Effects of different arbuscular mycorrhizal fungal backgrounds and soils on olive plants growth and water relation properties under well-watered and drought conditions

Monica Calvo-Polanco1,5, Iván Sánchez-Castro2, Manuel Cantos3, José Luís García3, Rosario Azcón1, Juan Manuel Ruiz-Lozano1, Carmen R. Beuzón1 & Ricardo Aroca1

1Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín (CSIC), C/Profesor Albareda, Granada 18008, Spain, 2Department of Microbiology, University of Granada, Av. Fuentenueva s/n, Granada 18071, Spain, 3Department of Plant Biotechnology, Instituto de Recursos Naturales y Agrobiología (CSIC), Av. Reina Mercedes, 10, Sevilla 41012, Spain, 4Department of Cellular Biology, Genetics and Physiology, Campus de Teatinos, University of Málaga, Málaga 29010, Spain and 5Biochimie et Physiologie Moléculaire des Plantes, SupAgro/INRA UMR 5004. 2, Place Viala, Montpellier Cedex 2 34060, France

ABSTRACT

The adaptation capacity of olive trees to different environments is well recognized. However, the presence of microorganisms in the soil is also a key factor in the response of these trees to drought. The objective of the present study was to elucidate the effects of different arbuscular mycorrhizal (AM) fungi coming from diverse soils on olive plant growth and water relations. Olive plants were inoculated with native AM fungal populations from two contrasting environments, that is, semi-arid – Freila (FL) and humid – Grazalema (GZ) regions, and subjected to drought stress. Results showed that plants grew better on GZ soil inoculated with GZ fungi, indicating a preference of AM fungi for their corresponding soil. Furthermore, under these conditions, the highest AM fungal diversity was found. However, the highest root hydraulic conductivity (Lp) value was achieved by plants inoculated with GZ fungi and growing in FL soil under drought conditions. So, this AM inoculum also functioned in soils from different origins. Nine novel aquaporin genes were also cloned from olive roots. Diverse correlation and association values were found among different aquaporin expressions and abundances and Lp, indicating how the interaction of different aquaporins may render diverse Lp values.

Key-words: Olea europaea; aquaporins; arbuscular mycorrhizal fungi; root hydraulic conductivity.

Abbreviations: AM, arbuscular mycorrhiza; FL, Freila (arid location); FW, fresh weight; gs, stomatal conductance; GZ, Grazalema (humid location); H', Shannon biodiversity index; HPFM, high-pressure flow meter; IB, indolbutyric acid; Kr, root hydraulic conductance; Lpr, root hydraulic conductivity; OTU, operational taxonomic unit; PIP, plasma membrane intrinsic protein; RWC, relative water content; S, specific richness; TIP, tonoplast intrinsic protein; ΦPSII, photochemical efficiency of photosystem II.

INTRODUCTION

Olive trees (Olea europaea L.) are among the most important crops for the Mediterranean area. They grow in very diverse environments (Parodi 1978) and are known for their resistance to water stress, especially during summer, when they face scarce precipitation and high temperatures (Connor 2005). Nevertheless, their response to water stress has been shown to be quite unpredictable, as their intraspecific genetic diversity is high (Guerfel et al. 2009). Even if olive tree is considered as a drought-resistant species, the number of irrigated cultivars is increasing (Carr 2013), and the balance between irrigation and productivity is a major issue as water is scarce in most countries growing this crop. With the current prediction of a decline in water availability and an estimation of crop production decline for the next years, the selection of olive trees with enhanced drought tolerance is needed to maintain and even improve their productivity. The effects of drought on plants vary among species considered, although one of the first responses of plants to the lack of water within the soil profile is to adjust their internal water balance by closing stomata (Gómez-del-Campo 2007; Schachtman and Goodger 2008) and by regulating their root hydraulic conductivity (Maurel et al. 2008; Aroca et al. 2012). Even though the regulation of root hydraulic conductivity has been mainly attributed to the action of aquaporins (Luu and Maurel 2005, Maurel et al. 2015), root and leaf osmotic adjustment as well as root architecture may be also playing a very important role in water uptake under prolonged period of drought stress. Three aquaporins have been described in olive trees, that is, OePIP1;1, OePIP2;1 and OeTIP1;1 (Secchi et al. 2007, Lovisolo et al. 2007). As in another tree species, these aquaporins have been reported to be differently regulated according to the stress and the intensity of the stresses (Secchi et al. 2007). Osmotic regulation in severely water stressed olive trees has been also described in experiments with
these trees and may be a complementary strategy to aquaporins in order to resist long-term exposure to soil water deficit (Chartzoulakis et al. 2000; Ennajeh et al. 2008). At the same time, Tataranni et al. (2015) found a close correlation between morpho-anatomical traits and $L_{pr}$ values in olive trees subjected to drought.

The use of AM fungi as relievers of the drought stress effects on plants has been studied for many years. AM fungi are believed to have a great capacity to resist fast environmental changes under drought conditions and long-term stresses, allowing the plants to have a wider range of possibilities to adapt and survive (Al-Karaki 2006; Smith and Read 2008; Ruiz-Lozano et al. 2012). Olive trees appear to be highly dependent on AM fungi under arid conditions (Mekahlia et al. 2013), although the effect of the AM symbiosis on plants varies according to the AM fungal strain used and the plant cultivars (Binet et al. 2007), as well as to the soil chemical composition (Burns et al. 2015). AM fungi establishes an extensive hyphal network in the soil, mobilizing soil nutrients, mainly phosphorus and nitrogen (Bonfante and Genre 2010, Bompadre et al. 2013) that would play a key role in plant survival under stress conditions. In general, AM fungi have been shown to increase plant resistance to drought (Azcon et al. 1996; Porcel et al. 2004) and to alleviate water stress (Sheng et al. 2008; Barzana et al. 2012), while improving plant productivity (Navarro-Fernández et al. 2011; Abbaspour et al. 2012) and nutrient status (Farzaneh et al. 2011; Lee et al. 2012). Furthermore, AM symbiosis causes significant changes in aquaporin abundance and activity in host plants (Aroca et al. 2007, Jahromi et al. 2008, Barzana et al. 2014). For example, a consistent diminution of PIP2 aquaporin phosphorylation has been observed in bean roots colonized by the AM fungus *Rhizophagus irregularis* under optimal conditions (Aroca et al. 2007; Benabdellah et al. 2009; Sanchez-Romera et al. 2016), suggesting less activity of such aquaporins (Maurer et al. 1995). These results support the idea that each aquaporin has a specific function under different environmental conditions (Aroca et al. 2007; Jang et al. 2007; Calvo-Polanco et al. 2014a) and that each plant will respond differently to each AM colonizing fungus. Therefore, the regulation of root hydraulic conductivity ($L_{pr}$) and aquaporin expression and abundance by AM symbiosis is far from being understood. In studies using the same plant and AM fungal species, the response of aquaporin expression to AM inoculation was different (Aroca et al. 2007; Sanchez-Romera et al. 2016), probably because the soil used was not the same. Furthermore, El-Meshahi et al. (2012) found that the response of $L_{pr}$ and aquaporin expression to AM symbiosis depended on the soil $K^+$ content. Furthermore, different AM fungal species had different effects on aquaporin expression in soybean and lettuce plants (Porcel et al. 2006).

