



Exploring the use of recombinant inbred lines in combination with beneficial microbial inoculants (AM fungus and PGPR) to improve drought stress tolerance in tomato



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ABSTRACT

At a world scale, tomato is an important horticultural crop, but its productivity is highly reduced by drought stress. Combining the application of beneficial microbial inoculants with breeding and grafting techniques may be key to cope with reduced tomato yield under drought. This study aimed to investigate the growth responses and physiological mechanisms involved in the performance under drought stress of four tomato recombinant inbred lines (RIL) after inoculation with the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* and the plant growth promoting rhizobacteria (PGPR) *Variovorax paradoxus* 5C-2. Results showed a variation in the efficiency of the different tomato RILs under drought stress and a differential effect of the microbial inoculants, depending on the RIL involved. The inoculants affected plant parameters such as net photosynthetic capacity, oxidative damage to lipids, osmolyte accumulation, root hydraulic conductivity or aquaporin abundance and phosphorylation status. RIL66 was the one obtaining maximum benefit from the microbial inoculants under drought stress conditions, due likely to improved CO₂-fixation capacity and root hydraulic conductivity. We propose that RIL66 could be selected as a good plant material to be used as rootstock to improve tomato growth and productivity under water limiting conditions. Since RIL66 is highly responsive to microbial inoculants, this grafting strategy should be combined with inoculation of *R. irregularis* and *V. paradoxus* in order to improve plant yield under conditions of drought stress.

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1. Introduction

Drought stress has a major impact on plant growth and development, limiting crop production throughout the world. It has been estimated that nearly one third of soils are too dry to support normal plant development and productivity (Golladack et al., 2014). Moreover, global climate change is spreading this problem of water deficit to regions where drought impacts were negligible in the past (Trenberth et al., 2014).

To cope with environmental stresses, plants have developed a variety of strategies (Dobra et al., 2010). Under drought stress plants regulate the permeability of tissues to water movement, use osmotic adjustment and enhance their antioxidant systems. The first of these processes is based on modifying membrane water permeability, a process in which aquaporins are involved (Maurel et al., 2008; Chaumont and Tyerman, 2014). Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient (Maurel et al., 2015), affecting directly the radial water flow through the cell-to-cell pathway. Under conditions of low transpiration, such as under drought stress, this pathway is predominant for water movement in plants (Steudle and Peterson, 1998). Among plant aquaporins, the plasma membrane intrinsic proteins subfamily (PIPs1 and

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PIPs2) is critical for whole plant water transport (Javot and Maurel, 2002; Chaumont and Tyerman, 2014). Since plants undergo frequent environmental changes, the activity of PIPs must be regulated by mechanisms that allow rapid responses to these changes. Post-translational modifications are necessary to achieve such rapid regulation (Vandeleur et al., 2014), including phosphorylation/de-phosphorylation of specific serine residues, the first post-translational regulation mechanism found in aquaporins. This generates conformational changes allowing aquaporin gating (Johansson et al., 1998; Prado et al., 2013) or modifying the subcellular localization of PIPs in the membrane (Prak et al., 2008) and may be a mechanism to prevent water loss (Bárzana et al., 2015).

The accumulation of compounds such as soluble sugars, proline, glycine betaine, pinitol or mannitol allows plants to osmotically adjust to maintain cell turgor (Morgan, 1984; Bheemareddy and Lakshman, 2011). Proline, a non-protein amino acid that accumulates in most plant tissues subjected to water stress, is one of the most common osmolytes accumulated (Kishor and Sreenivasulu, 2014) and can be readily metabolized upon recovery from drought (Singh et al., 2000). Besides acting as an osmoregulatory compound, proline also serves as a sink for energy, regulating redox potentials, as a scavenger of hydroxyl radicals, as a means of reducing acidity in the cell, and as a solute that protects macromolecules against denaturation (Kishor and Sreenivasulu, 2014).

Under drought stress, several metabolic pathways are uncoupled and electrons are transferred to molecular oxygen to form reactive oxygen species (ROS) (Noctor et al., 2014). ROS are toxic molecules capable of causing oxidative damage to lipids, proteins and DNA (Miller et al., 2010). However, at low levels, ROS can act as signalling molecules for stress responses and its generation is an early plant stress response (Singh et al., 2011). Antioxidant systems aim to eliminate excessive ROS production under stress conditions (Gill and Tuteja, 2010). The scavenging of ROS is achieved through the action of non-enzymatic compounds and different enzymatic systems. Non-enzymatic mechanisms include compounds able to scavenge directly several ROS, such as ascorbic acid (AsA), glutathione (GSH), or α -tocopherol. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), ascorbate- or thiol-dependent peroxidases, and the enzymes of the ascorbate-glutathione pathway (Scheibe and Beck, 2011).

At a world scale, tomato is the most important horticultural crop, and the second most important vegetable consumed after potato. Tomato is a major dietary component in many countries and constitutes an important source of vitamins, sugars, minerals, and antioxidant compounds. However, its productivity is highly reduced by abiotic stresses, including drought (Schwarz et al., 2010). While climate change is reducing crop productivity, world agriculture must increase its productivity by 60% to feed the expected population of 9.6 billion people in 2050 (Cabot et al., 2014). Therefore, drought tolerance is a target trait in breeding programs, particularly for rootstocks. Combining breeding techniques with grafting techniques and the application of beneficial microbial inoculants will play a key role in developing a more profitable horticulture to address this challenge (Asins et al., 2010; Albacete et al., 2015b). The rootstock effect to ameliorate abiotic stress tolerance in tomato was previously tested in a population of recombinant inbred lines (P-RILs) (Albacete et al., 2015a,c).

Grafting is a biotechnological tool to improve not only the amount and uniformity of crop yield, but also stress tolerance (reviewed by Albacete et al., 2015b). Nowadays, most fruit crops and many horticultural species are grown as scion-rootstock combinations. This strategy allows desired features such as stress tolerance to be conferred by a suitable rootstock, while retaining

excellent fruit yield and quality traits of a given scion (Asins et al., 2010). Thus, to start a grafting program to improve tomato drought tolerance, the selection of suitable genotypes to be used as rootstocks is the first necessary step.

Many studies have shown that the arbuscular mycorrhizal (AM) symbiosis and plant growth-promoting rhizobacteria (PGPR) may enhance host plant stress tolerance, including to drought (Azcón et al., 2013; Malusá et al., 2013; Zoppellari et al., 2014). Indeed, plant symbiotic relationships with mycorrhizal fungi greatly increase the surface area over which plant root systems take up water and nutrients. Soil bacteria on the root surface alter root phytohormone status thereby increasing growth, and can make nutrients more available to the plant. Studies have also shown that these beneficial microorganisms improve plant osmotic adjustment and antioxidant responses, as well as, water status throughout regulation of plant aquaporins (Marulanda et al., 2010; Dodd and Ruiz-Lozano, 2012; Azcón et al., 2013; Ruzzi and Aroca, 2015; Kaushal and Wani, 2016). Combining these two groups of microorganisms can increase crop resource use efficiency and productivity under stressful environmental conditions (Dodd and Ruiz-Lozano, 2012).

