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Research article

Autochthonous arbuscular mycorrhizal fungi and *Bacillus thuringiensis* from a degraded Mediterranean area can be used to improve physiological traits and performance of a plant of agronomic interest under drought conditions

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ABSTRACT

Studies have shown that some microorganisms autochthonous from stressful environments are beneficial when used with autochthonous plants, but these microorganisms rarely have been tested with allochthonous plants of agronomic interest. This study investigates the effectiveness of drought-adapted autochthonous microorganisms [*Bacillus thuringiensis* (Bt) and a consortium of arbuscular mycorrhizal (AM) fungi] from a degraded Mediterranean area to improve plant growth and physiology in *Zea mays* under drought stress. Maize plants were inoculated or not with *B. thuringiensis*, a consortium of AM fungi or a combination of both microorganisms. Plants were cultivated under well-watered conditions or subjected to drought stress. Several physiological parameters were measured, including among others, plant growth, photosynthetic efficiency, nutrients content, oxidative damage to lipids, accumulation of proline and antioxidant compounds, root hydraulic conductivity and the expression of plant aquaporin genes. Under drought conditions, the inoculation of Bt increased significantly the accumulation of nutrients. The combined inoculation of both microorganisms decreased the oxidative damage to lipids and accumulation of proline induced by drought. Several maize aquaporins able to transport water, CO_2 and other compounds were regulated by the microbial inoculants. The impact of these microorganisms on plant drought tolerance was complementary, since Bt increased mainly plant nutrition and AM fungi were more active improving stress tolerance/homeostatic mechanisms, including regulation of plant aquaporins with several putative physiological functions. Thus, the use of autochthonous beneficial microorganisms from a degraded Mediterranean area is useful to protect not only native plants against drought, but also an agronomically important plant such as maize.

1. Introduction

Plants are constantly confronted with environmental constraints of both biotic and abiotic origin. In particular, drought one of the most common environmental stresses experienced by soil plants (Shinozaki et al., 2003). Drought stress affects plant–water relations, as well as, specific and nonspecific physiological responses (Beck et al., 2007), causing an important detrimental effect on plant growth and nutrition and, thus, limiting crop production. In fact, drought is considered as major cause of declining crop productivity worldwide (Vinocur and Altman, 2005). There is consensus that global climate change is actually occurring and that its negative effects will probably increase in the coming years, imposing significant difficulties to plant and crop development in many areas of the world. These difficulties will be particularly important in current semi-arid agricultural zones (Denby and Gehring, 2005).

Plants usually interact with soil microorganisms that make them more efficient in coping with environmental limitations such as drought. Several strategies have been suggested to overcome the negative effects of drought (Warren, 1998). The most explored approaches have been the breeding for tolerant varieties and the use

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of genetic engineering. However, an alternative strategy is to induce drought stress tolerance by using beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR). There is ample information about the interactions occurring among AM fungi and PGPR, resulting in the promotion of key processes for plant nutrition, growth and health, particularly in stressed environment (Armada et al., 2014b; Marulanda-Aguirre et al., 2008; Vivas et al., 2006). Moreover, several studies have shown that using native AMF and PGPR, which appear to be physiologically and genetically adapted to the stress conditions of the environment of origin, provides a higher benefit for plant performance than non-native isolates (Armada et al., 2014b; Oliveira et al., 2005; Querejeta et al., 2006).

Plants can tolerate severe environmental conditions such as drought, and for that they need to adapt several physiological, biochemical and cellular/molecular processes in order to maintain cell homeostasis (Urano et al., 2010). Osmotic stress is a frequent consequence of plant tissues exposed to drought that induces plant water imbalance (Beck et al., 2007). The accumulation of some metabolites in plant tissues is an important mechanism to overcome the osmotic stress (Armada et al., 2014b; Bárzana et al., 2014). Several authors have reported that PGPR inoculation provides a better plant water balance under osmotic stress (Pereyra et al., 2012). Bacteria have developed mechanisms to cope with drought stress such as the ability to enhance indole-3-acetic acid (IAA) synthesis (Marulanda et al., 2009). Moreover, activities of several bacterial enzymes involved in the ascorbate–glutathione cycle are related with the severity of the stress (Kasim et al., 2013). In many cases, inoculated drought-stressed plants showed lower antioxidant activities than non-inoculated plants. These results are indicative of the bacterial capacity to reduce reactive oxygen species (ROS) levels in drought stressed plants and were also correlated with increased physiological parameters such as photosynthesis (Armada et al., 2014a, 2014b; Kasim et al., 2013; Rueda-Puente et al., 2010).

Under drought conditions, plants have to face with the problem of acquiring sufficient amount of water from the soil (Ouziad et al., 2006), and aquaporins participate in this process (Maurel et al., 2008). Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient (Maurel et al., 2008). These proteins are present in all kingdoms and belong to the major intrinsic protein (MIP) family of transmembrane proteins. In maize two major classes of plant aquaporins are located in the plasma membrane (PIPs) and in the tonoplast (TIPs). PIPs and TIPs isoforms have been recognized as central pathways for transcellular and intracellular water transport (Maurel et al., 2008).

In the last few years, much effort has been concentrated on investigating the function and regulation of aquaporins. High levels of aquaporin expression were shown in tissues with high water fluxes across membranes (Maurel et al., 2008; Otto and Kaldenhoff, 2000). Thus, aquaporins seem to play a specifically important role in controlling transcellular water transport in plant tissues (Javot and Maurel, 2002). In any case, the relationship between aquaporins and plant responses to water deficit is still elusive and with contradictory results (Aharon et al., 2003; Lian et al., 2004). In addition, although many aquaporins are highly selective for water, uptake experiments with *Xenopus laevis* oocytes clearly showed that certain aquaporins are permeable to small solutes such as glycerol, urea, amino acids, CO_2 and/or NH_3/NH_4 or even small peptides and ions (Kaldenhoff et al., 2007; Uehlein et al., 2007), which opens many questions about the physiological roles of aquaporins, especially in AM plants (Maurel and Plassard, 2011). Interestingly, several maize aquaporins have been shown to be regulated by the AM symbiosis under different drought scenarios, and their regulation has been related with the exchange of water and other molecules of physiological importance between the host plant and the AM fungus (Bárzana et al., 2014).

In a previous study we have shown that several native PGPRs from an arid and degraded Mediterranean area were effective in promoting plant growth and development in *Lavandula dentata* and *Salvia officinalis* growing under drought conditions in a natural soil containing also the native AM fungal population (Armada et al., 2014b). However, the question remains if these microorganisms can be also used to promote plant growth in a non-native plant of agronomic interest such as maize (*Zea mays* L.).

Maize is one of the most important crops both for human and animal consumption. According to the Maize CRP Annual Report (2013) (http://maize.org/wp-content/uploads/sites/5/2014/07/MAIZE-CRP-Annual-Report-2013-web.pdf), maize is cultivated on more than 142 million ha worldwide and it is estimated to produce around 913 million tonnes of grain per year, accounting for one third of the total global grain production. Although maize is originally from Mesoamerica, nowadays it is the third most important cereal crop and ranks first in countries with developing economies (Mejía, 2003). However, in arid and semi-arid regions and, particularly in Mediterranean areas, maize is vulnerable to adverse environmental conditions due to limited rainfall, high evapotranspiration, and high temperature (Azevedo Neto et al., 2006).

