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The arbuscular mycorrhizal symbiosis regulates aquaporins activity and improves root cell water permeability in maize plants subjected to water stress

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ABSTRACT

Studies have suggested that increased root hydraulic conductivity in mycorrhizal roots could be the result of increased cell-to-cell water flux via aquaporins. This study aimed to elucidate if the key effect of the regulation of maize aquaporins by the arbuscular mycorrhizal (AM) symbiosis is the enhancement of root cell water transport capacity. Thus water permeability coefficient (P_f) and cell hydraulic conductivity (L_{pc}) were measured in root protoplast and intact cortex cells of AM and non-AM plants subjected or not to water stress. Results showed that cells from droughted-AM roots maintained P_f and L_{pc} values of non-stressed plants, while in non-AM roots these values declined drastically as consequence of water deficit. Interestingly, the phosphorylation status of PIP2 aquaporins increased in AM plants subjected to water deficit, and P_f values higher than 12 $\mu m\ s^{-1}$ were found only in protoplasts from AM roots, revealing the higher water permeability of AM root cells. In parallel, the AM symbiosis increased stomatal conductance, net photosynthesis and related parameters, showing a higher photosynthetic capacity in these plants. This study demonstrate a better performance of AM root cells in water transport under water deficit, which is connected to the shoot physiological performance in terms of photosynthetic capacity.

An enhancement of the root cell water permeability was measured under drought both in intact cortex cells and protoplasts from AM maize plants, concomitantly with regulation of some plant aquaporins, increase of PIP2s phosphorylation status and enhanced photosynthetic capacity.

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KEYWORDS: aquaporins, arbuscular mycorrhizal symbiosis, cell hydraulic conductivity, cell pressure probe, photosynthesis, protoplasts, water permeability

INTRODUCTION

Maize is one of the most consumed crops worldwide (Lobell *et al.* 2008) with annual production of more than one trillion ton (FAOSTAT), and is expected to double its demand by 2050. However, its production is affected by a number of constraints, including an array of biotic and abiotic stresses (Daryanto *et al.* 2016). Drought stress has a high impact on plant growth and development, reducing crop production worldwide *(Lesk et al.* 2016), including maize (Daryanto *et al.* 2016). Thus, it seems necessary to elucidate the mechanisms that enhance maize drought tolerance, in order to guarantee food production in the near future (Trenberth *et al.* 2014; Lesk *et al.* 2016).

To this respect the arbuscular mycorrhizal (AM) symbiosis, the mutual association that naturally occurs between different fungal species of the Glomeromycota phylum and plant roots, is extensively reported in literature as beneficial for improving the resilience of the majority of crops to water stress (Augé 2001; Augé *et al.* 2015). In a climate change context, the AM symbiosis is likely to be a way to reduce water input in arid and semiarid soils being a feasible solution to overcome the stressful conditions. This symbiosis produces changes in soil properties (Augé 2004; Bedini *et al.* 2009) and in the plant that affect both the root and the shoot during drought stress, resulting in a better plant fitness (Ruiz-Lozano *et al.* 2012; Chitarra *et al.* 2016).

In plants, the water status of the shoot is determined by the balance between root water uptake and stomatal aperture. Root resistance to water is the highest within the soil-plant-atmosphere continuum (Steudle *et al.* 1987). Thus, to keep the stomata open, the root

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water conductivity (L_{pr}) must be high enough (Sack & Holbrook 2006). The AM association has been found to differently regulate root water transport both under well-watered and water deficit conditions, generally enhancing L_{pr} (Aroca *et al.* 2007; Bárzana *et al.* 2012, 2014, 2015; Sánchez-Romera *et al.* 2016; Quiroga *et al.* 2017). This effect has been related to the uptake of water through fungal hyphae from soil pores inaccessible to roots and to changes induced by the AM fungi affecting the hydraulic properties of the soil (Augé *et al.* 2004; Allen 2009; Hallet *et al.* 2009) and inside the roots (Ruiz-Lozano & Aroca 2017). Indeed, there is increasing evidence that mycorrhizal fungi transport water towards the host (Marulanda *et al.* 2003; Allen, 2009; Ruth *et al.* 2011; Li *et al.* 2013; Xu *et al.* 2015). Moreover, the AM symbiosis has been suggested to modulate the switching between apoplastic and cell-to-cell water transport pathways in roots (Bárzana *et al.* 2012), providing higher flexibility to these plants for reacting to water shortage depending on the demand of the aerial part.