In spite of the positive effects of AM fungi and PGPR on plant productivity, no studies have dealt with the use of breeding techniques in combination with these microorganisms to improve plant productivity under stressful conditions. Thus, we hypothesize that combining the use of a selected group of RILs having specific traits with microbial inoculants with a proved ability to improve drought tolerance will be useful to combine drought tolerance features coming from both the plant genotype and its interaction with AMF and PGPR and, thus, will improve tomato performance under drought. This study aimed to investigate the growth and physiological response of four P-RILs after root colonization by AMF and PGPR. The tomato lines obtaining maximum benefit from the microbial inoculants and performing best under drought stress conditions will be identified and selected as the most suitable in grafting programs directed toward improved tomato productivity under drought. The study also aims to understand the underlying physiological mechanisms involved in the improved plant performance.

The RILs used represent a valuable resource that has already been used to identify a specific QTL conferring salinity resistance (Asins et al., 2010, 2015). The AM fungus *Rhizophagus irregularis* DAOM 197198 was formerly known as *Glomus intraradices*. It was reassigned to *G. irregulare* by Stockinger et al. (2009) and then as *Rhizophagus irregularis* (Kruger et al., 2012). This fungus is widely used in abiotic stress studies, being one of the most effective in drought stress alleviation (Ruiz-Lozano et al., 2012; Azcón et al., 2013). *Variovorax paradoxus* 5C-2 is a PGPR that promotes tomato root length in vitro irrespective of bacterial load (Belimov et al., 2007) and stimulates root and shoot growth of another Solanaceae (potato) grown in both well-watered and drying soils (Belimov et al., 2015).

2. Materials and methods

2.1. Plant materials and experimental design

The experiment consisted of a complete randomized factorial design with four tomato recombinant inbred lines (RIL20, RIL40, RIL66, RIL100) plus one commercial cultivar (Boludo) used as reference (*Solanum lycopersicum* L. cv. Boludo F1, Monsanto). These RILs belong to a population of F10 lines (P population) derived by single seed descent from a cross between a salt sensitive genotype of *Solanum lycopersicum* var. *Cerasiforme* (formerly *L. esculentum*) and a salt tolerant line from *S. pimpinellifolium* L. (formerly *L. pimpinellifolium*) (Monforte et al., 1997). P population

has been extensively studied as rootstock of Boludo cultivar to ameliorate tolerance to several abiotic stresses (Albacete et al., 2015a,c; Asins et al., 2015).

Plants remained as uninoculated controls or were inoculated either with the AM fungus *Rhizophagus irregularis* MUCL 41833 – DAOM 197198 (AM fungus), the plant growth promoting bacteria *Variovorax paradoxus* 5C-2 (PGPR) or a combination of both microorganisms (AM fungus+PGPR). Ten replicates of each treatment totaled 200 pots (one plant per pot), with half of the plants cultivated under well-watered conditions throughout the entire experiment and the other half subjected to drought stress for four weeks before harvest.

2.2. Soil and biological materials

A loamy soil was collected at the grounds of Instituto de Investigación y Formación Agraria y Pesquera de Andalucía (IFAPA, Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100 °C for 1 h on 3 consecutive days). The soil had a pH of 8.1 (water); 1.5% organic matter, nutrient concentrations (g kg⁻¹): total N, 1; total P, 1 (NaHCO₃-extractable P); total K, 11. The soil texture comprised 38.3% sand, 47.1% silt and 14.6% clay.

Four RILs from the above-described P population were selected in previous assays for this study under drought stress conditions, on the basis of their good levels of root colonization by the AM fungus *R. irregularis* and the PGPR *V. paradoxus*, as well as, positive growth responses. Seeds from the different lines and the commercial cultivar (Boludo) were pre-germinated on sand for ten days and then transferred to 1.5 L plastic pots filled with 1200 g of the soil/sand mixture described above.

Mycorrhizal inoculum was provided by INOQ GmbH (<http://inoq.de/>) and consisted of sand containing spores, mycelia and AM fungi-colonized root fragments. The density of inoculum was estimated to 220000 propagules L⁻¹. Approximately 40 mL (circa 80 g) of the AM inoculum were applied to the appropriate pots, following manufacturer's recommendations. Plants that were not inoculated with the AM fungus, received the same amount of sand together with a 3 mL aliquot of a filtrate (<20 µm) of the AM inoculum to provide a general microbial population free of AM propagules.

The *Variovorax paradoxus* 5C-2 inoculum was also provided by INOQ GmbH in liquid medium (10⁸ cfu/mL), so that 1.5 mL of the purified bacterial culture was diluted with sterile water in a final volume of 15 mL and applied to the appropriate pots, according to manufacturer's recommendations. Thus, each pot received 1.5 × 10⁸ cfu.

2.3. Growth conditions

The experiment was carried out under greenhouse conditions with temperatures ranging from 19 to 25 °C, 16/8 h light/dark period, a relative humidity of 50–60% and an average photosynthetic photon flux density of 800 µmol m⁻² s⁻¹, as measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B).

Plants were cultivated for 9 weeks. After week 3, plants received weekly 15 mL per pot of Hoagland's nutrient solution (Hoagland and Arnon, 1950) containing only 25% of P, to prevent inhibition of AM root colonization. The plants were cultivated under well-watered conditions for 5 weeks. At that point, half of the pots per each of the inoculation treatments were left well-watered, and the other half subjected to drought stress for additional four weeks before harvest.

Soil moisture was controlled with the ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK). Water was supplied daily to maintain soil at 100% of field capacity (corresponding to 22%

volumetric soil moisture measured with the ThetaProbe, as determined experimentally in a previous experiment using a pressure plate apparatus) during the first 5 weeks after sowing. Then half of the plants were allowed to dry until soil water content reached 60% of field capacity (two days needed), while the other half were maintained at field capacity. This soil water holding capacity corresponds to 9% volumetric soil moisture measured with the ThetaProbe (also determined experimentally with a pressure plate apparatus in a previous assay). The level of drought stress (60% of field capacity) was selected on the basis of previous studies in order to subject tomato to a sharp drought stress (Ruiz-Lozano et al., 2016). The soil water content was measured daily with the ThetaProbe ML2 before rewatering (at the end of the afternoon), reaching a minimum soil water content around 55% of field capacity. The amount of water lost was added to each pot to keep the soil water content at the desired level (Porcel and Ruiz-Lozano, 2004). Plants were maintained under such conditions for 4 additional weeks before harvesting.