Thus, the aim of the present study was to analyse the effectiveness of drought-adapted autochthonous microorganisms (*Bacillus thuringiensis* and a consortium of AM fungi) to improve plant growth and physiology under two watering conditions of a non-native plant species which is an important cereal crop. The bacterium *B. thuringiensis* was selected as it was the most effective bacterial strain in the previous study (Armada et al., 2014b).

2. Materials and methods

2.1. Experimental design

The experiment had a 3×2 factorial design with four inoculation treatments: (1) non-inoculated control plants (C), (2) plants inoculated with *B. thuringiensis* (Bt), (3) plants inoculated with a consortium of AM fungi (AMF) and (4) plants dually inoculated with AMF + Bt. In addition, plants were cultivated either under well-watered conditions throughout the entire experiment, or were subjected to drought stress for 8 weeks. Each treatment had ten replicates to give a total of 80 pots.

2.2. Molecular identification of the bacterial strain

The autochthonous bacterium, identified as *B. thuringiensis*, was isolated from a semiarid soil at the Natural Ecological Park "Vicente Blanes" in Molina de Segura, (Murcia, Spain) (Armada et al., 2014b). This area suffers from drought and low nutrients availability and, as a result, desertification. Bt was the most abundant cultivable bacterial type in such arid soil. The bacterium was isolated from the above-mentioned soil (a mixture of rhizospheres from several autochthonous plant species). A homogenate of 1 g soil in 9 mL sterile water was diluted (10^{-2} to 10^{-4}), plated on three different media [Agar Yeast Mannitol, Dextrose Potato agar or Luria–Bertani agar (LB)] and then incubated at 28 °C for 48 h, to isolate bacteria from different taxonomic groups.

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 10X, 50 mM $MgCl_2$, 10 μM each primers: 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), (Rees et al., 2004) and 5 U/μL of

Taq polymerase (Platinum, Invitrogen). The PCR was performed in a thermal cycle with the following conditions: 5 min at 95 °C, followed by 30 cycles of 45 s at 95 °C, 45 s at 44 °C and 2 min at 72 °C, and finally one cycle of 10 min at 72 °C. The products of PCR were analysed by 1% agarose gel electrophoresis and DNA was extracted and purified with the QIAquick Gel extraction kit (QUIAGEN) for subsequent sequencing in an automated DNA sequencer (Perkin–Elmer ABI Prism 373). Sequence data were compared to gene libraries (NCBI) using BLAST program (Altschul et al., 1990).

2.3. Isolation and identification of the arbuscular mycorrhizal fungi (AMF)

AM fungal spores were separated 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 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).

2.4. Soil characteristics and inocula multiplication

The soil used was selected from an area located at the Natural Ecological Park "Vicente Blanes" in Molina de Segura, Murcia (southeastern Spain) (coordinates 38°12′ N, 1°13′ W, 393 m altitude). The climate is semiarid Mediterranean, with an average annual rainfall lower than 270 mm and the potential evapotranspiration (ETP) reaches approximately 1000 mm. The mean annual temperature is 19.2 °C with absence of frost period. The soil in the experimental area is a Typic Torriorthent, very little developed, with low organic matter content and a silty clay texture that facilitates the degradation of soil structure. The vegetation in the zone is dominated by *Piptatherum miliaceum* L. Cosson., *Trifolium repens* L., with some shrubs of *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *Retama sphaerocarpa* growing in a patchy distribution.

The main soil characteristics were pH 8.90, P $1.36 \cdot 10^{-3}$ g kg^{-1} (Olsen test), organic carbon 0.94%, total N 0.22%, and an electric conductivity of 1.55 dS m^{-1}. Soil was sieved (mesh diameter = 2 mm) and sterilized by steaming (100 °C for 1 h on 3 consecutive days). Sand and vermiculite were autoclaved. *Zea mays* seeds were sown in pots containing 1.5 kg of a 1:2:2 mixture of soil: sand: vermiculite (v/v/v). Plants were inoculated with the appropriate inocula at sowing time.

One milliliter of pure bacterial culture (10^7 cfu mL^{-1}), grown in LB medium for 48 h at 28 °C, was applied to the appropriate pots four days after sowing. The bacterial inoculum was applied again 15 days later. In control treatments, 1 mL of sterilized bacterial culture was added. The AM fungal consortium was multiplied in an open pot culture with sorghum. Five grams of AM fungal consortium, containing soil, root fragments and fungal spores and mycelia were applied to each one of the appropriate pots at sowing time, just below the maize seeds. Non-inoculated control plants received the same amount of autoclaved mycorrhizal inoculum together with a 3 mL aliquot of a filtrate (<20 μm) from the AM inoculum in order to provide a general microbial population free of AM propagules.

2.5. Plant growth conditions

Plants were grown for 2.5 months in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C and 50% relative humidity. The photosynthetic photon flux density (PPFD) was $700 \cdot 10^{-6}$ mol m^{-2} s^{-1}, as measured with a light-meter (LICOR, model LI-188B). During the first 2 weeks of plant growth, water was supplied daily to reach 100% of water-holding capacity. After this time, plants from the drought treatment were allowed to dry until soil water content was 50% of water holding capacity, and maintained under these conditions for additional 8 weeks. However, during the 24-h period comprised between each rewatering the soil water content was progressively decreasing until a minimum value of 30% of water holding capacity. Soil moisture was measured with ML2X ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil (Roth et al., 1992; White et al., 1994). During the growing period, Hewitt's nutrient solution was applied weekly (10 mL pot^{-1}) modified to have ½ N and ¼ P concentrations.

2.6. Parameters measured

2.6.1. Biomass production and nutrients acquisition

At harvest time, shoots were excised from the roots, and both shoots and roots were weighted to record fresh weights (ten replicates per treatment, n = 10). Root length was measured by scanning extended roots (five replicates per treatment, n = 5) with a HP Scanjet 5550c (Hewlett Packard, Palo Alto, CA, USA). The images were analysed and quantified with Adobe Photoshop CS (Adobe Systems, Inc., San Jose, CA, USA). After that, they were dried for 2 days at 75 °C to obtain dry weights.

Shoot mineral analysis of N, C, P, K, Mg and Ca (mg $plant^{-1}$), as well as, of B, Fe, Zn and Cu (μg $plant^{-1}$) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Mineral analyses were carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

2.6.2. Symbiotic development

Roots were carefully washed and stained as described in Phillips and Hayman (1970). The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples, using the gridline intersect method (Giovannetti and Mosse, 1980).