In the present study, we analysed the response to drought stress of 7-month-old olive plants (*O. europaea* cv. Picual) grown in natural soils from two locations with contrasting climatology: Freila (FL), a Mediterranean location in the south of Spain with low-average annual precipitation (380 mm), and Grazalema (GZ), whose average annual precipitation is high (2223 mm). Plants were inoculated with the native AM fungal population of each soil, including crossing of soils with non-autochthonous AM fungi. The plants were subjected to drought stress for 4 weeks. The objectives of the study were (1) to study how different combinations of soils, AM fungal communities and water regime modify root hydraulic properties of olive trees including aquaporin expression and abundance, (2) to identify novel aquaporin genes in olive plants taking advantage of the OLEAGEN database (Muñoz-Mérida et al. 2013) and (3) to find out which physiological or molecular traits are potential more determinants of $L_{pr}$ regulation.

### MATERIAL AND METHODS

#### Olive plants production and growth

We used olive (*Olea europaea* L. cv. Picual) plants to test the effect of two contrasting natural soils from the Mediterranean area in Spain combined with the natural AM population from those two soils on plant tolerance to severe drought stress. This particular olive cultivar is known for its moderate drought tolerance, because it has been selected for rain fed conditions (Tugendhaft et al. 2016). Olive explants were produced from mature olive trees growing at FL (37°31′43″N, 2°54′34″W), a Mediterranean location in the south of Spain. Olive branches were removed and transported in a humid piece of cloth to a greenhouse. Cuttings were produced as explained by Suárez et al. (1999). Briefly, 18 cm length and 4–6 cm diameter cuttings were treated with indolbutyric acid (IBA) 3500 ppm by immersing the cutting base for 10 s in a 1:1 (v:v) IBA hydro-alcoholic solution. The explants were immediately transferred to a mist propagation system for 90 d on a perlite substrate at 25 °C basal heating for rooting.

Once the explants were rooted, they were transferred into 2 L pots using two different natural soils; one originating from FL area and another one from the GZ area (36°46′4″N, 5°21′57″ W). These areas were chosen for their high olive oil production and their contrasting climatology. FL is a typical semi-arid Mediterranean location, with dry and hot summers, mild winters and low-annual total precipitation (380 mm), while the GZ area has more a continental climate with cold winters and hot summers and much higher total annual precipitation (2223 mm). Soil was collected from four different points (20 kg at each point), spaced 10 m each other in each olive field. The soil from the four points was mixed later. Only Horizon-A was collected. The soils were sieved (50 mm) and sterilized by steaming for 1 h, for three consecutive days at 100 °C. Plants were then transferred to a greenhouse at 22/18 °C, 65% relative humidity, and were grown for 7 months. Plants were irrigated with 50% modified Hoagland’s solution once per week (Epstein (1972)): 2.5 mM KNO$_3$, 0.5 mM KH$_2$PO$_4$, 2.5 mM Ca(NO$_3$)$_2$, 1 mM MgSO$_4$, 23 μM H$_2$BO$_3$, 5 μM MnCl$_2$, 0.3 μM ZnSO$_4$, 0.2 μM CuSO$_4$, 0.01 μM (NH$_4$)$_2$Mo$_7$O$_24$ and 90 μM EDTA-Fe.

Inoculation was carried out at the time of planting. The plants growing in each soil were divided into three different groups and the inoculation treatments were applied as follows:
(1) control, non-AM inocula; (2) natural AM fungal population from FL soil; and (3) natural AM fungal population from GZ soil. The AM fungal inocula from the natural soils were obtained by using the wet sieving and decanting technique (Navarro-Fernández et al. 2011). Briefly, 310 g of the corresponding 2 mm sieved soil (which is equivalent to 500 mL of each soil with the original texture) was suspended in 2 L of water. Then, the suspension was strongly stirred for sample homogenization and left for soil decantation. The supernatant was poured out through coupled sieves of 700, 500, 250 and 50 μm. This procedure was carried out twice with the same soil sample. Finally, the material from all sieves except from that of 700 μm was added to each pot in the planting hole, according to the corresponding treatment. Thus, the experiment consisted of 12 treatments arising from the combination of two different soils (FL and GZ), three AM inoculum (non-inoculated, native AM fungi from FL soil and native AM fungi from GZ soils) and two water regime treatments (well-watered and drought stress conditions). Each treatment was composed of six plants.

**Soil analyses and watering regime**

Elemental analyses of the natural soils from FL and GZ and from olive leaves were carried out by an inductively coupled plasma analyser at the Instrumental Service of the Estación Experimental del Zaidín. The results of soil analyses are presented in Table 1.

Six months after planting, plants from each fungal treatment were separated into two groups: half of them were well watered (95% field capacity) and the other half submitted to drought stress (55% field capacity). Soil moisture was controlled using a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK), and after that, the water content of the soil was maintained by weighing the pots every day and replacing the water lost to recover the desired level of soil water content (Porcel et al. 2004). The drought treatment lasted 4 weeks.

**Arbuscular mycorrhizal colonization rates and fungal mycelial length**

Mycorrhizal root colonization was estimated by analysing four roots (n = 4) per 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).

Fungal mycelial length was determined as explained in Abbott et al. (1984). For the calculation of the total length of the mycelia, the following equation was used as $R = \pi AN / (2H) \times F_d \times F_{ps}$, where $R$ is the hyphal length, $A$ is the area of the filter (m$^2$), $N$ is the number of intersections, $H$ the total length of the reticulum used, $F_d$ is the dilution factor and $F_{ps}$ is the dry-weight factor.