2.4. Parameters measured

2.4.1. Biomass production and symbiotic development

The shoot dry weight (SDW) was measured as an integrative index of plant performance under the growing conditions assayed. At harvest time (9 weeks after transplanting), shoots were detopped from roots, and fresh weights recorded. Samples were kept to measure dry weight after drying in a forced hot-air oven at 70 °C for two days.

The percentage of mycorrhizal root colonization was estimated in five roots per RIL line and treatment combination. Approximately 0.5 g of root tissues were cleared in 10% KOH and stained with 0.05% trypan blue in lactic acid (v/v). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

To quantify bacterial root colonization as previously described (Belimov et al., 2015), fresh tomato root samples were weighed and homogenized in sterile tap water with a sterile mortar and pestle. Homogenates were serially diluted in 10-fold steps and 20 µL aliquots were plated in three replicates on LB agar supplemented with 30 µg mL⁻¹ kanamycin and 20 µg mL⁻¹ rifampicin, to which *V. paradoxus* 5C-2 naturally shows resistance, and 40 µg mL⁻¹ nystatin to prevent fungal growth. The characteristic colonies of *V. paradoxus* 5C-2 were counted after incubation at 28 °C for 3 days.

2.4.2. Plant CO₂ assimilation rate and leaf chlorophyll content

The CO₂ assimilation rate was measured 2 h after sunrise on the second youngest leaf from each plant. We used a portable infrared gas analyzer LI-6400 (LI-COR Biosciences, Inc., Lincoln, NE, USA), which allows environmental conditions inside the chamber to be precisely controlled, with 400 ppm CO₂ concentration, a humidity of 50% and a light intensity of 1000 µE m⁻² s⁻¹. The photosynthetic parameters were calculated by using LI-6400 6.1 software.

Leaf chlorophyll contents were estimated 4 h after sunrise using a SPAD, model 502 (Minolta, München, Germany) on the second youngest leaf for each plant.

2.4.3. Oxidative damage to lipids and proline content

Oxidative damage to lipids was measured by grinding 500 mg of fresh leaf tissues with an ice-cold mortar and 6 mL of 100 mM potassium phosphate buffer (pH 7). Homogenates were filtered through one Miracloth layer and centrifuged at 15,000g for 20 min. The chromogen was formed by mixing 200 mL of supernatants with 1 mL of a reaction mixture containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl hydroxytoluene, 0.25 N HCl and by incubating the mixture at 100 °C for 30 min. After cooling at room temperature, tubes were

centrifuged at 800g for 5 min and absorbance of the supernatant 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). The calibration curve was made using MDA in the range of 0.1–10 nmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25 N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artefactual formation of 2-thiobarbituric acid-reactive substances (TBARS) during the acid-heating step of the assay.

Free proline was extracted from 1 g fresh tissues in sulfosalicylic acid 5% (w/v). Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. (1973).

2.4.4. Root hydraulic conductivity

Root hydraulic conductance (K_r) was measured with a high pressure flow meter (HPFM Dynamax Inc., Houston). K_r measurements utilized the transient mode, where the pressure increases over a range and K_r is calculated from the slope of flow versus pressure. For that, detached tomato roots were connected to the HPFM using compression couplings, and water was perfused at increasing pressures ranging from 0 to 500 kPa. Root volume was calculated after the measurements as in Calvo-Polanco et al. (2012) and hydrostatic root hydraulic conductivity ($L_{p,r}$) determined by dividing K_r by the root volume.

2.4.5. PIP aquaporins abundance and phosphorylation status

We analyzed PIP1 and PIP2 proteins abundance and the PIP2 phosphorylation state in root samples. We checked accumulation of these proteins since aquaporin gene expression is not always correlated with protein abundance, and aquaporin activity can be regulated by phosphorylation events (Prado et al., 2013). The phosphorylation of PIP2 aquaporins was quantified by the use of two different antibodies that recognize a phosphorylated Serine residue at position 280 (PIP2₂₈₀) or two phosphorylated Serine residues at positions 280 and 283 (PIP2₂₈₀₋₂₈₃) in the C-terminal end (Calvo-Polanco et al., 2014a,b). It has been previously shown that the phosphorylation of PIP2 aquaporins at Ser280 and Ser283 was linked to the regulation of hydraulic conductivity in plants (Prado et al., 2013).

Microsomes were isolate from three different tomato roots per RIL and treatment combination, as described in Hachez et al. (2006). Two micrograms of the protein extracts were used for ELISA analyses to determine the abundance of the different tomato proteins, as described in Calvo-Polanco et al. (2014a,b). We used four different primary antibodies (at a dilution of 1:1000), two antibodies that recognize several PIP1s and PIP2s, and two antibodies that recognize the phosphorylation of PIP2 proteins in the serine residue at the C-terminal end, Ser280 (PIP2₂₈₀) or in two serine residues at the C-terminal end (Ser 280 and Ser 283 – PIP2_{280/283}) (Prado et al., 2013; Calvo-Polanco et al., 2014a,b). All antibodies were designed against the most conservative regions of these aquaporin groups. To detect PIP1 aquaporins, we used the first 26 amino acids of the N-terminal part of the PvPIP1;3 protein (accession No. DQ855475). To detect PIP2 aquaporins, we used the last 12 amino acids of the C-terminal part of the PvPIP2;1 protein (accession No. AY995195). To detect phosphorylated PIP2, we used the same protein PvPIP2;1 as the amino acid sequence but with one or two serine groups phosphorylated, AIKALG{pSER}FR{pSER}NA (Abyntek Biofarma SL, BiotechSpain), as described by Calvo-Polanco et al. (2014b). A goat anti-rat IgG coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as secondary antibody at 1:10,000 for PIP1. Goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as

secondary antibody at 1:10,000 for PIP2 and PIP2₂₈₀, and PIP2_{280/283}. Protein quantification was carried out in three different independent root samples per treatment ($n=3$), replicated three times each. The specificity of the PIP2 and phosphorylated antibodies PIP2₂₈₀, and PIP2_{280/283} is described in Calvo-Polanco et al. (2014b). The equal loading of proteins in the different treatments was confirmed by staining a gel blot loaded with the same quantities used for the ELISA measurement with Coomassie brilliant blue and also by Bradford quantification (Bradford, 1976).

2.5. Statistical analysis

Within each recombinant inbred line, data were subjected to analysis of variance (ANOVA) with the Proc MIXED procedure in SAS (version 9.2, SAS institute Inc., NC, USA) together with the post-hoc Tukey's test to detect significant differences among treatment means. The different inoculation treatments and water regimes were the sources of variation (Supplementary Table S1 in the online version at DOI: [10.1016/j.envexpbot.2016.06.015](https://doi.org/10.1016/j.envexpbot.2016.06.015)).