2.6.3. Photosynthetic efficiency

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

2.6.4. Stomatal conductance

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

2.6.5. Shoot water potential

Mid-day leaf water potential (Ψ) was determined one day before harvest with a C-52 thermocouple psychrometer chamber and a HR-33T microvoltmeter (Wescor Inc., Logan, UT, USA). Leaf discs were cut, placed inside the psychrometer chamber and allowed to reach temperature and water vapor equilibrium for 15 min before measurements were made by the dew point method.

2.6.6. Electrolyte leakage

Leaf electrolyte leakage was determined in six plants per treatment (n = 6). Leaf samples were washed with deionized water to remove surface-adhered electrolytes. The samples were placed in closed vials containing 10 mL of deionized water and incubated at 25 °C on a rotary shaker for 24 h, and the electrical conductivity of the solution (L_0) was determined using a conductivity meter (Metler Toledo AG 8603, Switzerland). Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (L_f) was obtained after cooling at 25 °C. The electrolyte leakage was defined as follows: $(L_o - L_{water})/(L_f - L_{water})$ X 100, where L_{water} is the conductivity of the deionized water used to incubate the samples.

2.6.7. Leaf photosynthetic pigment contents

Photosynthetic pigments were extracted in 100% methanol from leaf samples (0.2 g). Extinction coefficients and equations reported by Lichtenthaler (1987) were used to calculate the pigment concentrations.

2.6.8. Oxidative damage to lipids and hydrogen peroxide content

Lipid peroxides were extracted by grinding 0.5 g of shoots and roots with an 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) 2-thiobarbituric acid (TBA), and by incubating the mixture at 95 °C for 30 min (Minotti and Aust, 1987). After cooling at room temperature, absorbance was measured at 532 nm. Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1989). The calibration curve was made using MDA in the range of 0.1–100 μmol. The blank for all samples was prepared by replacing the sample with extraction medium.

Hydrogen peroxide content in shoots and roots was determined by Patterson's method (1984), with slight modifications as described by Aroca et al. (2003). Five hundred milligrams of shoot fresh weight was homogenized in a cold mortar with 5 mL 5% (w/v) TCA containing 0.01 g of activated charcoal and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 3070 *g* for 10 min. The supernatant was filtered through a Millipore filter (0.22 μm). A volume of 1.0 mL of 100 mM potassium phosphate buffer (pH 8.4) and 1.0 mL of the colorimetric reagent were added to 100 μL of the supernatant. The colorimetric reagent was freshly made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4–2 (2-pyridylazo) resorcinol (disodium salt). The samples were incubated at 45 °C for 1 h and the absorbance at 508 nm was recorded. The calibration curve was made using H_2O_2 in the range of 50–1000 μmol. The blank was made by replacing plant extract by TCA 5%.

2.6.9. Shoot proline content

The proline was extracted in 100 mM phosphate buffer (pH 7.8) from 0.5 g of fresh shoots and roots. Proline was determined by spectrophotometric analysis at 520 nm using the ninhydrin reaction according to Bates et al. (1973).

2.6.10. Total ascorbate and glutathione content

Total ascorbate was quantified photometrically by the reduction of 2,6-dichlorophenolindophenol (DCPIP) as described by Leipner et al. (1997). Five hundred milligrams of the youngest fully developed leaves of each plant group were homogenized in 5 mL ice-cold 2% (w/v) metaphosphoric acid in the presence of 1 g NaCl. The homogenate was filtered through a filter paper. An aliquot of 3 μL was mixed with 20 μL 45% (w/v) K_2HPO_4 and 10 μL homocysteine 0.1%. After 15 min incubation at 25 °C, 100 μL citrate-phosphate buffer 2 M (pH 2.3) and 100 μL DCPIP 0.003% (w/v) were added. The absorbance was measured at 524 nm. Total ascorbate is expressed in mmol ascorbate g^{-1} shoot or root dry weight.

Glutathione content was measured as described by Smith (1985). Five hundred milligrams of the youngest fully developed leaves of each plant group were homogenized in a cold mortar with 5 mL 5% (w/v) sulfosalicylic acid and the homogenate was filtered and centrifuged at 10,000 rpm for 10 min. One milliliter of supernatant was mixed with 1.5 mL 0.5 M K-phosphate buffer (pH 7.5). The standard incubation medium was a mixture of: 0.5 mL 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.2 mL 6 mM 5, 5′-dithiobis-(2-nitrobenzoic acid), 0.1 mL 2 mM NADPH, and 0.1 mL (1 unit) glutathione reductase. The reaction was initiated by the addition of 0.1 mL glutathione standard or of extract. The absorbance was measured at 412 nm and expressed mmol glutathione g^{-1} shoot or root dry weight.

2.6.11. Root hydraulic conductivity (L_{pr})

The L_{pr} was determined in six plants per treatment (n = 6), using a high pressure flow meter (HPFM, Dynamax, Inc.), between 3 and 4 h after sunrise. The roots were detached from the shoot with a razor blade and, immediately after excision, connected to the HPFM. Water was pressurized into the roots from 0 to 0.5 MPa in the transient mode to calculate root hydraulic conductance (K_r). L_{pr} was determined by dividing K_r by the root fresh weight (Calvo-Polanco et al., 2014).

2.6.12. Molecular analyses

Total RNA was isolated from maize shoots and roots by a phenol/chloroform extraction method, followed by LiCl precipitation (Kay et al., 1987). DNase treatment of total RNA and cDNA synthesis were done with Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany). The expression of the PIP aquaporin subfamily from maize was determined by means of real-time quantitative RT-PCR (iCycler system Bio-Rad, Hercules, CA, U.S.A.), adjusting protocols to optimize the PCR reaction to each gene. The primer sets used to amplify each gene in the synthesized cDNAs were designed in the 3′ and 5′ untranslated regions of each gene (the less conserved regions) in order to avoid unspecific amplifications of the different PIPs (Hachez et al., 2006). The efficiency of the primer sets was evaluated as described by Bárzana et al. (2014).

Standardization was carried out by measuring the expression levels of four different housekeeping genes from maize: poliubiquitin (gi:248338), tubulin (gi:450292), GAPDH (gi:22237) and elongation factor 1 (gi:2282583). After analyses, the best scoring genes were selected. Thus, poliubiquitin gene was chosen in shoots and GAPDH gene was chosen for roots, as the most stable genes in all the treatments. Real-time PCR experiments were carried out in three independent RNA samples and at least three times for each sample, with the threshold cycle (CT) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions.

2.6.13. Statistical analyses

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

3. Results

3.1. Identification and characteristics of microorganisms used as inocula

Each bacterial sequence was compared with the 16S rDNA database. Similarity searches at NCBI using BLAST program, unambiguously identified the bacterium as *B. thuringiensis* (Accession NR 043403.1, similarity >98%).

The more predominant AMF species identified in the native consortium used in this 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 EEZ 198 to EEZ 202, respectively).

B. thuringiensis (Bt) grown under osmotic stress [induced with 40% polyethylene glycol (PEG) (equivalent to −3.99 MPa)], decreased cell growth and certain plant growth promoting abilities (data not shown). In fact, the stress increased proline and ACC production, but did not change the levels of IAA and reduced slightly the phosphate solubilisation ability. Thus, the stress applied in the culture medium to test the bacterial stress tolerance and its PGPR abilities did not reduce significantly the bacterial potential to improve plant growth by mechanisms such as IAA and ACC production or phosphate solubilisation.