<table>
<thead>
<tr>
<th>Location</th>
<th>EC (1:5) (ms cm$^{-1}$)</th>
<th>EC (1:2.5) (ms cm$^{-1}$)</th>
<th>pH (extract KCl 2 N)</th>
<th>CaCO$_3$ (%)</th>
<th>AC (%)</th>
<th>OM (%)</th>
<th>N (%)</th>
<th>C/N</th>
<th>N-NO$_3$ (mg L$^{-1}$)</th>
<th>N-NH$_4$ (mg L$^{-1}$)</th>
<th>NH$_4$ (extract KCl 2 N; mg kg$^{-1}$)</th>
<th>Cl$^{-}$ (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grazalema</td>
<td>4.65 221.90</td>
<td>4.65</td>
<td>8.33</td>
<td>4.14</td>
<td>81.5</td>
<td>34.40</td>
<td>317</td>
<td>8.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.08</td>
<td>2.20</td>
</tr>
<tr>
<td>Freila</td>
<td>4.50 4785</td>
<td>4.50</td>
<td>8.55</td>
<td>5.90</td>
<td>75.8</td>
<td>34.40</td>
<td>340</td>
<td>0.57</td>
<td>0.04</td>
<td>0.07</td>
<td>0.04</td>
<td>3.50</td>
</tr>
</tbody>
</table>

EC: electrical conductivity; AC: active carbonates; OM: organic material.
Molecular identification of arbuscular mycorrhizal fungi colonizing the roots

For AM fungal identification, genomic DNA was extracted from 120 to 140 mg of fine roots from each treatment combination by using the DNeasy Plant Mini Extraction Kit (Qiagen Inc., Mississauga, ON, Canada) following manufacturer’s instructions. A nested polymerase chain reaction (PCR) approach was used to amplify a partial LSU rRNA gene region (approx. 370 bp) of the AM fungal DNA from the root samples. The primer combinations LR1/FLR2 and FLR3/FLR4 were employed as explained in Gollotte et al (2004). All amplifications were performed on a Mastercycler Nexus PCR cycler (Eppendorf, Hamburg, Germany) by using Canvax Biotech Taq polymerase (Canvax Biotech, Cordoba, Spain). PCR products were separated by gel electrophoresis on a 1% agarose gel in TAE buffer, and DNA was visualized under UV light after being stained with ethidium bromide.

Four to five replicates of both PCR amplifications were performed for each sample and the resulting amplicons pooled to yield a composite sample (Renker et al. 2006). The mixed products from the second PCR were cloned into the p-GEM® T-Easy Vector (Promega, Madison, USA) and used to yield a composite sample (Renker et al 2006). The mixed fungi colonizing the roots

Physiological parameters

Root and leaf fresh weight (FW) were determined at the time of harvest in six ($n=6$) plants per treatment combination. Relative height increments and relative number of leaves were determined from the measurements taken at the beginning and at the end of drought treatment.

Stomatal conductance ($gs$) was measured before harvesting, in the last fully developed mature leaves with a portable AP4 Porometer (Delta-T Devices Ltd, Cambridge) 4 h after sunrise. The photochemical efficiency of photosystem II ($Φ_{PSII}$) and leaf relative water content (RWC) were determined in six mature leaves per treatment combination ($n=6$), using the same leaves as for $gs$. For $Φ_{PSII}$, we used a FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows the measurement of chlorophyll a fluorescence and hence plant photosynthetic performance. To determined RWC, mature, fully developed leaves were excised from the main shoot, weighed ($W_0$) and introduced into 15 mL centrifuge tubes (BD Falcon, Fisher Scientific) with a piece of wet cotton for 24 h at 4°C. Leaves were weighed again ($W_b$), dried at 75°C for 2 d and then weighed ($W_d$) a third time, and the relative water content was calculated as $\text{RWC} = (W_0 - W_d)/(W_h - W_d) \times 100$. Leaf chlorophyll contents were extracted in 100% methanol and concentration calculated using the coefficients and equations reported in Lichtenthaler (1987).

Root hydraulic conductance ($K_r$) was determined for six complete roots ($n=6$), per each of the 12 treatments. We used a high-pressure flow meter (Dynamax, Inc., Houston), and the measurements were taken in the same plants used for $gs$, between 3 and 4 h after sunrise. Detached roots were connected to the high-pressure flow meter and water was pressurized into the roots from 0 to 0.5 MPa in the transient mode to calculate root hydraulic conductance ($K_r$; Calvo-Polanco et al. 2012). Root hydraulic conductivity ($L_p$) was determined by dividing $K_r$ by the root volume (Calvo-Polanco et al. 2012).

Olive aquaporins identification

For olive aquaporins identification, total RNA was isolated from roots by a phenol/chloroform extraction method followed by LiCl precipitation (Kay et al. 1987). cDNA was synthesized from 2 μg of total RNA using oligo(dT)$_{12-18}$ as a primer and M-MLV as reverse transcriptase (Invitrogen). For the identification of aquaporins, cDNA was amplified by PCR using the degenerate primers from Park et al. (2010). PCR reactions were performed as described for AM fungal identification, except that the annealing temperature was changed to 60°C. After gel electrophoresis of the PCR products, visible bands of the expected size (approximately 400 kb) were recovered from the gel, eluted with a QIAquick Gel Extraction Kit (Qiagen) and cloned as explained previously. To determine the sequences of the full-length cDNAs, we used the SRS database from the OLEAGEN consortium (Muñoz-Mérida et al. 2013).
Amino acid sequences predicted for the olive aquaporin genes were used for phylogenetic analysis along with those predicted for other plant aquaporin genes using MEGA4 software (Tamura et al. 2007). The reliability of the branches in each resulting tree was supported with 1000 bootstrap resampling. Sequences generated in this study have been deposited at the European Molecular Biology Laboratory database under the accession numbers KT380900 to KT380908.

Root aquaporin expression analysis
Aquaporins expression was determined in the roots of three plants of each treatment combination (n = 3). Total RNA was isolated as described earlier. DNase treatment of total RNA and reverse transcription were performed following the instructions provided by the manufacturer (Quantitect Reverse Transcription Kit Cat#205311, Qiagen, CA). We used, for the root aquaporin expression analyses, the three known O. europaea aquaporins (OePIP1;1 GeneBank accession no. DQ202708, OePIP2;1 GeneBank accession no. DQ202709 and OeTIP1;1 GeneBank accession no. DQ202710), plus the new ones described in this study (OePIP1;2 GeneBank accession no. KT380904; OePIP1;3 GeneBank accession no. KT380905; OePIP2;2 GeneBank accession no. KT380990; OePIP2;3 GeneBank accession no. KT380993; OePIP2;4 GeneBank accession no. KT380908; OePIP2;5 GeneBank accession no. KT380906; OeTIP1;2 GeneBank accession no. KT380902 and OeTIP1;3 GeneBank accession no. KT380907). The expression of the different aquaporins was determined using a real time quantitative PCR (iCycler-Bio-Rad, Hercules, CA) as explained in Calvo-Polanco et al. (2014a, 2014b). We could not detect any expression from OePIP1;1 and OePIP2;1 in the roots of our olive plants. For the aquaporins analysed, the annealing temperature was 58°C, and the primers used are described in the Supporting Information. The specificity of the PCR amplification procedure was confirmed using a heat dissociation protocol (from 60 to 100°C) after the final cycle of the PCR. The relative abundance of transcription was calculated using the 2^ΔΔCt method (Livak and Schmitthen 2001). To normalize aquaporin expression, we tested different olive housekeeping genes: actin, ubiquitin and elongation factor. The elongation factor was the one chosen after RT-qPCR as it displayed stable mRNA levels throughout all treatments. Elongation factor-specific primers were used for standardization by measuring the expression of elongation factor gene in each sample. Negative controls without cDNA were used in all the PCR reactions.

