methods based on DNA or RNA (Ramsey *et al.* 2006). However, PLFA lacks specificity since many different (and unknown) groups of organisms may present the same biomarker. Thus, molecular methods allow obtaining more accurate information on the soil microbial community by detecting not only the active microbes, but also the whole soil microbiota including the seedbank (Philippot *et al.* 2013). Based on that, we confirm that strain IAM 12077 did not induce a significant shift in the rhizozpshere microbial community after one year of inoculation.

Moreover, the co-inertia results point to significant interactions between rhizosphere microbial activity and plant nutrition, and we later identified part of this covariance as single variable correlations. The β-glucosidase activity that was highest in *L. dentata* suggests carbohydrate transformation, which is important as an alternative energy source for microorganisms. Indeed, β-glucosidase activity is positively associated with plant K, Ca and Fe contents, which suggest that metabolically active microbes may directly contribute to plant nutrient uptake. *S. chamaecyparissus* had the greatest phosphorus content in shoot biomass and the increase of alkaline phosphatase activity. The cycling of N, C and P are controlled by hydrolase enzymes such as urease (N), β-glucosidase (C) and phosphatases (P), which are mainly synthesized by soil microorganisms (Ros *et al.* 2006). These hydrolases are involved in the mineralization of compounds that provide nutrients that include N, P and C. Therefore, rhizosphere active microbes contribute to plant nutrient uptake, while the strain IAM 12077 improved nutrient uptake. As our reported differences in microbial activities are plant-specific, we infer that plants might shift their microbial rhizosphere composition by activating microbes able to mediate soil nutrient uptake in plants. The Rv coefficient suggested that part of plant nutrient content results from microbial activity which according to the BCA analysis is a plant-specific selection. Furthermore, ANOVA results also highlighted the role of strain IAM 12077 inoculation in plant nutrients. Therefore, beyond the role of strain IAM 12077 in the rhizosphere, the plants likely drive the microbial activity towards their nutrient necessities. Plant nutrient uptake probably occurred as a combination of plant selection of active microbes together with the beneficial roles of strain IAM 12077.

The strongest link found in our study relates the rhizosphere bacterial and fungal community covariance for plants under water stress. Here we found major positive associations that were nearly double the number of negative microbial interactions. According to the stress-gradient hypothesis (SGH), species interactions increase their importance due to shifts from competition to facilitation with respect to stress (He and Bertness 2014). Our results provide support for this hypothesis, the strong covariance between bacterial and fungal communities indicates that the variance of some bacterial groups links with fungal community changes. In fact, fungal groups were positively associated with many of the evaluated bacterial groups the (*e.g.*, the genus *Paraglomus* was positively associated with *Pseudomonadaceae* and *Microvirga*). Furthermore, the bacterial community likely employs several physiological modifications in response to changing soil moisture, such as the production of exopolysaccharides (Kohler *et al.* 2009), sporulation (Landesman and Dighton 2010) and adjustment of internal water potential to match that of the external environment.

Effects of microbial inoculum on the rhizosphere microbial community structure have often been reported for only short-term experiments ranging from 30 to 90 days (Cipriano *et al.* 2016) and usually for short-cycle crops (Schreiter *et al.* 2014). Since we aimed to describe the longer-term impact of strain IAM 12077 in rhizosphere community by evaluating their effects after one year, this may explain why we found weak changes induced by inoculation with respect to the total rhizosphere microbial community (results of DNA analysis). In addition, it is likely that the strain IAM 12077 produced a primer effect on plants (Cipriano *et al.* 2016) that resulted in positive effect on plant growth after one year of inoculation.

Previous studies have shown the potential of PGPB as a strategy for successful ecosystem recovery strategies (Bashan *et al.* 2008; Bashan *et al.* 2012) and our study goes beyond by showing the dismal impact of strain IAM 12077 in rhizosphere microbial community. Thus, strategies for land recovery might be more effective by the use and application of existent PGPB present in soil.

In conclusion, in greenhouse conditions, inoculation using native bacterial strain with PGPB properties enhanced the growth of three autochthonous shrubs species and nutrient uptake without changing the rhizosphere microbial diversity and did not affect AMF groups after one year of inoculation under water stress conditions. Plants possess ecological advantages by fostering soil microbial seedbank and assembly their rhizosphere microbial populations (Mendes *et al.* 2014; Barbosa Lima *et al.* 2015; Cipriano *et al.* 2016; Schlemper *et al.* 2017a,b). The inoculation of autochthonous shrubs species with strain IAM 12077 may be a sustainable option for recovering degraded

soils without harming and impacting the rhizosphere microbial community structure and activity over the long-term. However, future studies in field conditions are needed to evaluate the responses of plants inoculated with this strain.