3.2. Plant growth and symbiotic development

Under well-watered conditions, the applied microbial treatments did not affect significantly shoot or root biomass production. In contrast, it had an effect on root length. Thus, dual AM + Bt inoculations increased root length by 20% as compared to non-inoculated control plants. Under drought conditions the greatest maize shoot development were achieved in plants singly inoculated with Bt or in those dually inoculated (over 30% of increase in shoot dry weight as compared to non-inoculated control plants). Drought stress had a negative effect in reducing shoot and root growth (Table 1). However, no negative effect of drought was observed in plants dually inoculated with AM + Bt, which did not reduce their biomass production. Plants dually inoculated with AM + Bt showed the highest root length under drought stress conditions (Table 1).

AM-colonization was not observed in non-inoculated plants at harvest time, 75 days after inoculation. No differences in the percentage of root colonization were observed between well-watered and drought-stressed maize plants (Table 1). Similarly, no significant differences on this parameter were observed in dually inoculated plants.

3.3. Accumulation of macro and micronutrients

The different treatments applied had an important effect on plant nutrients acquisition. Results showed that microbial treatments were the main source of variation in the uptake of nutrients. Plants inoculated with microorganisms increased nutrients acquisition as compared to control plants, both under well-watered and under drought conditions (Tables 2 and 3). Drought reduced the uptake of N, C, P, K, Mg and Ca as well as B, Zn and Cu. In general, under drought conditions the inoculation of *B. thuringiensis* induced an increase in the tissue contents of nutrients. The single inoculation of this microorganism in drought-stressed plants increased N, C, P and K by 51%, 23.6%, 37% and 38%, respectively (Tables 2 and 3). A similar trend was observed for micronutrients, which were maximized by the bacterial inoculation and increased by 58% (Mg), 35% (Ca), 43% (B), 95% (Fe), 65.8% (Zn) and 76% (Cu) (Tables 2 and 3). Under well-watered conditions the bacterium did not significantly affect these nutritional values.

3.4. Leaf photosynthetic efficiency, stomatal conductance, water potential, electrolyte leakage and photosynthetic pigments

The effectiveness of AM fungi in increasing photosynthetic efficiency and stomatal conductance was more relevant under well-watered than under drought conditions (Table 4). Microbial treatments did not significantly affect shoot water potential at any water level applied (Table 4).

Regarding the membrane electrolyte leakage, the drought stress treatment increased this value in control plants, while AM colonization highly reduced this value regardless of the watering regime. Such decrease was more important under drought than under well-watered conditions (Table 4).

The leaf content of carotenoids was reduced by drought conditions. However, under stress conditions, plants dually inoculated with AM + Bt had the highest carotenoids content (Table 2). The chlorophyll content was not significantly enhanced by the inoculation with microorganisms, and it decreased as a consequence of drought.

3.5. Root hydraulic conductivity (L_{pr})

We measured root hydraulic conductivity (L_{pr}) in order to analyse the influence of the different treatments on root water transport capacity (Table 4). Under well-watered conditions inoculation of microorganisms did not significantly affect the hydraulic conductivity of maize root. In contrast, under drought conditions, AM plants increased L_{pr} by 192% and AM + Bt plants by 117%, when compared to non-inoculated control plants. The single inoculation

Table 1
Shoot and root dry weights, root length and AMF colonization in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		Shoot dry weight (g)	Root dry weight (g)	Root length (cm)	AMF (%)
Well-watered	C	1.00 ± 0.05 a	0.71 ± 0.03 a	391 ± 20.4 a	0 ± 0.00 a
	Bt	1.02 ± 0.07 a	0.74 ± 0.04 a	442 ± 20.9 ab	0 ± 0.00 a
	AM	0.97 ± 0.06 a	0.65 ± 0.03 a	441 ± 10.7 ab	22 ± 0.02 b
	AM + Bt	0.94 ± 0.07 a	0.64 ± 0.05 a	470 ± 20.5 b	23 ± 0.05 b
Drought	C	0.77 ± 0.04 a	0.66 ± 0.04 a	410 ± 20.4 b	0 ± 0.00 a
	Bt	1.00 ± 0.08 b	0.62 ± 0.07 a	342 ± 30.1 a	0 ± 0.00 a
	AM	0.87 ± 0.05 ab	0.64 ± 0.04 a	411 ± 20.2 b	16 ± 0.02 b
	AM + Bt	1.02 ± 0.07 b	0.74 ± 0.07 a	480 ± 20.4 c	23 ± 0.04 b

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (for shoot and root dry weights and AMF colonization n = 10. For root length n = 5).

Table 2
Contents of N and C in shoots and total chlorophylls and carotenoids in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		N (mg plant^{-1})	C (mg plant^{-1})	Total chlorophylls (mg g^{-1} DW)	Total carotenoids (mg g^{-1} DW)
Well-watered	C	12.57 ± 1.4 a	392.4 ± 17.8 a	4.08 ± 1.1 a	285.08 ± 73.8 a
	Bt	13.27 ± 1.0 a	414.4 ± 21.2 a	4.07 ± 0.7 a	289.10 ± 53.3 a
	AM	11.13 ± 1.2 a	395.3 ± 16.8 a	4.93 ± 1.4 a	337.47 ± 92.6 a
	AM + Bt	11.66 ± 0.7 a	398.6 ± 34.9 a	4.47 ± 0.9 a	308.26 ± 65.6 a
Drought	C	9.23 ± 1.2 a	320.9 ± 15.4 a	2.61 ± 0.4 a	186.36 ± 40.8 a
	Bt	13.95 ± 0.7 b	396.7 ± 19.5 b	3.35 ± 0.7 a	223.41 ± 46.2 a
	AM	8.18 ± 0.5 a	366.0 ± 13.6 ab	3.19 ± 0.8 a	227.89 ± 63.4 ab
	AM + Bt	8.64 ± 0.4 a	410.2 ± 20.6 b	4.04 ± 1.2 a	275.27 ± 83.2 b

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 5).