Aquaporins are thought to be the main pathway for water movement through the cell membranes (Maurel *et al.* 2015), providing the capacity of rapidly modify membrane water permeability, which help the plant with the maintenance of the water balance during stress episodes, and affecting root hydraulic conductivity (Hachez *et al.* 2006, 2012; Maurel *et al.* 2008; Moshelion *et al.* 2009; Zarrouk *et al.* 2016). Among higher plant aquaporins, the plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs), have been highlighted for their involvement in the control of radial transcellular water transport and also of cell osmoregulation and, in general, PIP and TIP aquaporin expression seems to be more abundant in roots than in leaves (Chaumont & Tyerman 2014). Moreover, the expression of PIP aquaporins in roots has been correlated to the presence of apoplastic barriers, suggesting an essential role in the transmembrane water diffusion when its movement is hindered (Shatil-Cohen *et al.* 2011; Prado *et al.* 2013). Water is also transported by TIPs, but they display a great diversity of substrates and Ar/R selectivity filter

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configurations. Thus, besides water, TIPs also play roles in glycerol, urea, ammonia and H_2O_2 transport, as well as, in abiotic stress responses (Afzal *et al.* 2016; Fox *et al.* 2017). It has also been proposed that TIPs may provide a quick way for cellular osmotic balance by controlling the exchange of water between vacuole and cytosol (Forrest & Bhave 2007). In any case, there is increasing evidence of a higher contribution of aquaporin-mediated water transport to global root water uptake than previously thought, even under high transpiration conditions (Knipfer & Fricke 2010, 2011). Furthermore, their relevance for plant physiology is emphasized by the fact that, apart from water, some aquaporin isoforms can facilitate membrane diffusion of other small solutes such as CO_2, metalloids, urea, ammonia, H_2O_2, oxygen or even ions (Li *et al.* 2014; Byrt *et al.* 2017; Zwiazek *et al.* 2017). Hence, although the role of aquaporins in the maintenance of water homeostasis in the whole plant and in the stress responses has been well established (Afzal *et al.* 2016; Chaumont & Tyerman 2014), their numerous functions in plant growth and development seem to be essential but not well understood yet (Chaumont & Tyerman 2014; Li *et al.* 2014; Afzal *et al.* 2016).

The importance of aquaporins for both nutrient and water exchanges during mycorrhizal symbiosis was recognized by Maurel & Plassard (2011) and supported by the results obtained by Bárzana *et al.* (2014), who found that 16 out of the 36 maize aquaporins (Chaumont *et al.* 2001) were regulated by the AM fungus *R. irregularis* during drought stress. However, results obtained so far on aquaporins regulation by the AM symbiosis show that the effects of the symbiosis on aquaporin gene expression are complex and depend on the intrinsic properties of the osmotic stress. Under drought stress conditions, the AM symbiosis usually decreases or anticipates the decrease of aquaporin gene expression. Under salt stress, the trend is just the opposite since the AM symbiosis enhanced the expression of most of the aquaporin genes analyzed (Aroca *et al.* 2007). In addition, the regulation of the plant aquaporins also depends on the severity and duration of the stress applied, as

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evidenced by Bárzana *et al.* (2014) for maize aquaporins. Moreover, given the diversity of substrates that can be transported by the AM-regulated aquaporin isoforms, they may have a role in the regulation of leaf and root hydraulics, as well as, in other physiological processes such as nutrient uptake and translocation, stomatal movement and carbon fixation (Uehlein *et al.* 2007) or signalling processes (Fox *et al.* 2017). Thus, the elucidation of *in planta* transport capacities of the AM-regulated aquaporins is required in order to understand their role in the AM-induced drought tolerance.

The above mentioned results suggested that additional studies should elucidate the specific function of aquaporin isoforms regulated by the AM symbiosis in order to know how the AM symbiosis alters the plant fitness under stressful conditions. Thus, we aimed to elucidate if the key effect of the regulation of maize aquaporins by the AM symbiosis is indeed the enhancement of root cell membrane water transport capacity. One approach to quantify the permeability of plant cells to water consists in the isolation of protoplasts and measuring their osmotic water permeability coefficient (P_f) upon an osmotic challenge (Moshelion *et al.* 2004) and is a convenient way to measure the role of aquaporins in cell water transport (Shatil-Cohen *et al.* 2014). This method overcomes the limitation of measuring only root hydraulic permeability that can be mediated by cell-to-cell pathway (determined in part by aquaporins activity, but also by plasmodesmata) and by apoplastic pathway, which is unrelated to aquaporins activity (Steudle 2000). Besides, the cell pressure probe is also used as a technique to do direct and accurate measurements of cell turgor pressure, cell wall elasticity and cell hydraulic conductivity (L_{pc}) in intact cells (Husken *et al.* 1978). Using both approaches to determine permeability of cells may provide better information about the water transport ability of AM root cells.

We hypothesized that if the aquaporins regulated by the AM symbiosis are mainly involved in cell to cell water transport, the values of these parameters measured in

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protoplasts and intact cells from AM plants should be different from those of non-AM plants, and regulated by the watering conditions. We also aimed to determine if the expected changes in root cell membrane water permeability have a significant effect on plant fitness and alter important physiological parameters such as plant photosynthetic capacity. For that, we performed two independent experiments, one focused on the effect of AM on root cell membrane water permeability, and the other one focused on the effect of AM on the water transport from soil to leaves in order to be used in CO_2 exchange at stomatal level, leading to greater photosynthetic efficiency.