Conflict of Interest

The authors declare no conflict of interest.

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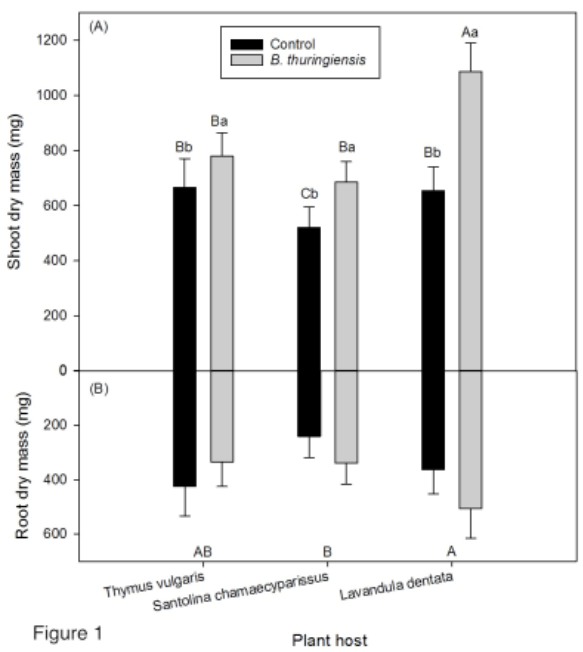

Figure 1 – Influence of a native *B. thuringiensis* strain IAM 12077 on both shoot and root biomass of three autochthonous plant species native to natural arid Mediterranean soil under drought stress conditions. Means + $CI_{95\%}$, data followed by the same capital letter do not differ statistically among plants, while small-case letters refer to differences between inoculated and control plants. Tukey's test at 5% probability.

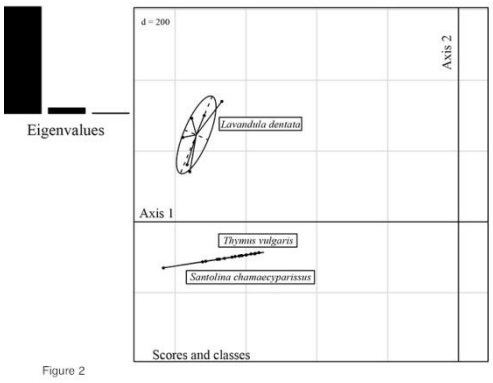

Figure 2

Figure 2 – Effect of three autochthonous plant species of natural arid Mediterranean under drought stress condition on rhizosphere microbial activity and abundance (enzymatic activity and PFLA composition). The interclass inertia was 98.5%, and the Monte Carlo permutation level of significance was P=0.001 after 999 permutations.

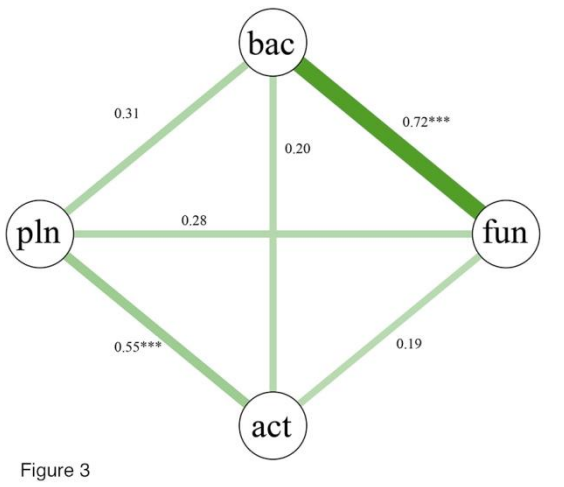

Figure 3

Figure 3 – Co-inertia analysis results from plant biomass and nutrients (pln), microbial activity and abundance (enzymatic activity and PFLA composition) (act), and bacterial (bac) and fungi (fun) community datasets.