Proteins isolation and enzyme-linked immunosorbent assay analysis
Microsomes were isolated as described in Hachez et al (2006). For enzyme-linked immunosorbent assay analysis, two micrograms of the protein extracts were processed as described in Calvo-Polanco et al. (2014a, 2014b). We used, as primary antibodies (at a dilution of 1:1000), the two antibodies that recognize several PIP1 and PIP2 and three antibodies that recognize the phosphorylation of PIP2 proteins at their C-terminal region at Serine 280 (PIP2_280), Serine 283 (PIP2_283) and both at Serine 280 and 283 (PIP2_280_283) as described in Calvo-Polanco et al. (2014a, 2014b). A goat anti-rat IgG coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as secondary antibody at 1:10000 for PIP1. Goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as secondary antibody at 1:10000 for PIP2 and PIP2_280, PIP2_283 and PIP2_280_283. Protein quantification was carried out in three different independent root samples per treatment (n = 3), replicated three times each. PIP2 antibodies antigens were aligned to see which O. europaea aquaporins could be recognized by each antibody (Supporting Information). The specificity of the PIP2 and phosphorylated antibodies PIP2_280, PIP2_283 and PIP2_280_283 is described in Calvo-Polanco et al. (2014b).

Statistics
Data were analysed using 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 all treatment means. When the ANOVA P-values for the three-way interaction were not significant, we proceed to run t-test for the significant interactions. The aquaporin expression and abundance data, mycorrhizal colonization rates and length, stomatal conductance, together with the Lp, data were firstly sorted out using a principal components analyses and posterior Pearson’s correlations to determine which of the aquaporins may have contributed to Lp, and if gs was correlated with Lp. Pearson correlations were also calculated among Lp, and stomatal conductance and leaf N, P and K contents.

RESULTS
Soil conditions and plant growth
Grazalema and FL soil analyses showed high pH values, between 8 and 9, and low-electrical conductivity (Table 1). GZ soil was richer in nutrients (higher N, P, K and Ca) and also in organic matter, which should favour plant growth. Significant lower values of N, P, K and S contents were observed in leaves of plants growing in FL soil, mostly in non-inoculated plants (Table 2). Inoculation with AM fungi from FL soil increased P and K contents of leaves of plants growing in FL soil (Table 2). The difference in K content between leaves from plants growing in both kinds of soils was only significant under well-watered conditions (Table 2).

When plant growth was analysed using either leaf or root FW as a parameter, the impact of the soil origin become clear as GZ soils supported larger growth (P < 0.0001 and P = 0.044, respectively) (Table 3). However, drought stress induced a significant reduction of the leaf FW (P = 0.0008), in both non-inoculated plants and plants inoculated with...
Table 2. Nutrient content of leaves of *Olea europaea* plants growing into two different types of soils (GZ and FL), inoculated with different mycorrhizal communities from GZ and FL soils and cultivated under well-watered conditions or subjected to drought stress for 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>Grazalema soil</th>
<th>Freila soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Myc</td>
<td>Myco GZ</td>
</tr>
<tr>
<td>N (mg g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>1.09 ± 0.12</td>
<td>1.21 ± 0.03*</td>
</tr>
<tr>
<td>Drought</td>
<td>0.92 ± 0.07</td>
<td>1.02 ± 0.02</td>
</tr>
<tr>
<td>P (mg g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>1.09 ± 0.17*</td>
<td>2.12 ± 0.11*</td>
</tr>
<tr>
<td>Drought</td>
<td>0.65 ± 0.12</td>
<td>1.23 ± 0.04</td>
</tr>
<tr>
<td>K (mg g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>7.37 ± 0.68abc</td>
<td>7.99 ± 0.36a</td>
</tr>
<tr>
<td>Drought</td>
<td>6.03 ± 0.32cde</td>
<td>6.28 ± 0.34bcd</td>
</tr>
<tr>
<td>Mg (mg g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>1.22 ± 0.18</td>
<td>1.33 ± 0.19</td>
</tr>
<tr>
<td>Drought</td>
<td>1.07 ± 0.12</td>
<td>1.01 ± 0.05</td>
</tr>
<tr>
<td>S (mg g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>0.96 ± 0.07</td>
<td>1.08 ± 0.05</td>
</tr>
<tr>
<td>Drought</td>
<td>0.81 ± 0.04</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td>Ca (mg g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>10.11 ± 0.95</td>
<td>11.32 ± 1.48</td>
</tr>
<tr>
<td>Drought</td>
<td>9.15 ± 1.1</td>
<td>9.07 ± 0.42</td>
</tr>
</tbody>
</table>

ANOVARs plus post hoc Tukey’s test were run when significant three-way interaction P-values allowed it (only in the case of K contents), and then different letters mean significant differences (P < 0.05) among treatments in the same row. When the three-way interaction was non-significant, t-test was used to indicate significant differences (P < 0.05) between treatment means at the same column indicated by an asterisk.

ANOVA P-values for the different parameters measured: soil – soil type; Myc – non-inoculated, inoculated with AM from GZ or inoculated with AM from FL; and well-watered and drought stress.

ANOVAs, analyses of variance; GZ, Grazalema; FL, Freila.