Table 3
Contents of P, K, Mg, Ca, B, Fe, Zn and Cu in shoots in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		P (mg plant^{-1})	K (mg plant^{-1})	Mg (mg plant^{-1})	Ca (mg plant^{-1})	B (μg plant^{-1})	Fe (μg plant^{-1})	Zn (μg plant^{-1})	Cu (μg plant^{-1})
Well-watered	C	0.53 ± 0.02 a	39.81 ± 2.5 a	4.40 ± 0.4 a	5.62 ± 0.6 a	7.54 ± 1.3 b	35.79 ± 8.1 a	11.69 ± 1.1 a	6.17 ± 0.5 b
	Bt	0.57 ± 0.02 a	42.01 ± 2.2 a	5.62 ± 0.4 b	6.48 ± 0.6 a	8.79 ± 0.5 b	30.82 ± 4.3 a	13.82 ± 1.5 ab	7.42 ± 0.5 b
	AM	0.74 ± 0.04 b	39.36 ± 2.5 a	3.96 ± 0.3 a	4.81 ± 0.4 a	4.45 ± 0.5 a	26.57 ± 3.5 a	15.13 ± 1.4 ab	3.67 ± 0.3 a
	AM + Bt	0.80 ± 0.03 b	35.37 ± 1.7 a	4.23 ± 0.4 a	5.01 ± 0.4 a	5.05 ± 0.5 a	29.10 ± 4.6 a	17.79 ± 1.5 b	4.74 ± 0.4 a
Drought	C	0.35 ± 0.02 a	27.81 ± 1.8 a	3.39 ± 0.3 a	4.53 ± 0.5 a	5.75 ± 0.5 a	42.08 ± 5.9 a	9.87 ± 1.1 a	4.48 ± 0.5 a
	Bt	0.48 ± 0.03 b	38.42 ± 1.3 b	5.35 ± 0.4 c	6.17 ± 0.6 a	8.21 ± 0.6 b	82.14 ± 3.2 b	16.37 ± 0.8 b	7.88 ± 0.6 b
	AM	0.65 ± 0.03 c	31.91 ± 1.5 a	4.12 ± 0.4 ab	5.40 ± 0.5 a	4.89 ± 0.4 a	33.67 ± 3.3 a	13.90 ± 0.9 b	4.02 ± 0.4 a
	AM + Bt	0.73 ± 0.05 c	37.13 ± 1.8 b	4.96 ± 0.4 bc	5.86 ± 0.5 a	6.05 ± 0.7 a	36.94 ± 9.7 a	13.92 ± 0.7 b	4.66 ± 0.7 a

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 5).

Table 4
Photosynthetic efficiency, stomatal conductance, shoot water potential, root hydraulic conductivity (L_{pr}) and leaf electrolyte leakage in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		Photosynthetic efficiency (Fv/Fm)	Stomatal conductance (mmol H_2O m^{-2} s^{-1})	Shoot water potential (MPa)	L_{pr} (mg H_2O g^{-1}RFW MPa^{-1} h^{-1}×10^{-6})	Leaf electrolyte leakage (%)
Well-watered	C	0.22 ± 0.03 a	14.80 ± 0.8 ab	−3.31 ± 0.3 a	3.22 ± 6.9 a	6.41 ± 0.5 a
	Bt	0.21 ± 0.04 a	14.20 ± 0.8 a	−3.43 ± 0.2 a	2.99 ± 5.0 a	13.43 ± 3.1 b
	AM	0.35 ± 0.02 b	19.08 ± 2.1 b	−3.72 ± 0.1 a	3.49 ± 3.7 a	4.49 ± 0.8 a
	AM + Bt	0.39 ± 0.02 b	23.57 ± 1.8 c	−3.35 ± 0.1 a	2.14 ± 2.4 a	3.16 ± 0.4 a
Drought	C	0.26 ± 0.02 a	12.80 ± 0.4 a	−3.77 ± 0.4 a	2.38 ± 7.2 a	8.46 ± 0.9 b
	Bt	0.33 ± 0.03 a	14.47 ± 2.3 a	−3.69 ± 0.1 a	2.58 ± 1.4 a	8.10 ± 1.7 b
	AM	0.34 ± 0.02 a	17.52 ± 1.5 a	−4.26 ± 0.5 a	6.95 ± 9.4 b	3.57 ± 0.5 a
	AM + Bt	0.30 ± 0.02 a	18.23 ± 2.1 a	−3.26 ± 0.2 a	5.18 ± 1.3 ab	4.05 ± 0.8 ab

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 10). For L_{pr}: n = 6.

with Bt did not significantly affect this parameter.

3.6. Oxidative damage to lipids, H_2O_2, proline, ascorbate and glutathione accumulation in shoot and root tissues

The most significant effect of the inocula applied on these parameters was found in root tissue under drought conditions (Tables 5 and 6).

In roots under drought stress, MDA, H_2O_2, proline and glutathione accumulation were considerably lower in the inoculated treatments than in the control one (Table 6). The mycorrhizal association highly decreased the values of MDA by 71% and H_2O_2 by 58%. Mycorrhizal plants decreased proline in shoots (by 36%) compared with control ones, but such decrease was greater in roots (88.6%), and dual inoculation reduced proline by 55%. In root of Bt-inoculated plants proline was lowered by 79% as compared to control plant.

Glutathione accumulation resulted more affected by inoculants than ascorbate. In shoots, inoculation of AM and AM + Bt increased glutathione levels by 63% and by 54% under well-watered conditions. In roots it decreased by 20% and by 37%, respectively. Under drought conditions glutathione decreased in roots, particularly after dual AM + Bt inoculation (by 56%) (Tables 5 and 6).

3.7. Aquaporin gene expression

The maize PIP aquaporin subfamily, comprising the genes *ZmPIP1; 1, ZmPIP1; 2, ZmPIP1; 3, ZmPIP1; 4, ZmPIP1; 5, ZmPIP1; 6, ZmPIP2; 1, ZmPIP2; 2, ZmPIP2; 3, ZmPIP2; 4, ZmPIP2; 5, ZmPIP2; 6* and *ZmPIP2; 7* was analyzed both in shoots and in roots. Several of these genes resulted unaffected by the treatments applied (data not shown). The expression of genes presented in Figures 1 to 4 resulted regulated by the treatments applied. Thus, under well-watered conditions, the expression of *ZmPIP1*; *2*, *ZmPIP1*; *5* and *ZmPIP1*; *6* was inhibited in shoot tissues by inoculation with Bt, while the gene *ZmPIP2*; *1* was up-regulated (Fig. 1). The genes

Table 5
Shoot oxidative damage to lipids (measured as malondialdehyde equivalents, MDA) and contents of hydrogen peroxide, proline, ascorbate and glutathione in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		MDA (μmol g^{-1} DW)	H_2O_2 (μmol g^{-1} DW)	Proline (μmol g^{-1} DW)	Ascorbate (mmol g^{-1} DW)	Glutathione (mmol g^{-1} DW)
Well-watered	C	193.5 ± 5.9 a	319.0 ± 11.1 a	553.5 ± 142.9 a	292.2 ± 12.7 ab	130.3 ± 7.8 a
	Bt	184.3 ± 17.0 a	321.6 ± 11.3 a	784.3 ± 165.3 a	300.5 ± 10.9 ab	136.0 ± 16.1 a
	AM	194.8 ± 19.5 a	330.1 ± 36.8 a	520.4 ± 58.5 a	324.6 ± 15.9 b	213.7 ± 14.8 b
	AM + Bt	260.3 ± 54.5 a	393.4 ± 46.8 a	491.4 ± 200.3 a	286.3 ± 11.4 a	200.4 ± 10.7 b
Drought	C	611.3 ± 140.0 a	395.4 ± 83.2 a	889.2 ± 64.8 b	364.3 ± 28.6 a	179.6 ± 39.3 ab
	Bt	315.2 ± 53.7 a	273.2 ± 66.1 a	982.1 ± 122.9 b	373.0 ± 22.2 a	132.1 ± 7.1 a
	AM	365.2 ± 69.9 a	287.1 ± 70.9 a	571.7 ± 82.0 a	381.4 ± 12.4 a	241.7 ± 20.8 b
	AM + Bt	447.5 ± 129.1 a	348.3 ± 97.5 a	524.0 ± 45.1 a	384.4 ± 8.9 a	187.3 ± 36.6 b

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 4).