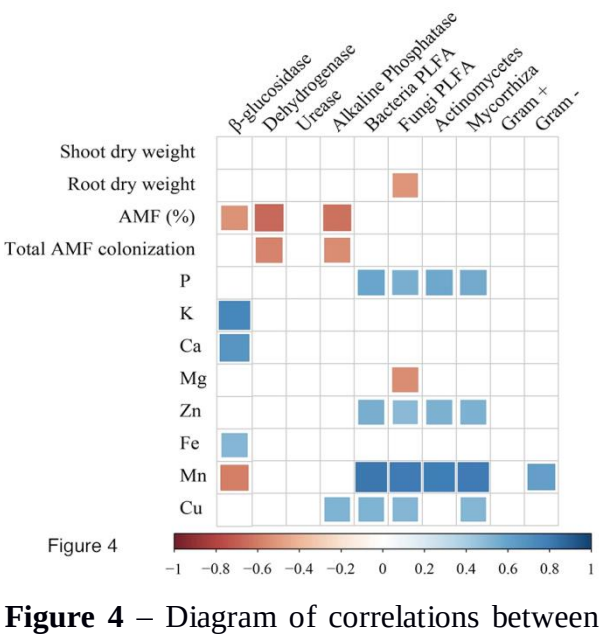

Figure 4

Figure 4 – Diagram of correlations between microbial activity, acidic lipid abundance, fatty acid composition, and plant biomass and nutrients. Blue squares represent positive correlations, and red squares represent negative correlations. Darker/lighter colors indicate stronger/weaker correlations. White squares are non-significant relationships.

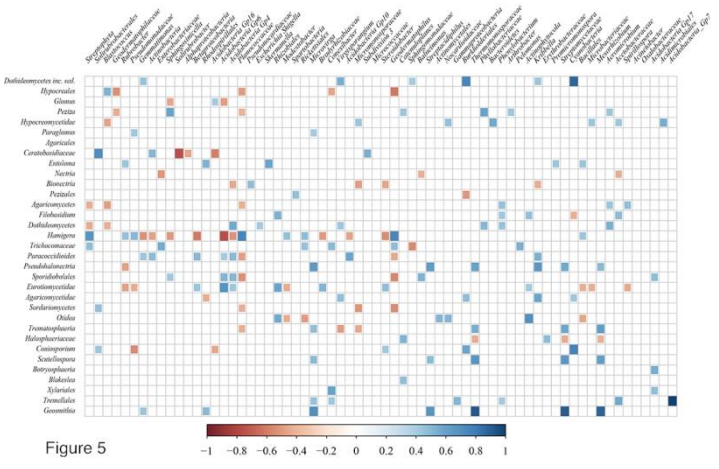

Figure 5

Figure 5 – Diagram of correlations between bacterial and fungal community inhabiting the rhizosphere of the three plant species. The shades of blue squares represent positive correlations, and the shades of red squares represent negative correlations, white squares are non-significant relationships.

Table 1. Total content of macro- (P, K, Ca, Mg) and micronutrients (Zn, Fe, Mn and Cu) in the shoot of three autochthonous plants species *Thymus vulgaris* (T), *Santolina chamaecyparissus* (S) and *Lavandula dentata* (L) grown in natural arid Mediterranean soil under drought stress conditions as affected by the inoculation of the autochthonous bacteria strain *Bacillus thuringiensis* (Bt).

	P (mg plant^{-1})	K (mg plant^{-1})	Ca (mg plant^{-1})	Mg (mg plant^{-1})	Zn (µg plant^{-1})	Fe (µg plant^{-1})	Mn (µg plant^{-1})	Cu (µg plant^{-1})
T(-)	0.57 ±0.11Ab	7.07 ±0.25Bb	6.01 ±1.85Bb	1.70 ±0.62Ab	33.28 ±1.69Ba	42.79 ±3.67ABb	40.35 ±14.51Ba	4.21 ±0.27Bb
TBt	0.86 ±0.09Aa	10.42 ±0.07Ba	7.94 ±0.28Ba	2.21 ±0.24Aa	54.06 ±5.90Bb	115.22 ±17.83ABa	46.49 ±0.74Ba	6.38 ±1.04Ba
S(-)	0.87 ±0.07Ab	10.10 ±0.45Ba	8.45 ±1.00Bb	1.01 ±0.06Bb	60.93 ±3.78Ab	87.16 ±20.55Ba	100.19 ±3.02Ab	11.05 ±0.59Ab
SBt	1.01 ±0.08Aa	12.93 ±0.70Bb	11.95 ±0.67Bb	1.43 ±0.10Ba	89.72 ±7.46Aa	78.43 ±19.76Ba	121.93 ±2.57Aa	13.17 ±0.27Aa
L(-)	0.62 ±0.09Ba	13.51 ±0.45Ab	13.30 ±1.68Ab	2.14 ±0.13Ab	38.50 ±4.69Ab	104.25 ±16.89Aa	13.41 ±1.32Cb	5.19 ±0.48Bb
LBt	0.64 ±0.11Ba	21.97 ±1.48Aa	16.83 ±0.51Aa	2.91 ±0.04Aa	47.39 ±2.42Aa	100.21 ±15.00Aa	20.60 ±0.69Ca	7.23 ±0.69Ba

Standard Errors are given. Within each parameter, values followed by the same capital letter did not differ between plants while the values followed by the same small letter did not differed between inoculation within plant species ($p \leq 0.05$) as determined by Tukey's test (n= 3).