Table 3. Leaf and root FW, leaf RWC, photochemical efficiency of ΦPSII and chlorophyll content in *Olea europaea* plants growing into two different types of soils (GZ and FL), inoculated with different mycorrhizal communities from GZ and FL soils and cultivated under well-watered conditions or subjected to drought stress for 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>Grazalema soil</th>
<th>Freila soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Myc</td>
<td>Myco GZ</td>
</tr>
<tr>
<td>Leaf FW (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>5.6 ± 1.0*</td>
<td>7.1 ± 0.8</td>
</tr>
<tr>
<td>Drought</td>
<td>3.4 ± 0.4</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>Root FW (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>4.7 ± 0.9</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Drought</td>
<td>3.3 ± 0.8</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>ΦPSII (r.u)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>0.61 ± 0.01*</td>
<td>0.61 ± 0.01*</td>
</tr>
<tr>
<td>Drought</td>
<td>0.16 ± 0.07</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>RWC (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>94 ± 1*</td>
<td>92 ± 1*</td>
</tr>
<tr>
<td>Drought</td>
<td>85 ± 1</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>Chlorophyll content</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-watered</td>
<td>7.4 ± 1.1*</td>
<td>7.3 ± 0.2*</td>
</tr>
<tr>
<td>Drought</td>
<td>4.7 ± 0.2</td>
<td>5.4 ± 0.4</td>
</tr>
</tbody>
</table>

As the three-way interaction was non-significant for any parameter, t-test was used to indicate significant differences (P < 0.05) between treatment means at the same column indicated by an asterisk (n = 6).

ANOVA P-values for the different parameters measured: soil – soil type; Myc – non-inoculated, inoculated with AM from GZ or inoculated with AM from FL; and drought- Well-watered and drought stress.

ANOVA, analysis of variance; GZ, Grazalema; FL, Freila; FW, fresh weight; RWC, relative water content; ΦPSII, photosystem II.

the FL-inocula growing in GZ soil (Table 3). On the other hand, root FW was significantly reduced by drought in all plants growing in FL soil, while no effect could be detected on GZ soil (Table 3). There was also a significant positive effect of the mycorrhizal inoculation in leaf FW ($P = 0.01$; Table 3).

Drought treatment also affected relative plant height ($P = 0.04$) (Fig. 1b), as well as the relative number of leaves produced during the drought treatment ($P < 0.0001$) (Fig. 1a). Plants growing in GZ soil with the GZ inocula were the only ones that displayed a significant increase in their relative heights under well-watered conditions, while the relative height did not change in any of the other treatments considered (Fig. 1b). For the relative number of leaves, drought caused a significant loss of leaves in the GZ soil plants in either non-inoculated plants or those inoculated with FL-AM fungi (Fig. 1a). It is noteworthy that under these conditions, inoculation with GL-AM fungi prevented such decrease.

**Fungal mycelium length, root mycorrhization rates and fungal diversity**

The highest extension of external mycelia under well-watered conditions was found in plants growing in FL soil and inoculated with AM fungi from GZ (Fig. 2a, $P < 0.0001$). When drought was applied, the highest external fungal development was found in FL soils with FL AM fungi. In GZ soils, hyphae within the soil were longer in the presence of GZ fungi, despite the water regime (Fig. 2a). However, the fungal root colonization within the roots only varied when the plants were subjected to drought stress and the FL inoculum was applied (Fig. 2b).

---

**Figure 1.** Relative plant height (a), relative number of leaves (b) and ANOVA P-values table in *Olea europaea* cv. Picual plants growing into two different types of soils (Grazalema – GZ and Freila – FL) and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for 4 weeks. Asterisks above bars indicate significant differences ($P < 0.05$) between well-watered and drought treatments after t-student test ($n = 6$).
A total of nine OTUs in the roots of all analysed samples were found (Fig. 2c). Only one of them, OTU1, likely affiliated to an undescribed species from the genus Dominikia, was found in all treatments. In addition, this OTU1 resulted to be dominant in all samples except in roots grown on GZ soil with the GZ inoculum and cultivated under well-watered conditions. Certain OTUs were detected exclusively in one of the root treatments related to a specific water regime or soil-inoculum association. Maximum AM fungal diversity values corresponded to well-watered treatments and if AM fungal inoculum was applied to its original soil, while the lowest diversity (0) was found in roots involving FL inoculum on GZ soil under well-watered conditions or in FL soil under drought conditions (Fig. 2c).

Leaf stomatal conductance, relative water content, photochemical efficiency of photosystem II, chlorophyll content and root hydraulic conductivity

Drought treatment caused a massive reduction of gs in all treatments except in non-inoculated plants growing in FL soil, as their initial values were already very low (Fig. 3a). In FL soil, AM inoculation significantly increased gs under well-watered conditions, especially with the GZ inoculum (Fig. 3a). This massive reduction of gs by drought could indicate a strong drought stress.

Leaf RWC and ΦPSII were reduced by the drought treatment ($P < 0.0001$, Table 3), with no effect of the different inoculation treatments or soil (Table 3). Leaf chlorophyll contents were significantly reduced by drought in GZ soils in
non-inoculated plants or those inoculated with GZ-AM fungi, as well as in non-inoculated plants growing in FL soil (Table 3). Under drought condition, AM inoculation (with both GZ and FL) significantly increased chlorophyll contents of plants growing in FL soil (Table 3). The trends observed in gs were not the same as the trends found in root hydraulic conductivity (Lp) (Fig. 3), in fact a significant negative correlation was found between gs and Lp (R = −0.320 P = 0.0249). Our study showed that the plants having higher Lp values were found in FL soil, with a significant increase under drought in plants inoculated with GZ-AM fungi (Fig. 3b). However, in FL soil both AM inocula reduced Lp under well-watered conditions. In GZ soil, we did not observed any effects on Lp by any treatment (Fig. 3b).

Molecular identification, expression and abundance of olive aquaporins

From the molecular approach used, nine new olive aquaporins were described: OePIP1;2, OePIP1;3, OePIP2;2, OePIP2;3, OePIP2;4, OePIP2;5, OePIP2;6, OeTIP1;2 and OeTIP1;3. A phylogenetic tree was built with the currently known PIPs and TIPs aquaporins (OePIP1;1, OePIP2;1 and OeTIP1;1) from O. europaea and Poplar (Fig. 4).

We next proceeded to analyse expression in the roots of the 12 aquaporins (the three already described and the nine new ones). RNA accumulation could not be detected for the previously known aquaporins OePIP1;1 and OePIP2;1 using qRT-PCR analyses. To analyse aquaporin expression, we firstly ran an examination of principal components to elucidate which
aquaporins were more related with the \( L_p \) and contributed the most to water transport (Fig. 5a). We found that expression of \( OePIP1;2, OePIP2;2, OePIP2;3, OePIP2;5, OePIP2;6 \) and \( OeTIP1;1 \) were related to whole \( L_p \), while the expression of aquaporins \( OePIP1;3, OePIP2;4, OeTIP1;2 \) and \( OeTIP1;3 \) explained the higher sources of variation within the data. At the same time, soil mycelial hyphal length and proportion of root length colonized also are closely related to \( L_p \). Stomatal conductance (\( gs \)) also explained the variation observed in \( L_p \) data. We ran Pearson correlations with the different aquaporins and \( L_p \), and found that \( L_p \) was correlated positively with \( OePIP1;2 \) and \( OeTIP1;2 \); and negatively correlated with \( OePIP1;3, OePIP2;4 \) and \( OeTIP1;3 \) (Table 3).