Table 6
Root oxidative damage to lipids (measured as malondialdehyde equivalents, MDA) and contents of hydrogen peroxide, proline, ascorbate and glutathione in non-inoculated maize plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered or drought conditions.

		MDA (μmol g^{-1} DW)	H_2O_2 (μmol g^{-1} DW)	Proline (μmol g^{-1} DW)	Ascorbate (mmol g^{-1} DW)	Glutathione (mmol g^{-1} DW)
Well-watered	C	36.3 ± 10.4 a	173.1 ± 45.8 a	457.9 ± 123.8 a	926.2 ± 104.9 a	1318.6 ± 94.6 c
	Bt	22.9 ± 5.2 a	364.3 ± 71.2 ab	682.3 ± 101.7 a	848.6 ± 53.6 a	1368.2 ± 63.5 c
	AM	16.8 ± 2.1 a	496.4 ± 100.8 ab	561.4 ± 96.7 a	838.3 ± 55.1 a	1060.4 ± 54.2 b
	AM + Bt	21.4 ± 3.8 a	540.1 ± 170.5 b	662.2 ± 141.1 a	800.3 ± 30.1 a	827.9 ± 22.4 a
Drought	C	113.7 ± 8.6 bc	560.9 ± 122.1 b	1754.7 ± 129.8 c	572.6 ± 86.3 a	1623.5 ± 250.5 c
	Bt	153.0 ± 39.4 c	406.3 ± 61.2 ab	366.6 ± 141.2 ab	423.7 ± 66.7 a	1116.7 ± 113.0 b
	AM	33.3 ± 20.4 a	237.1 ± 45.4 a	200.1 ± 10.0 a	511.5 ± 84.8 a	1141.2 ± 90.9 b
	AM + Bt	48.1 ± 11.5 ab	272.4 ± 57.7 a	782.0 ± 263.9 b	541.6 ± 24.8 a	714.4 ± 155.0 a

For each parameter the means ± standard errors are given. Within each parameter and watering condition, values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test (n = 4).

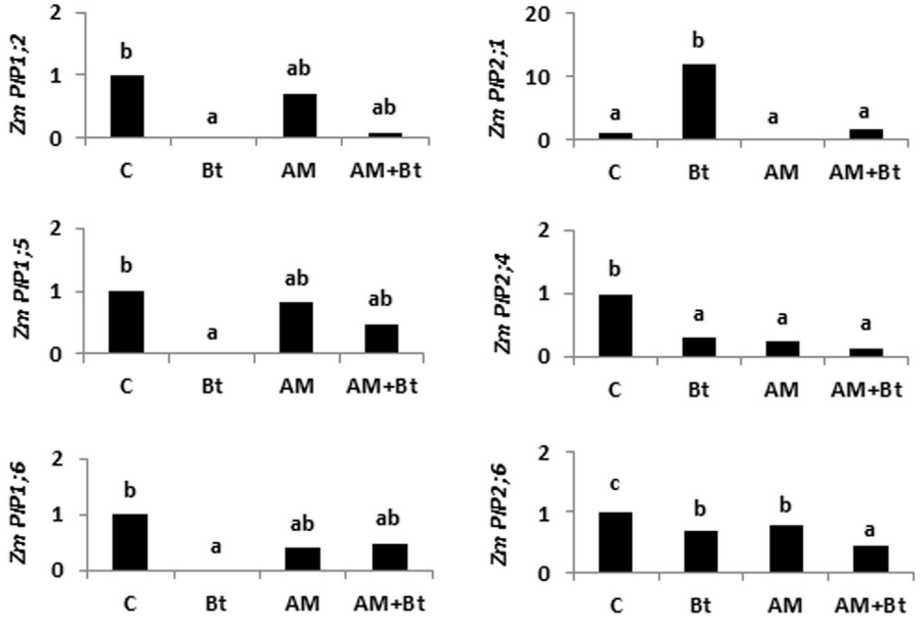

Fig. 1. Shoot gene expression (in relative units) of maize aquaporins in non-inoculated plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under well-watered conditions. Values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test.

ZmPIP2; *4* and *ZmPIP2*; *6* were inhibited by either AM, Bt or their combination (AM + Bt).

In roots, a different effect was observed, since AM inoculation alone or in combination with Bt enhanced the expression of *ZmPIP1*; *2*, *ZmPIP2*; *1*, *ZmPIP2*; *2*, *ZmPIP2*; *5* and *ZmPIP2*; *6* (Fig. 2). Inoculation with Bt alone inhibited the expression of several aquaporins, while it increased the expression of *ZmPIP2*; *3* (both alone and in combination (AM + Bt)).

Under drought stress conditions, the inoculation with the AM fungi up-regulated the expression of *ZmPIP1*; *6* and *ZmPIP2*; 5 in

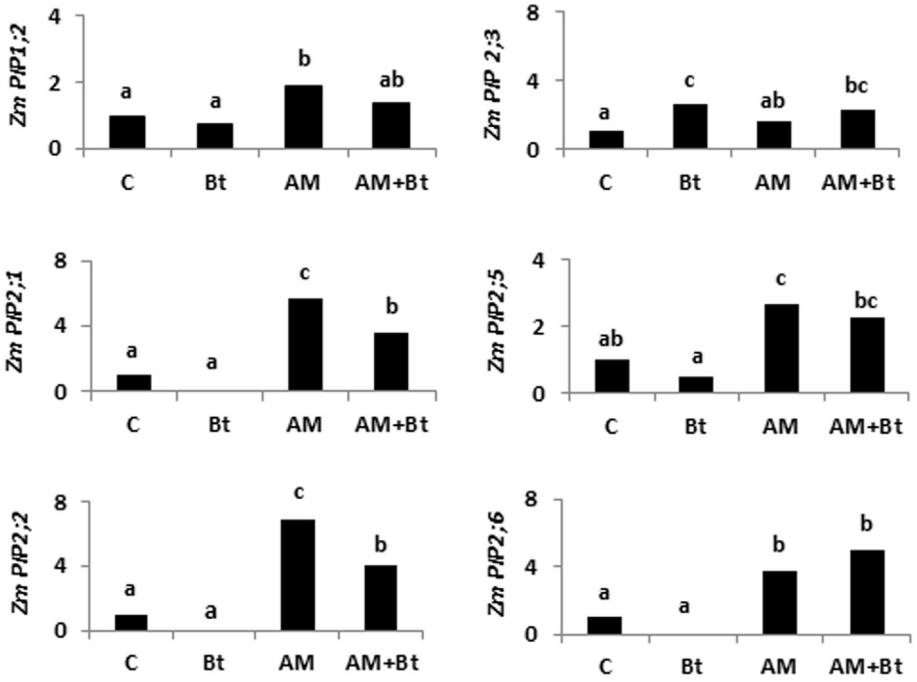

Fig. 2. Root gene expression (in relative units) of maize aquaporins. See legend for Fig. 1.