3. Results

3.1. Microbial root colonization

The percentage of root length colonized by the AM fungus ranged from 26% in RIL100 to 67% in RIL66 when co-inoculated with the PGPR bacterium (Supplementary Fig. S1 A in the on line version at DOI: [10.1016/j.envexpbot.2016.06.015](https://doi.org/10.1016/j.envexpbot.2016.06.015)). The commercial line Boludo, also exhibited high mycorrhizal root colonization, reaching 62% of root length colonized. However, the co-inoculation of Boludo plants with the PGPR decreased the AM root colonization as compared to plants inoculated with the AM fungus alone. In RILs 66 and 100, the co-inoculation of the PGPR had a positive effect on AM root colonization under well-watered conditions. No AM root colonization was observed in uninoculated plants.

The bacterial colonization of inoculated tomato roots in the different RILs was estimated as the number of colony-forming units (CFU) g^{-1} root fresh weigh (RFW) and ranged from 5×10^5 CFU g^{-1} RFW in RIL20 to 30×10^7 CFU g^{-1} RFW in cv. Boludo, with most plants having an average of 6×10^6 CFU g^{-1} RFW (Fig. S1 B in the online version at DOI: [10.1016/j.envexpbot.2016.06.015](https://doi.org/10.1016/j.envexpbot.2016.06.015)). No colonies were recovered from non-inoculated plants.

3.2. Plant biomass

The inoculation of the different tomato lines had different effects on SDW according to the genotype studied (Fig. 1). Under well-watered conditions, the different inoculation treatments did not affect the SDW of Boludo and RIL40 plants (Fig. 1). However, the AM fungus and AM fungus + PGPR treatments increased the SDW of RIL66 plants and decreased the SDW of RIL20 and RIL100 plants (Fig. 1).

After 4 weeks of drought treatment, there was a general reduction of SDW in Boludo and RIL20 plants, with no significant differences between the various inoculation treatments and the uninoculated control plants. Drought decreased SDW of RIL66 plants by 79%, but plants treated with the different microbial inoculants had significantly higher SDW than the control plants, with the maximum values in the AM and AM fungus + PGPR treatments (Fig. 1). Therefore, RIL66 clearly obtained beneficial effects from PGPR and AM inoculation under drought conditions. In RIL40, drought stress and PGPR treatment reduced SDW by 71% and 75%, respectively, while the presence of AM fungus recovered the SDW values as in well-watered control plants (Fig. 1). A negative effect was observed for RIL20 only under well-watered conditions.

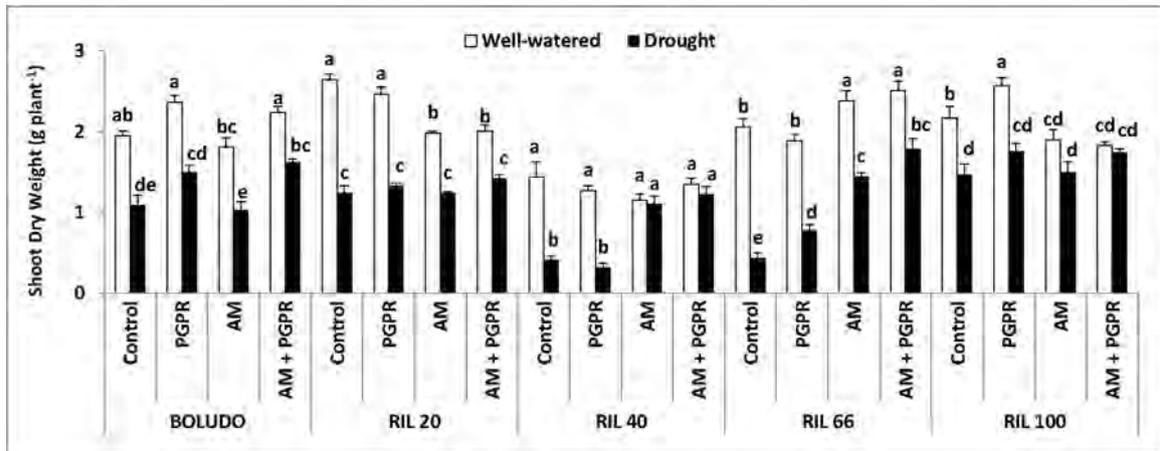


Fig. 1. Shoot dry weight of tomato plants. A commercial tomato cultivar (Boludo) and four tomato recombinant inbred lines (RIL20, RIL40, RIL66 and RIL100) remained as uninoculated controls (Control) or were inoculated either with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (AM), the plant growth promoting rhizobacteria *Variovorax paradoxus* 5C-2 (PGPR) or a combination of both microorganisms (AM + PGPR). Plants were cultivated under well-watered conditions or subjected to drought stress for four weeks. Bars represent means \pm standard error ($n = 5$). Within each recombinant inbred line, different letters indicate significant differences ($P < 0.05$), as determined by Tukey's test.

3.3. Plant CO_2 assimilation rate and leaf chlorophyll content

Drought stress considerably decreased CO_2 -assimilation rate in most of the RILs (Fig. 2A), but this effect was counteracted in some RILs. Thus, inoculating RIL20 with the AM fungus enhanced the CO_2 -assimilation rate under drought stress. Plants from RIL40 exhibited a similar CO_2 -assimilation rate under drought stress conditions, regardless of the microbial treatment. The PGPR inoculation enhanced this parameter only under well-watered conditions. In plants from RIL66, both the AM fungus and the AM fungus + PGPR treatments maintained a high CO_2 -assimilation rate,

which was similar to that under well-watered conditions. Plants from RIL100 exhibited similar CO_2 -assimilation rate under well-watered and under drought stress conditions, regardless of the microbial treatment, but this rate was lower than in the other RILs.

Leaf chlorophyll content increased under drought stress treatment in Boludo and RIL20 plants, with no significant changes as compared to control plants in RIL40, RIL66 and RIL100 (Fig. 2B). In any case, under drought stress, there was little effect of the different inoculation treatments, except for the increase in leaf chlorophyll content in the AM plants from RIL40 and RIL66 (Fig. 2B).