Analysis of variance analyses were run to detect significant differences between treatment means at the different treatments considered. The \( P \)-values showed, as in previous studies (Barzana et al. 2014), that the expression of all the aquaporins did not follow the same trend at the different treatments considered (Table 4 – ANOVA \( P \)-values, and Supporting Information). However, plants growing in FL soil showed a positive increase in \( OeTIP1;3 \) mRNA accumulation under drought conditions (Fig. 5b).

**Protein analyses**

The PIP1 proteins were undetected with the methodology applied. Amounts of the PIP2 proteins, as well as the different phosphorylated proteins changed as with the different treatments. The ANOVA \( P \)-values showed significant differences between treatment means for all the proteins studied (Table 4). We also ran Pearson’s correlations to test possible links between our \( L_p \) data and the PIP2 protein abundance and phosphorylation state. It was found that a general positive
Figure 5. Principal component analyses (A) for the expression of 10 aquaporin genes, PIP1 and PIP2 abundances, PIP2 phosphorylation state, root hydraulic conductivity ($L_{pr}$), stomatal conductance ($g_s$), root colonization rate (MycP) and soil hyphal length (MycL). Relative mRNA expression of OeTIP1;3 (B) in roots of *Olea europaea* cv. Picual plants growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for four weeks. Asterisks above bars indicate significant differences ($P < 0.05$) between well-watered and drought treatments after t-student test ($n = 3$).

Table 4. ANOVA $P$-values for expression of 10 *Olea europaea* aquaporins and the PIP2 root protein abundance and phosphorylation state (PIP2<sub>280</sub> – proteins phosphorylated at Ser-280, PIP2<sub>283</sub> – proteins phosphorylated at Ser-283 and PIP2<sub>280-283</sub> – proteins phosphorylated at both Ser-280 and Ser-283)

<table>
<thead>
<tr>
<th></th>
<th>Soil</th>
<th>Myc</th>
<th>Soil × Myc</th>
<th>Drought</th>
<th>Soil × drought</th>
<th>Myc × drought</th>
<th>Soil × Myc × drought</th>
</tr>
</thead>
<tbody>
<tr>
<td>OePIP1;2</td>
<td>0.6948</td>
<td>0.6960</td>
<td>0.2279</td>
<td>0.0099*</td>
<td>0.7861</td>
<td>0.2295</td>
<td>0.5441</td>
</tr>
<tr>
<td>OePIP1;3</td>
<td>0.0247*</td>
<td>0.0028*</td>
<td>0.0002*</td>
<td>0.1258</td>
<td>0.1597</td>
<td>0.0746</td>
<td>0.0482*</td>
</tr>
<tr>
<td>OePIP2;2</td>
<td>0.9796</td>
<td>0.0077*</td>
<td>0.0026*</td>
<td>&lt;0.0001*</td>
<td>0.4775</td>
<td>0.0075*</td>
<td>0.1077</td>
</tr>
<tr>
<td>OePIP2;3</td>
<td>0.0369*</td>
<td>0.1614</td>
<td>0.4516</td>
<td>0.5105</td>
<td>0.9305</td>
<td>0.1303</td>
<td>0.0443*</td>
</tr>
<tr>
<td>OePIP2;4</td>
<td>0.5710</td>
<td>0.0143*</td>
<td>0.4131</td>
<td>0.0005*</td>
<td>0.9357</td>
<td>0.1137</td>
<td>0.0606</td>
</tr>
<tr>
<td>OePIP2;5</td>
<td>0.0243*</td>
<td>0.1782</td>
<td>0.0133*</td>
<td>0.7510</td>
<td>0.0898</td>
<td>0.1810</td>
<td>0.1267</td>
</tr>
<tr>
<td>OePIP2;6</td>
<td>0.6627</td>
<td>0.6126</td>
<td>0.3966</td>
<td>0.3079</td>
<td>0.0066*</td>
<td>0.0271*</td>
<td>0.0167*</td>
</tr>
<tr>
<td>OeTIP1;1</td>
<td>0.5439</td>
<td>0.6830</td>
<td>0.4257</td>
<td>0.6290</td>
<td>0.8697</td>
<td>0.5195</td>
<td>0.6225</td>
</tr>
<tr>
<td>OeTIP1;2</td>
<td>0.9458</td>
<td>0.6177</td>
<td>0.0060*</td>
<td>0.0037*</td>
<td>0.8828</td>
<td>0.8179</td>
<td>0.0203*</td>
</tr>
<tr>
<td>OeTIP1;3</td>
<td>0.9647</td>
<td>0.1395</td>
<td>0.0356</td>
<td>&lt;0.0001*</td>
<td>0.0015*</td>
<td>0.3041</td>
<td>0.5517</td>
</tr>
<tr>
<td>PIP2&lt;sub&gt;280&lt;/sub&gt;</td>
<td>0.0007*</td>
<td>&lt;0.0001*</td>
<td>0.0104*</td>
<td>&lt;0.0001*</td>
<td>0.0271*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>PIP2&lt;sub&gt;283&lt;/sub&gt;</td>
<td>0.6411</td>
<td>0.1466</td>
<td>0.0887</td>
<td>0.5913</td>
<td>0.0380*</td>
<td>0.5233</td>
<td>0.0162*</td>
</tr>
<tr>
<td>PIP2&lt;sub&gt;280-283&lt;/sub&gt;</td>
<td>0.6581</td>
<td>0.0067*</td>
<td>0.0112*</td>
<td>&lt;0.0001*</td>
<td>0.0061*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

correlation between $L_p$ and the abundance of PIP2 proteins, but a negative correlation with the abundance of phosphorylated PIP2$_{\text{Ser280}}$ and PIP2$_{\text{Ser280-283}}$ proteins (Table 5). However, at the treatment level, the higher values of $L_p$ in drought plants in FL soil inoculated with the native mycorrhizas from GZ was not correlated with the abundance of PIP2 proteins (Fig. 6a) or the phosphorylation of these proteins at PIP2$_{\text{Ser280}}$ (Fig. 6b), but with the increase on the abundance of phosphorylated proteins PIP2$_{\text{Ser283}}$ and PIP2$_{\text{Ser280-283}}$ (Fig. 6c,d).