Table 2. Soil enzymatic activities and AMF root colonization in the rhizosphere of three autochthonous plants *Thymus vulgaris* (T), *Santolina chamaecyparissus* (S), *Lavandula dentata* (L) grown in natural arid Mediterranean soil under drought stress conditions as affected by the inoculation of the autochthonous bacteria strain *Bacillus thuringiensis* (Bt).

	Dehydrogenase (µg INTF g^{-1})	β-glucosidase (µmol PNF g^{-1} soil h^{-1})	Urease (µmol N-NH_3 g^{-1} h^{-1})	Alkaline Phosphatase (µmol PNF g soil^{-1} h^{-1})	AMF (%)	Total AMF colonization
T(-)	71.9 ±0.49Ba	137.8 ±0.00Ba	606.5 ±19.96Aa	181.2 ±26.90Ba	54 ±3.0Aa	231 ±37.6Aa
TBt	66.5 ±3.19Ba	177.8 ±0.02Ba	577.3 ±21.56Aa	141.7 ±28.96Ba	48 ±3.5Aa	162 ±24.2Aa
S(-)	88.6 ±3.36Aa	201.5 ±0.02Ba	526.5 ±16.95Ab	302.4 ±17.71Aa	24 ±4.1Bb	64 ±22.4Bb
SBt	72.9 ±5.11Aa	186.9 ±0.04Ba	687.2 ±71.87Aa	215.1 ±5.00Aa	46 ±4.6Aa	157 ±25.7Aa

L(-)	87.3 ±6.65Aa	367.6 ±56.37Aa	622.1 ±35.97Ab	221.4 ±36.02Aa	27 ±2.1Ba	97 ±16.2Ba
LBt	80.6 ±4.08Aa	346.2 ±37.05Aa	679.6 ±12.09Aa	224.5 ±12.58Aa	30 ±1.7Aa	153 ±1.8Aa

Standard Errors are given. Within each parameter, values followed by the same capital letter did not differ between plants while the values followed by the same small letter did not differed between inoculations within plant species ($p \leq 0.05$) as determined by Tukey's test.

Table 3. Content of phospholipid acid (μg PLFA g^{-1} sed) and neutral lipids acid biomarkers (μg NLFA gr^{-1} sed) in the rhizosphere of the three autochthonous plants species [*Thymus vulgaris* (T), *Santolina chamaecyparissus* (S) and *Lavandula dentata* (L)] inoculated (Bt) or not (-) with bacteria strain *Bacillus thuringiensis* grown in natural arid Mediterranean soil under drought stress conditions.

	Bacteria	Fungi	Actinomycetes	Mycorrhiza	Gram+	Gram-	Total PLFA	Total NLFA
T(-)	0.307 ±0.11ABa	0.143 ±0.052ABa	0.059 ±0.018Aa	0.042 ±0.012Aa	0.015 ±0.005Aa	0.020 ±0.006Aa	0.552 ±0.19ABa	14.37 ±2.7Ab
TBt	0.601 ±0.48ABa	0.237 ±0.187ABa	0.207 ±0.111Aa	0.098 ±0.078Aa	0.046 ±0.020Aa	0.041 ±0.008Aa	1.144 ±0.84ABa	18.48 ±0.8Aa
S(-)	0.581 ±0.25Aa	0.266 ±0.101Aa	0.126 ±0.054Aa	0.069 ±0.025Aa	0.028 ±0.010ABa	0.036 ±0.013Aa	1.042 ±0.43Aa	9.91 ±5.2Ab
SBt	0.754 ±0.10Aa	0.323 ±0.054Aa	0.165 ±0.021Aa	0.110 ±0.019Aa	0.026 ±0.013ABa	0.039 ±0.018Aa	1.353 ±0.18Aa	19.42 ±4.7Aa
L(-)	0.015 ±0.004Ba	0.012 ±0.007Ba	0.001 ±0.000Ba	0.003 ±0.000Aa	0.001 ±0.000Ba	0.002 ±0.000Ba	0.032 ±0.01Ba	10.34 ±2.7Ab
LBt	0.093 ±0.003Ba	0.046 ±0.017Ba	0.020 ±0.000Ba	0.022 ±0.000Aa	0.006 ±0.000Ba	0.012 ±0.000Ba	0.181 ±0.02Ba	22.77 ±2.5Aa

Standard Errors are given. Within each parameter, values followed by the same capital letter did not differ between plants while the values followed by the same small letter did not differed between inoculations within plant species ($p \leq 0.05$) as determined by Tukey's test (n= 3).