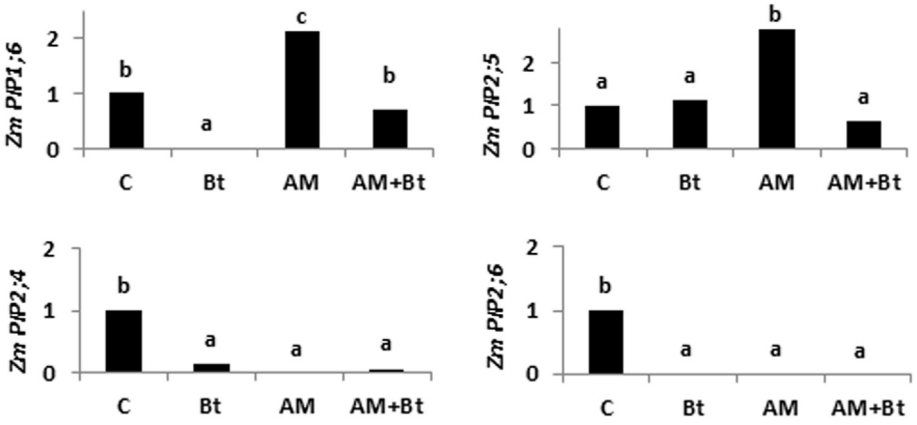

Fig. 3. Shoot gene expression (in relative units) of maize aquaporins in non-inoculated plants (C), plants singly inoculated with *Bacillus thuringiensis* (Bt) or with AM fungi (AM) or coinoculated with both microorganisms (AM + Bt) under drought conditions. Values having a different letter are significantly different ($p \leq 0.05$) as determined by Duncan's multiple-range test.

shoots, but such up-regulation was abolished when Bt was co-inoculated with the fungi (Fig. 3). The genes *ZmPIP2*; *4* and *ZmPIP2*; *6* were down regulated by either Bt or AM inoculation. In roots, the inoculation of the AM fungi up-regulated the expression of *ZmPIP2*; *3* and *ZmPIP2*; *4* and the double inoculation AM + Bt up-regulated *ZmPIP1*; *1*, *ZmPIP1*; *3*, and *ZmPIP2*; *3* (Fig. 4).

4. Discussion

The microbial inoculants applied affected several physiological and molecular processes such as nutrients acquisition, plant biomass production, antioxidative plant responses and expression of aquaporins genes. Indeed, these processes could be particularly regulated according to the inoculant abilities, resulting in a better root development and enhanced nutrition and physiological/biochemical plant values, which represent adaptations to support and counteract the water limiting conditions (Aroca et al., 2012).

In this study, drought highly reduced growth in non-inoculated maize plants but such biomass reduction was smaller in inoculated plants, particularly in those dually inoculated. Also, C, P, K, Ca and Mg were not reduced by drought in these AM + Bt inoculated plants. The non-significant reduction of C in inoculated plants under drought means that the bacterium had a non-limited energy source for its growth and for its activities, allowing its maximum potential. As well, the C fungal requirements resulted compensated by the benefits provided by the symbiotic association (Gianinazzi et al., 2010).

The enhancement of P nutrition is considered an important mechanism of AM-colonized plants to improve growth and water status (Marulanda-Aguirre et al., 2008). As data show, P was the only nutrient increased in shoots by AMF under well-watered conditions (7% by AM and 51% by AM + Bt). Nevertheless, under drought stress such mycorrhizal effect increased up to 86% (AM) and 108% (AM + Bt). Bt inoculation alone also enhanced the uptake

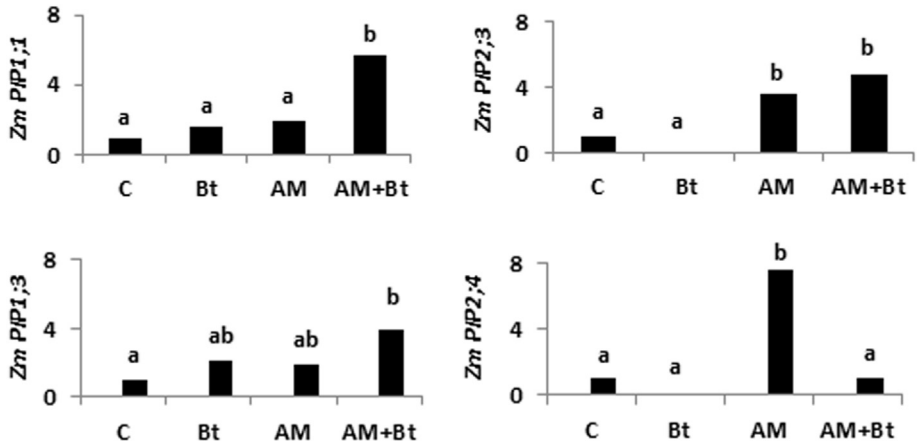

Fig. 4. Root gene expression (in relative units) of maize aquaporins. See legend for Fig. 3.

of this nutrients by 37%. This mycorrhizal effect on P acquisition could be linked to a lower electrolyte leakage and higher glutathione accumulation in these colonized plants (Ruíz-Sánchez et al., 2011). These treatments stimulated root growth and, probably the lateral root formation, thus increasing the water uptake capacity of inoculated plants. This may also affect the switching of water flow through apoplastic or symplastic pathways, thereby improving plant stress tolerance (Bárzana et al., 2012).

Maize is sensitive to water shortages and maize plants are able to maintain C assimilation during drought by decreasing transpiration (Ghannoum, 2009). To improve maize productivity under drought, it may be advantageous to consider whole plant strategies such as root traits, uptake and distribution of nutrients into plant tissues, accumulation of compatible solutes, reduction of oxidative damage, and regulation of water uptake and transport by means of aquaporins (Boomsma and Vyn, 2008). In addition, the maintenance of physiological values such as photosynthesis is also important (Türkan and Demiral, 2009). Photosynthesis performance is the most important factor influencing the plant growth and survival. In this study, dually inoculated plants showed the greatest stomatal conductance and a better functioning of the photosynthetic machinery, which may explain why dually inoculated plants were less affected by drought stress in spite of the higher plant biomass (increased by 111%) of these plants.