**DISCUSSION**

Olive plants were generated from explants of mature olive trees growing in a typically Mediterranean area. The plants were grown for 7 months into two contrasting Mediterranean soils and were inoculated with fungal communities obtained from those soils. In addition, plants were subjected to well-watered and drought stress conditions for 4 weeks. Under well-watered conditions, the plants growing on GZ soils and inoculated with the GZ fungal community were the only ones which increased their relative height during the 4 weeks of the stress period. It is well known that AM fungi can exhibit a considerable level of selectivity in their association with different plants species or plant ecological groups (Öpik et al. 2009, Varela-Cevero et al. 2015). However, these studies have not related the AM associations with the physical and chemical characteristics of the soil, only with the presence of certain AM fungi species. In our study, the higher relative heights of the plants were obtained with fungi from GZ and soils also from GZ, which have the highest content in organic matter, K, P and N. It has been previously found that soil chemistry influences soil microbial community composition, diversity and activity (Ehrenfeld et al. 2005). Greater soil fertility increases bacterial biomass and activity in grassland mesocosms, and these effects interact with plant species identity in some systems (Innes et al. 2004). Thus, effects of soil chemistry are often system-dependent. Whether or how these soil chemistry effects may interact with plant species identity or plant relatedness is not generally known. In our study, it seems to be crucial for the development of the AM fungi within the roots (that was similar in both well-watered and drought stressed plants), as well as, with the development of the external mycelia within the soil. This combination had also the higher diversity in the OTUs found within the roots under well-watered conditions.

It is also remarkable that plants growing in FL soil without inoculation had the lowest leaf FW, root FW and chlorophyll content, but these values were increased by AM inoculation. These results confirm that under some environmental conditions AM fungi are essential for plant growth and development (Estrada et al. 2013). Moreover, these plants, even under well-watered conditions, had gs values similar to plants under drought stress conditions. The cause could be the lower values of leaf P and K content. It is known that nutrient deficiency, including P and K, causes reduction of stomatal conductance (Flores et al. 2015). In fact, we found a positive correlation

<table>
<thead>
<tr>
<th>$L_p$</th>
<th>OePIP1;2</th>
<th>OePIP1;3</th>
<th>OePIP2;2</th>
<th>OePIP2;3</th>
<th>OePIP2;4</th>
<th>OePIP2;5</th>
<th>OePIP2;6</th>
<th>OeTIP1;1</th>
<th>OeTIP1;2</th>
<th>OeTIP1;3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
<td>PIP2</td>
</tr>
<tr>
<td>Lpr</td>
<td>0.357</td>
<td>0.378</td>
<td>0.208</td>
<td>-0.524</td>
<td>0.401</td>
<td>0.399</td>
<td>-0.534</td>
<td>0.492</td>
<td>-0.401</td>
<td>0.492</td>
</tr>
<tr>
<td></td>
<td>0.084</td>
<td>0.3831</td>
<td>0.223</td>
<td>0.00021</td>
<td>0.00021</td>
<td>0.00024</td>
<td>0.00024</td>
<td>0.00021</td>
<td>0.00021</td>
<td>0.00021</td>
</tr>
<tr>
<td>p</td>
<td>0.0219*</td>
<td>0.0125*</td>
<td>0.1860</td>
<td>0.5342</td>
<td>0.0417*</td>
<td>0.1869</td>
<td>0.9418</td>
<td>0.3603</td>
<td>0.0013*</td>
<td>0.0070*</td>
</tr>
</tbody>
</table>
between leaf P and K contents and gs ($P < 0.05$). Furthermore, no significant correlation was found between any leaf nutrient content and $L_{\text{pr}}$, except a negative correlation ($P > 0.05$) with K leaf contents, because plants from FL soil had less K contents but higher $L_{\text{pr}}$ values.

After 4 weeks of drought treatment, there was a general reduction in growth and gs in all treatments. These are typical responses of plants exposed to severe drought stress. However, there were some responses of plants that reinforce the previous idea of fungal and soil chemistry action on roots. We found that plants lost less leaves when growing in the GZ soils in combination with GZ-inocula, in agreement with the previous idea of the advantages of certain communities in soils of a certain chemistry and structure (Burns et al. 2015). Olive trees are known to have a tight control of gs under different watering regimes (Torres-Ruiz et al. 2013), as we found under drought stress conditions regardless of the soil or fungal community applied. This control of gs did not hamper the different responses in $L_{\text{pr}}$, as these plants seem to have a suitable hydraulic efficiency to yield water potentials that maintains the photosynthetic apparatus hydrated under different water demands (Raimondo et al. 2009). An increase of $L_{\text{pr}}$ under drought conditions was only found in the FL soil, and in plants inoculated with the GZ inocula, being this time the dominant fungi present in the roots the OTU1 ($\text{Dominikia} \text{ sp.}$) in conjunction with OTU5 ($\text{Funneliformis} \text{ sp.}$). Furthermore, plants growing on FL soil had a higher $\text{OeTIP1;3}$ expression and higher phosphorylated PIP2Ser280 and PIP2Ser280/283. Plant and fungal aquaporin expression, abundance and phosphorylation state can play a major role in root water transport (El-Mesbah et al. 2012). Plant aquaporins are usually responsible for the majority of radial root water transport under severe drought conditions (Barzana et al. 2012). There are many studies trying to understand how the presence of AM fungi regulates plant aquaporins (Barzana et al. 2014). It is known that AM symbiosis results in altered rates of water transfer in and out of the host plants (Auge 2001), and that also modifies $L_{\text{pr}}$ (Barzana et al. 2014, Calvo-Polanco et al. 2014a, Sanchez-Romera et al. 2016). Aquaporins provide a low-resistance pathway for the movement of water across membranes. Furthermore, as aquaporins can be gated, this provides greater control for the movement of water along plant tissues (Nyblom et al. 2009, Maurel et al. 2015). PIP and TIP isoforms have been recognized as central pathways for transcellular and intracellular water transport (Maurel et al. 2015). Thus, it seems likely that mycorrhizal symbiosis causes significant changes in aquaporin activity of host plants by changes in their phosphorylation status (Calvo-Polanco et al. 2014a, Sanchez-Romera et al. 2016). On the other hand, it has also been suggested that water could be absorbed by the external AM

Figure 6. Quantification of PIP2 proteins (A), PIP2 phosphorylated proteins at Ser-280 (B), Ser-283 (C) and both Ser-280 and 283 (D) in roots of Olea europaea cv. Picual plants growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for four weeks. Different letters means significant differences ($P < 0.05$) among treatments after ANOVA and Tukey’s test ($n=3$).
mycelium and delivered to the cortical apoplast, at the symbiotic interfaces, where it would join water taken up via the root apoplastic pathway (Smith et al. 2010; Barzana et al. 2012). Hyphal water uptake and transfer to the host plants has been demonstrated in several studies (Marulanda et al. 2003; Khalvati et al. 2005; Ruth et al. 2011). The increased water uptake by hyphae will be critical when soil dries, and water is retained in the smaller pores where fungal hyphae can grow (Marulanda et al. 2003), although the role of this hyphae in relation to the internal fungal development is not very well known yet under severe drought stress. However, a close relation between $L_p$, and mycorrhizal hyphal growth in both soil and roots was observed.  