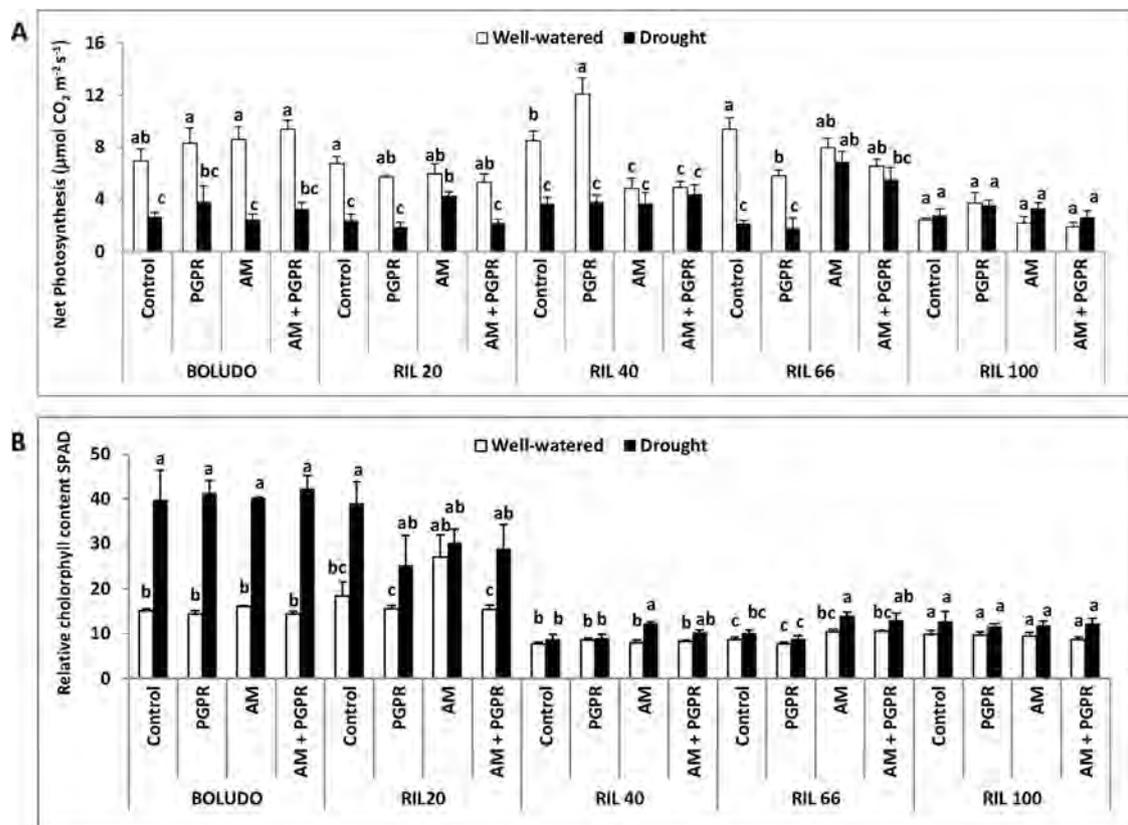


Fig. 2. (A) Net photosynthetic activity and (B) relative chlorophyll content in tomato plants. See legend for Fig. 1.

3.4. Shoot oxidative damage to lipids and proline content

Oxidative damage to lipids was considerably enhanced by drought stress in Boludo plants, in plants from RIL20 inoculated with the PGPR and in those from RIL100 (Fig. 3A). However, plants from RIL40 and RIL66 exhibited the lowest values of oxidative damage either under well-watered or under drought stress conditions. No protective effect by the microbial inoculants was observed on this parameter at any of the RILs assayed.

Drought stress induced a general increase of shoot proline content in the entire RILs studied (Fig. 3B). Under drought stress conditions, Boludo plants accumulated higher amounts of proline but it was significantly reduced by the different inoculation treatments (Fig. 3B). The same trend was observed in RIL66 plants, where PGPR and AM fungus treatments (alone or in combination) halved proline content. In RIL20 and RIL40, only the dual inoculation of PGPR plus AM fungus decreased proline accumulation under drought stress.

3.5. Root hydraulic conductivity

The root hydraulic conductivity (L_{pr}) of plants that were cultivated under well-watered conditions showed little variation between RILs and microbial treatments (Fig. 4). Under drought stress, the inoculation with the PGPR, the AM fungus or combination of both microorganisms decreased L_{pr} in Boludo plants, as compared to the uninoculated control. In RIL20 only the inoculation with the PGPR alone enhanced L_{pr} under drought stress conditions, as compared to the uninoculated control. In contrast, the dual inoculation of the PGPR plus the AM fungus decreased this parameter to values similar to uninoculated control plants. In plants from RIL40, L_{pr} was enhanced only by the

inoculation with the AM fungus alone. In RIL66, L_{pr} was enhanced over uninoculated control when plants were inoculated with the AM fungus in combination with the PGPR, reaching the highest values of L_{pr} under such conditions. In contrast, microbial treatments did not affect L_{pr} of RIL100 plants.

3.6. Accumulation of PIPs in roots of tomato plants

Abundance of PIP1 aquaporins proteins in the Boludo cultivar was little affected by the microbial treatments or the watering conditions (Fig. 5A). Only a slight decrease was observed in AM plants, both under well-watered or under drought stress conditions. In RIL20 and RIL40 the accumulation of PIP1s was significantly enhanced by drought stress, mainly in uninoculated control plants. However, under drought stress, the accumulation of these proteins decreased after inoculation with either the PGPR or the AM fungus. In RIL66 and RIL100, the trend was different since PIP1s accumulated more in plants that were inoculated with the PGPR or the AM fungus and subjected to drought stress.

The abundance of non-phosphorylated PIP2s proteins in Boludo cultivar was also little affected by the microbial or watering treatments (Fig. 5B). Again, only a decrease in the abundance of these proteins was observed in AM plants, both under well-watered or under drought stress conditions. In RIL20 the presence of PIP2s was induced by drought stress only in plants inoculated with the PGPR. However, in AM plants (alone or in combination with the PGPR) these proteins were less abundant. In uninoculated RIL40 and RIL66 plants, the accumulation of PIP2s was enhanced by drought. However, in RIL40 the inoculation of the PGPR further enhanced the abundance of PIP2s, while in RIL66 it reduced the accumulation as compared to droughted uninoculated plants. In both RILs the inoculation with the AM fungus avoided the drought-