The photosynthetic efficiency values increased by microbial inoculation under well-watered conditions, and can be considered as an important process to increase physiological status in these non-stressed plants. Drought stressed maize plants synthesized more proline in shoot and root tissues than well-watered plants. Proline enables the plant to maintain an osmotic balance under low water potential by adjusting osmotic potential and stabilizing membranes and proteins (Mäkelä et al., 2000; Yoshiba et al., 1997). Concomitantly, stressed plants showed the highest oxidative damage to lipids and glutathione accumulation (in shoots and roots) and ascorbate in shoots. Curiously, the lowest enhancements in proline (shoots and roots) and glutathione in roots were found in mycorrhizal plants. The results obtained evidenced that the plant growth promoting microorganisms applied alleviated the oxidative stress generated in maize plants by the water limitation, causing a decrease of MDA and H_2O_2 levels. These effects may contribute to maintain membrane integrity and function (Evelin et al., 2009). Changes in physiological plant parameters seem more important in AMF-colonized plants, while those related to nutrition were more affected by Bt inoculation. Thus, dual inoculation was highly effective exerting beneficial effects related to drought tolerance in maize.

B. thuringiensis was the most effective treatment increasing Fe, Zn and Cu and particularly Fe, probably through production of siderophores (Dimkpa et al., 2009a, 2009b). It is well known that PGPR may improve the plant growth by several mechanisms such as stimulating the synthesis of phytohormones, solubilizing non-available nutrients, optimizing the supply of nutrients and by reducing the levels of ethylene in plants (ACC deaminase production) under stress conditions (Azcón et al., 2013; Yang et al., 2009).

Roots play an important role in drought adaptation and modifying their anatomical and morphological characteristics can contribute to drought tolerance (Kashiwagi et al., 2005). Here mycorrhizal plants had a better root development under drought, which may be further enhanced by the associated extraradical fungal mycelia, allowing the plant to take up more nutrients from deeper soil layers and helping the plant to cope with drought. These nutrients acquisition may be a useful trait for plant resistance to drought.

In this study, the bacterial IAA production did not improve the root development. Thus, the enhanced nutrients uptake by bacterial inoculation under drought conditions cannot be attributed to this cause. However, microorganisms in soil play a major role enhancing the availability of nutrients in the rooting medium.

Regarding L_{pr} values, drought reduced this parameter in control plants, but it was increased in the presence of microorganisms used as inoculants [by 192% (AM) and by 117% (AM + Bt)]. Regulation of aquaporins expression may play important roles to compensate drought effects on root hydraulic conductivity (Bárzana et al., 2014). Indeed, aquaporins provide a low resistance pathway for the movement of water across membranes and their gating ability provides greater control for the movement of water along plant tissues (Maurel et al., 2008). Thus, in this study we analyzed the expression pattern of the whole PIP aquaporin subfamily, as the most relevant for regulation of water transport in maize (Chaumont and Tyerman, 2014). Some of these PIP genes resulted regulated by the microorganisms used as inoculant, but results varied in shoots and roots and also depending on the watering conditions. In shoot tissues, most of the PIPs were down-regulated under well watered conditions, except *ZmPIP2;1* that was up-regulated by Bt application. Under drought stress conditions, single AMF inoculation up-regulated *ZmPIP1;6* and *ZmPIP2;5,* while inoculation with Bt or AM + Bt inhibited the expression of most of the aquaporins.

In root tissues most of the PIPs were up-regulated in AMF-inoculated plants (singly or dually inoculated) under well-watered conditions. This includes *ZmPIP2;5*, which is one of the most expressed aquaporins in maize roots (Hachez et al., 2006). Under drought stress conditions, two PIP genes were up-regulated

by single AMF inoculation and three PIP genes by dual AM + Bt inoculation. Curiously, ZmPIP2; 4 was only up-regulated by single AMF inoculation, in agreement with recent results by Bárzana et al. (2014), but this effect disappeared when the AMF was co-inoculated with Bt. This suggests that the function that this specific aquaporin plays *in planta* may be compensated by the bacterial activity.

The function and regulation of aquaporins is quite intensively integrated to explain the remarkable hydraulic properties of plants (Maurel et al., 2008). According to the composition of their selectivity filters (Hove and Bhave, 2011), all the PIPs genes regulated by the microbial inoculants applied in this study have the potential for water transport. Moreover, *ZmPIP2*; *2* and *ZmPIP2*; *5* have been shown to transport high amounts of water (Bárzana et al., 2014; Hachez et al., 2008). Thus, the effects of the microbial inoculants on the PIP genes in maize may be related to a possible role of these aquaporins in root water uptake from soil. Indeed, under drought stress conditions the root hydraulic conductivity of AM- or AM + Bt-inoculated plants was significantly higher than that of uninoculated control plants, and this correlated with the up-regulation of several PIP genes in roots of these plants. Nevertheless, it has become increasingly clear that some aquaporins (including PIPs) do not exhibit a strict specificity for water and can transport also other small neutral molecules such as glycerol, urea, carbon dioxide (CO_2), hydrogen peroxide (H_2O_2) or boric acid (Bienert et al., 2014; Fitzpatrick and Reid, 2009; Heinen et al., 2014; Uehleln et al., 2003), highlighting the potential relevance of aquaporins for plant physiology (Li et al., 2014).

The ability of aquaporins to transport urea has pointed to important roles for aquaporins in nitrogen metabolism. The diffusion of CO_2 through aquaporins suggests their involvement in carbon fixation and photosynthesis. The ability of aquaporins to transport H_2O_2 points to important roles in stress signaling and responses. Silicon seems to be crucial for responses to biotic and abiotic stresses (Maurel et al., 2008; Miwa et al., 2009). Indeed, (Li et al., 2014) have recently shown that silicon induced an up-regulation of certain aquaporins in sorghum and this translated into higher root hydraulic conductivity under osmotic stress. Thus, it is possible that the regulation of several PIP aquaporins by the microbial inoculants used in this study may also affect the uptake and/or transport *in planta* of these compounds, with subsequent effects on plant physiology (Li et al., 2014). For instance, *ZmPIP1*; *6* and *ZmPIP2*; *5* were considerably induced in shoots of AMF-inoculated plants under drought stress. *ZmPIP1*; *6* can transport CO_2 (Heinen et al., 2014) and *ZmPIP2*; *5* has been shown to be involved in leaf radial water movement (Hachez et al., 2008) and can also transport H_2O_2 (Bienert et al., 2014). Thus, their activity may have contributed to a high transpiration and photosynthetic rates in these plants, as well as, to a better signaling of the drought stress responses, resulting in enhanced growth.

In conclusion, results show that the bacterium used (*B. thuringiensis*) has a strong impact on plant nutrition, while the AM fungi were more active improving stress tolerance/homeostatic mechanisms, including regulation of plant aquaporins with several putative physiological functions. Thus, the combination of morphological, metabolic and physiological effects obtained using both microorganisms (AM + Bt) allowed maize plants to gain tolerance against drought.

This study demonstrated that the use of beneficial microorganisms is a promising approach to alleviate drought stress damage in maize plants. The present results support those reported in previous studies, using shrubs exposed to different environmental conditions (natural soil) but the same bacterial strain (Armada et al., 2014b). In addition, our results validate the benefits of using autochthonous plant growth promoting microorganisms from a degraded Mediterranean area not only to protect native plants against drought, but also an agronomically important plant such as maize.

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