Aroca et al. (2009) observed that the expression of one AM fungal aquaporin gene was higher in anti-sense tobacco plants with lower expression of tobacco aquaporins than in wild-type plants. This compensatory mechanism could also explain the negative correlation found here between $L_p$ and the expression and phosphorylation state of some olive aquaporins, where the AM fungal aquaporins could be the main pathway for water uptake.

Most recently, Tataranni et al. (2015) found that $L_p$ in olive trees subjected to drought stress was close correlated with the amount of suberin and root-cell density. It is known that AM fungi may increase lignin contents of colonized roots, especially in endodermis cells (Dehne and Schonbeck 1979). Furthermore, most recently, Almeida-Rodriguez et al. (2016) found that AM symbiosis modifies xylem vessels diameter in Salix purpurea plants, modifying also $L_p$, values under copper stress conditions. Anatomical changes caused by AM symbiosis in olives plants cannot be ruled out, and they deserve further investigations. At the same time, gs explain much of the variation observed in $L_p$, and a significant ($P < 0.05$) negative correlation between gs and $L_p$, was found. Such negative correlation could be indicative of a preferential water transport through the cell-to-cell path in olive trees, because this path is not dependent on leaf transpiration (Steudle & Peterson 1998).

The contribution of the different aquaporins studied was addressed with their mRNA expression and protein abundance. The combined expression of different PIP1, PIP2 and TIP1 aquaporins (Fig. 5a) gave as a combined $L_p$, response of the plant to the different treatments, and each of them responding differently to the several factors that were affecting the plants (Table 4). Different aquaporins are either up-regulated or down-regulated by the same stress and those in the same subgroup may have distinct expression patterns under different stress conditions (Guo et al. 2006; Barzana et al. 2014). The contribution of all these aquaporins to $L_p$, allowed a fine regulation of water uptake under the GZ soils (Fig. 3b) and contributed to the increase of $L_p$, under drought conditions in FL soil with GZ inocula. Among all studied aquaporins, the one that clearly increased under drought stress was OeTIP1.3. The interest on the role of ‘TIPS’ in water transport has increased in the last few years. Wang et al. (2014) showed that the expression of TsTIP1.2 increased in response to various stresses in Thellungiella salsuginea, and ectopic overexpression of TsTIP1.2 enhanced tolerance to drought, salt and oxidative stresses in transgenic Arabidopsis. Most interesting are the results of Henry et al. (2012) who found a good correlation between TIP expression and $L_p$, in rice roots subjected to drought stress and by Kuwagata et al. (2012) in rice roots under different air humidity conditions. Furthermore, Boursiac et al. (2005) found that the decrease of $L_p$, caused by salt stress in Arabidopsis was correlated with a down regulation of PIP and TIP expression as well to a relocalization to internal membranes. On the other hand, the effect of the phosphorylation of PIP2 proteins at Ser283 residue may have contributed to the increase of water uptake in olive plants. Aquaporin phosphorylation is considered as one of the major processes affecting water transport (Suga and Maeshima 2004) and may have also contribute to the adaptation of plant to different stresses, including drought.

Our results show negative correlations between the expression and abundance of some aquaporins and $L_p$. Similar results were found by Sutka et al. (2011) analysing the natural variation in $L_p$, of different accessions of Arabidopsis. Most recently, Li et al. (2016) also found a negative correlation between $L_p$, and the gene expression of some Arabidopsis PIPs (AtPIP1;4 and AtPIP2;6). It is known that aquaporins can interact each other with positive or negative results in terms of membrane water permeability, mostly because internalization processes (Bellati et al. 2010; Chaumont and Tyerman 2014). Recently, Hachez et al. (2014) found that the plasma membrane amount of ZnPIP2;7 proteins is regulated by autophagic degradation when interact with tryptophan-rich sensory protein/translocator. Furthermore, it has been shown that aquaporin mRNA expression does not always matches aquaporin protein abundance (Marulanda et al. 2010).

In conclusion, we have confirmed that the different characteristics of the soil affect the development of plants and their responses to drought stress. Furthermore, we have established that the fitness among AM fungal communities, plant species and soil origin is crucial for the development of a proper plant-fungus symbiosis. In our case, the inocula from GZ soils had a higher impact in olive trees under well-watered conditions and also these inocula had a higher impact in FL soils under drought treatment. So, the AM fungi originated from a wet climate had a better performance with olive plants especially in endodermis cells (Dehne and Schonbeck 1979). Furthermore, most recently, Almeida-Rodriguez et al. (2016) found that the plasma membrane amount of ZnPIP2;7 proteins is regulated by autophagic degradation when interact with tryptophan-rich sensory protein/translocator. Furthermore, it has been shown that aquaporin mRNA expression does not always matches aquaporin protein abundance (Marulanda et al. 2010).

ACKNOWLEDGMENTS

We would like to thank Sonia Molina, Jose Luis Manella and Olga M. Lopez for their technical support. The study was supported by the Ministry of Economy and Competitiveness of Spain (Juan de la Cierva Program) and Junta de Andalucia (PI0-CVI-5920 project) for research funding.
REFERENCES


Received 30 October 2015; revised in received form 14 July 2016; accepted for publication 15 July 2016.
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Supplemental File S1.** Primers for aquaporin expression

**Supplemental File S2.** Multiple alignment C-terminal regions $OIP2;2$, $OIP2;3$, $OIP2;4$, $OIP2;5$, and $OIP2;6$ proteins, with *Phaseolus vulgaris* $PvPIP2;1$, respectively. The consensus amino acids are underlined. The $PvPIP2;1$ sequences correspond to the peptide used to make the respective antibody.

**Supplemental File S3.** Aquaporins expression in roots of *O. europaea* cv. Picual seedlings growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for four weeks. Different letters means significant differences ($p < 0.05$) among treatments after ANOVA and Tuckey’s test. Asterisks above bars indicate significant differences ($p < 0.05$) between well-watered and drought treatments after $t$-student test ($n = 6$).