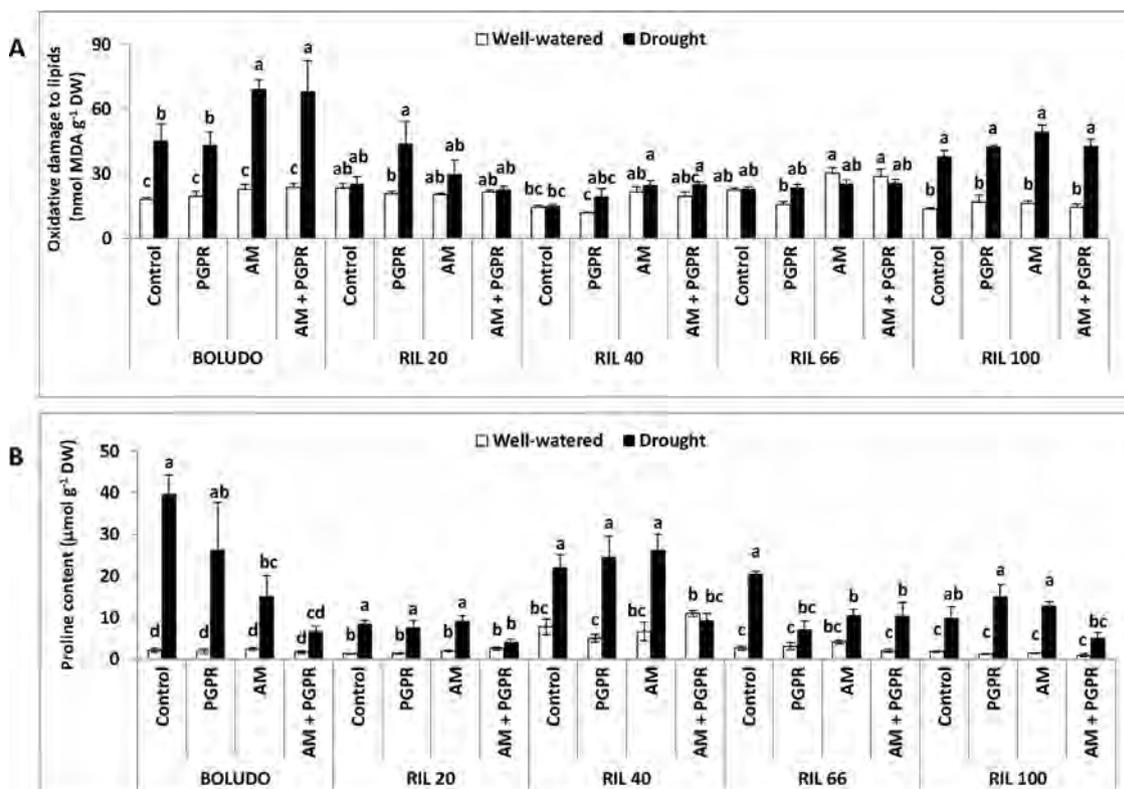


Fig. 3. (A) Oxidative damage to lipids and (B) proline content in shoots of tomato plants. See legend for Fig. 1.

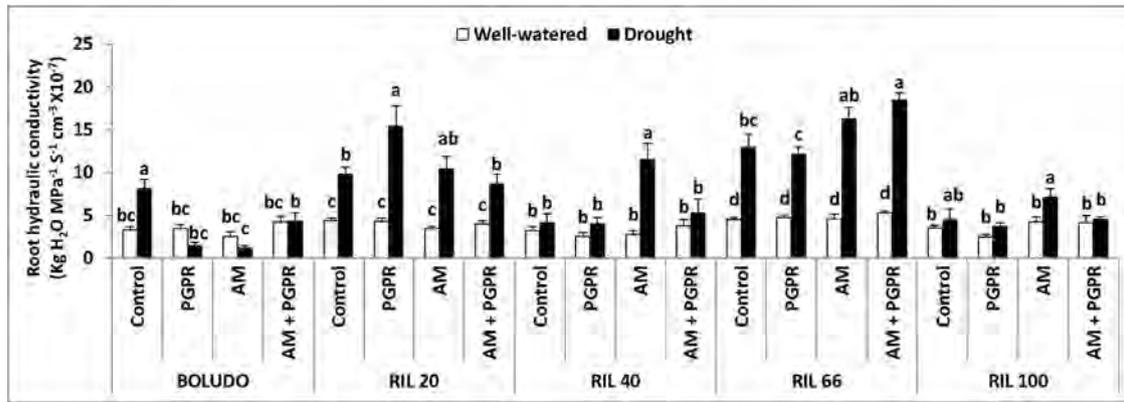


Fig. 4. Root hydraulic conductivity in tomato plants. See legend for Fig. 1.

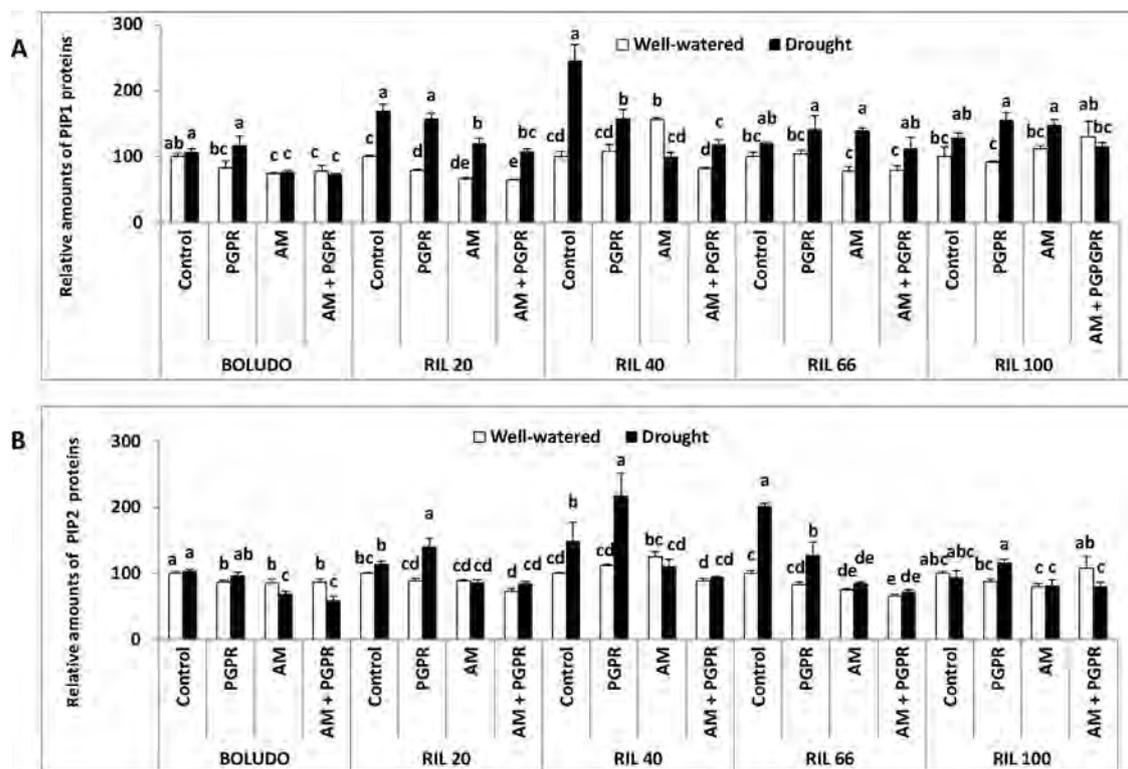


Fig. 5. (A) Relative amounts of un-phosphorylated PIP1 proteins and (B) un-phosphorylated PIP2 proteins in roots of tomato plants (n=3). See legend for Fig. 1.

induced accumulation of these aquaporins. In RIL100 there was almost no effect of the microbial or the watering treatments on the accumulation of non-phosphorylated PIP2s (Fig. 5B).

In the case of phosphorylated PIP2s, the patterns of protein accumulation were similar for both kinds of antibodies used (Figs. 6A and B). Thus, drought stress induced the accumulation of phosphorylated PIP2s in uninoculated control plants or in plants singly inoculated with the PGPR in RILs 20, 40 and 66. Such an effect was not observed in plants from Boludo cultivar and was no consistent in plants from RIL100. A common effect was observed in plants from all lines. Indeed, drought-induced accumulation of phosphorylated PIP2s was avoided when plants were inoculated with the AM fungus, either alone or in combination with the PGPR. Furthermore, these treatments exhibited lower abundance of phosphorylated PIP2s than uninoculated control plants. This reduction of protein abundance in AM roots was observed in

most RILs even under well-watered conditions and was particularly evident in RIL66.

4. Discussion

The reduction in plant biomass production caused by drought stress has been linked to direct effects on the plant photosynthetic capacity due to reduced stomatal conductance. This, in turn, results in low CO₂ supply to Rubisco. Thus, maintaining a high stomatal conductance allows the plant a higher CO₂ uptake for photosynthesis (Davies et al., 1993; Sheng et al., 2008).

Although inoculating tomato plants with the AM fungus *R. irregularis* MUCL 41833 or the PGPR *V. paradoxus* 5C-2 resulted in high root colonization rates in the different RILs considered, the responses of these RILs to the presence of the AM fungus and the PGPR (alone or in combination) varied considerably. One of the

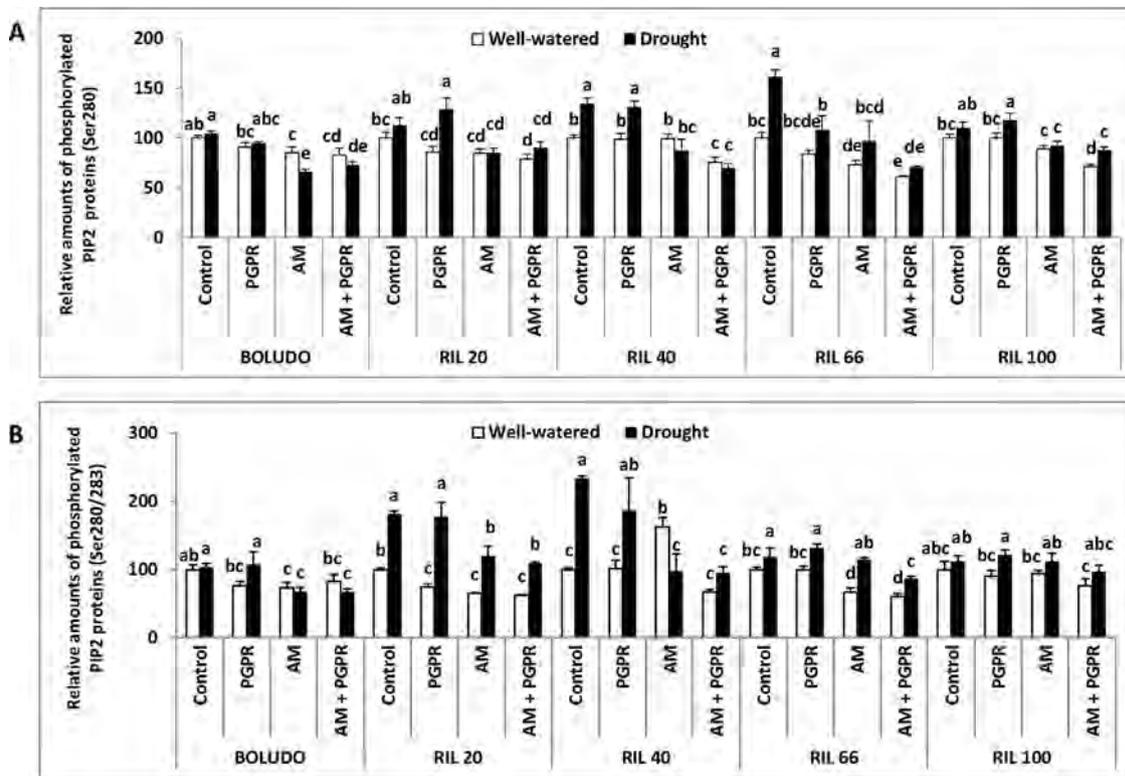


Fig. 6. (A) Relative amounts of phosphorylated PIP2 proteins at Ser280 and (B) phosphorylated PIP2 proteins at Ser280/Ser283 in roots of tomato plants ($n = 3$). See legend for Fig. 1.

main benefits of AM or AM+PGPR inoculation for RIL66 plants under drought stress conditions was the maintenance of high photosynthetic rates as compared to non-inoculated plants. Increased photosynthetic activity or water use efficiency have been reported in AM plants growing under drought stress (Birhane et al., 2012; Liu et al., 2015), which was attributed to mycorrhizal enhancement of plant water status, rather than to a direct influence on the efficiency of photosystem II (Sheng et al., 2008). However, in salinized tomato and rice plants, mycorrhization improved photosynthetic activity by both elevating stomatal conductance and protecting PSII photochemical processes (Hajiboland et al., 2010; Porcel et al., 2015). Stomatal changes in AM plants have been linked to altered plant hormone status (Augé, 2000) or to a higher capacity for CO₂ fixation. Indeed, mycorrhizal grapevines showed higher Rubisco activity than non-AM ones during drought episodes (Valentine et al., 2006), as did salinized rice plants (Porcel et al., 2015).

Drought decreases both soil water potential and the soil-root gradient of water potential favouring root water uptake, thereby reducing the water flow toward roots. To counter this effect, many plants increase their osmotic potential by synthesizing and accumulating compatible osmolytes such as proline, to maintain root water uptake (Porcel and Ruiz-Lozano, 2004; Flowers and Colmer, 2008). Under drought stress, proline accumulation was higher in most of the RILs, but this accumulation was reduced by the presence of microorganisms, especially in RIL66 plants. This suggests that AM- and PGPR-inoculated plants were less strained by the drought stress applied, due to other drought-avoidance mechanisms such as water uptake by fungal hyphae or hormonal-mediated regulation of stomatal conductance, and that they had a lower need for osmotic adjustment. While non-AM lettuce plants accumulated more proline in their shoots than AM plants under drought, AM plants accumulated more proline in the roots than non-AM plants (Ruiz-Lozano et al., 2011). Thus, in root tissues, AM

plants accumulate more proline in order to cope with the low water potential of drying soil and to keep a water potential gradient favourable to water entrance into the roots, as was also found in soybean (Porcel and Ruíz-Lozano, 2004). Proline homeostasis may be important to sustain growth under long-term stress, since proline accumulated during a stress episode can be degraded to provide a supply of energy to drive growth once the stress is relieved (Kishor and Sreenivasulu, 2014).

The differences in proline accumulation among RILs and microbial treatments may also be related to hormonal changes in these plants or due to the microbial treatments. ABA, auxins and salicylic acid are known to up-regulate proline synthesis, while cytokinin down-regulates proline accumulation (Kishor and Sreenivasulu, 2014).

Drought stress generates a secondary oxidative stress in plant tissues due to the accumulation of ROS. Cytotoxic ROS can destroy normal metabolism through oxidative damage of lipids, proteins and nucleic acids (Miller et al., 2010; Noctor et al., 2014). In the present study drought significantly increased the level of oxidative damage to lipids, which was accentuated when cv. Boludo received the AM and AM+PGPR treatments. Generally, the RILs showed lower levels of drought-induced oxidative damage, except in RIL100. Generally, the microbial treatments applied did not alter the rates of oxidative damage in the RILs. The differences observed are only due to the own RIL used. Thus, RIL66 must have additional mechanisms to respond to the drought-induced oxidative stress as compared to Boludo, which seems quite sensible. It is known that ROS accumulation depends on the balance between its production and its elimination (Miller et al., 2010; Scheibe and Beck, 2011). Thus, the ROS scavenging systems may be more effective in plants from RIL40 or RIL66 than in those from RIL100 or the Boludo cultivar.

A fine control of water transport is of key importance for plant survival under drought stress conditions, as it decreases L_p_r (Aroca

et al., 2012), a process in which PIPs play a fundamental role (Maurel et al., 2015). In addition, AM fungi can affect the L_p of host plants through regulation of plant aquaporins (Bárcana et al., 2014; Calvo-Polanco et al., 2014a; Sánchez-Romera et al., 2016), being this effect considered as an important factor in the regulation of water relations in mycorrhizal plants (Lee et al., 2010; Ruiz-Lozano et al., 2012). PIP2s are usually considered as the main aquaporins responsible for the major water transport capacity in plants (Chaumont et al., 2000). However, PIP1 aquaporins have also been shown to play a role in water transport in plants (Zou et al., 2010) in combination with PIP2 proteins via heteromerization (Zelazny et al., 2007; Li et al., 2013a,b), and in the trafficking of PIP proteins to the plasma membrane (Zelazny et al., 2007; Hachez et al., 2013).

Generally, the microbial treatments did not alter L_p under well-watered conditions, but more pronounced effects were seen in droughted plants. Taking RIL20 and RIL66 as lines with contrasting responses to the microbial inoculants it is evident that L_p was unaltered by microbial treatments under well-watered conditions. In contrast, under drought stress conditions, plants from RIL20 and RIL66 also exhibited contrasting effects of the microbial inoculants on L_p . Indeed, plants from RIL20 enhanced the L_p only when inoculated with the PGPR alone, but the dual inoculation with the AM fungus avoided this increase (Fig. 4). In contrast, in RIL66, the highest L_p values were achieved under drought stress conditions in plants dually inoculated with the PGPR and the AM fungus. Inhibiting root ethylene production reversed the limiting effect of P-deprivation on L_p (Li et al., 2009) and it is plausible that the impact of the ACC-deaminase containing PGPR in decreasing root ethylene production (Belimov et al., 2015) enhanced L_p . Why this effect should occur only in RIL20 is not clear, although PGPR root colonization under drought was lowest in this line, and physiological impacts of ACC-deaminase containing PGPR can be dose-dependent (Belimov et al., 2007).

The correlation between the measured L_p values and PIP accumulation patterns was not evident for most of the RILs analyzed, as previously observed (Boursiac et al., 2005; Aroca et al., 2007; Ruiz-Lozano et al., 2009). This is not surprising since symplastic movement of water via plasmodesmata may also contribute significantly to hydraulic conductivity (Galmés et al., 2007), and aquaporin regulation occurs at both transcriptional and post-transcriptional levels (Zelazny et al., 2007).

In any case, in RIL20 L_p showed a significant statistical correlation with the accumulation of PIP1s, non-phosphorylated PIP2s and both phosphorylated PIP2s (Table 1). Thus, in RIL20 the variation in L_p by the PGPR and the AM fungus under drought stress seems to be directly related to the regulation of PIPs aquaporins (Chaumont and Tyerman, 2014). Conversely, in RIL66 L_p followed a significant negative correlation with accumulation of non-phosphorylated PIP2s and both phosphorylated PIP2s (Table 1). This suggests that in RIL66 the enhanced root hydraulic conductivity of plants inoculated with the PGPR and the AM fungus was rather related to altered apoplastic water flow in these plants. It must be taken into account that the values of L_p measured in this study includes both apoplastic and symplastic water flow. In

Table 1
Pearson correlations between root hydraulic conductivity (L_p) and PIP root protein abundance and phosphorylation state in plants from RIL20 and RIL66.

RIL20	PIP1	PIP2	PIP2 ₂₈₀	PIP2 ₂₈₀₋₂₈₃
L_p	0.687	0.590	0.637	0.629
P	0.0001*	0.0004*	0.0001*	0.0001*
RIL66	PIP1	PIP2	PIP2 ₂₈₀	PIP2 ₂₈₀₋₂₈₃
L_p	-0.012	-0.693	-0.688	-0.562
P	0.9678	0.0059*	0.0064*	0.0364*

* means statistically significant correlation.

this regards, the presence of mycorrhizal fungi within the roots may have greatly contributed to the increase of the apoplastic water flow within the roots as previously reported (Lehto and Zwiazek, 2011; Bárcana et al., 2012). Increased water uptake by mycorrhizal plants under drought has been related to the increased absorbing surface of growing hyphae, and mycorrhizal ability to take up water from soil pores inaccessible to roots, as AM hyphae represent a low-resistance way for water movement until root cells (Ruiz-Lozano, 2003; Allen, 2009; Lehto and Zwiazek, 2011). Thus, water movement through AM fungal hyphae under such conditions can be critical to improve the water supply to the plant and, therefore, cell-to-cell and apoplastic pathways increase (Bárcana et al., 2012). On the other hand, AM fungal aquaporins have been related to water transport in the extraradical mycelium and in the periarbuscular membrane (Li et al., 2013a,b). Thus, in AM plants, the enhanced root hydraulic conductivity could be also due to the activity of the own fungal aquaporins (Bárcana et al., 2014, 2015).

5. Conclusions

Results obtained clearly demonstrate a variation in the performance of the different tomato RILs under conditions of drought stress, as well as, a differential effect of the microbial inoculants (AM fungus and/or PGPR) on plant performance, depending on the RIL involved. Thus, RIL66 is the one obtaining the maximum benefit from inoculation with the AM fungus and the PGPR. In contrast, RIL20 or RIL100 received little benefit from the microorganisms applied under conditions of drought stress. This genetic diversity in microbial response may be exploited commercially, particularly if a selected RIL is used as stress-tolerant rootstock.

We propose that RIL66 could be selected as a good plant material to be used as rootstock in a grafting program to improve tomato growth and productivity under water limiting conditions. Since RIL66 is highly responsive to microbial inoculants, this grafting strategy should be combined with inoculation of the AM fungus *R. irregularis* MUCL41833 and the PGPR *V. paradoxus* 5C-2 in order to improve plant productivity while reducing water and fertilizer inputs under conditions of drought stress.

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