MATERIALS AND METHODS

Experimental design and statistical analysis

Two independent experiments with the same design were performed. The first was used for measurements of root cell membrane water permeability and the second one for measurements of plant photosynthesis-related parameters.

The two experiments consisted of a factorial design with two factors: (1) inoculation treatment, including plants inoculated with the AM fungus *Rhizophagus irregularis*, strain EEZ 58 (Ri) and non-inoculated control plants (C); (2) water regime, so that one half of the plants were cultivated under well-watered conditions (WW) throughout the entire experiment and the other half of the plants were subjected to water deficit (WD) for two weeks just before harvest. Each treatment had 15 replicates giving a total of 60 pots for each experiment. The experiments were repeated twice.

Statistical analyses for both experiments were performed in SPSS Statistics (Version 23, IBM Analytics) using two way analysis of variance (ANOVA), with AM inoculation (M),

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water regime (W) and their interaction (M X W) as sources of variation. Duncan's or Student-T were used as posthoc tests to find out differences between means at α=0.05.

Biological material and growth conditions

The growing substrate consisted of a mixture of soil and sand (v/v 1:1). Soil was collected at the grounds of IFAPA (Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm) and sterilized by steaming (100°C for 1 h on 3 consecutive days). Soil had a pH of 8.1 (water); 0.85% organic matter, nutrient concentrations (mg kg^{-1}): N, 1; P, 10 ($NaHCO_3$-extractable P); K, 110. The soil texture was made of 38.3% sand, 47.1% silt and 14.6% clay.

Seeds of *Zea mays* L. (cv PR34B39, Pioneer Hi-Bred, Spain) were sown in 1.5 L pots containing 1250 g of the substrate described above.

Mycorrhizal inoculum was bulked in an open-pot culture of *Z. mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM fungus was *Rhizophagus irregularis* (Schenck and Smith), strain EEZ 58. Ten grams of inoculum with about 60 infective propagules per gram (according to the most probable number test), were added to appropriate pots at sowing time. Non inoculated control plants received the same amount of autoclaved mycorrhizal inoculum together with a 10 ml aliquot of a filtrate (<20 µm) of the AM inoculum in order to provide a general microbial population free of AM propagules. For experiment 1 maize plants were grown under greenhouse conditions (25/20ºC, 16/8 light dark period, 50-60% RH and average photosynthetic photon flux density 800 µmol m^{-2} s^{-1}) during the first 5 weeks and during the last 3 weeks plants were maintained in a controlled environmental chamber with the following conditions: 25/18 ºC, 16h/8h light/dark period, and photosynthetic photon flux density 300 µmol m^{-2} s^{-1}.

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For experiment 2 plants were grown under greenhouse conditions (25/20ºC, 16/8 light dark period, 50-60% RH and average photosynthetic photon flux density 800 µmol m^{-2} s^{-1}) for 8 weeks, until harvest.

For both experiments soil moisture was measured with the ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK) as described previously (Quiroga *et al.* 2017). Water was supplied daily to maintain soil at 100% of field capacity during the first 6 weeks after sowing. Then, half of the plants were allowed to dry (DS treatments) until soil water content reached 60% of field capacity (2 days needed), while the other half were maintained at field capacity (WW treatments). Plants were maintained under such conditions for 14 additional days. The water stress imposed was similar to that described by Quiroga *et al.* (2017).

Common measurements

Biomass production and symbiotic development

At harvest, the shoots and roots of 10 replicates per treatment were collected and used for fresh weight recording.

In order to visualize and quantify AM fungal structures, roots were stained with trypan blue according to Phillips & Hayman (1970). The percentage of mycorrhizal colonization was calculated by the gridline intersect method according to Giovannetti & Mosse (1980) in 3 replicates per treatment.

Measurements in experiment 1

Stomatal conductance

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Stomatal conductance was measured two hours after the onset of photoperiod with a porometer system (Leaf porometer, model SC-1, Decagon devices, WA, USA) following the user manual instructions. Measurements were taken one day before harvest in the second youngest leaf from eight plants per treatment.

Cell Pressure Probe measurement

Cell pressure probe measurements were done as described by Moshelion *et al.* (2009) with some modifications. Briefly, an oil-filled microcapillary was inserted in cortex cells of the medium part of intact roots with the aid of a micromanipulator (Leica, Wetzlar, Germany) under a stereomicroscope (magnification: 130x). When the cell was punctured, cell sap started entering the capillary and formed a meniscus between sap and oil. Cell turgor pressure (P) was measured when returned to its original level by pushing the meniscus back to the cell side by means of a motor-driven metal rod. Then, peaks of pressure relaxations were used to see how the pulses affected the cell permeability by monitoring the change in the half time of water exchange ($T_{1/2}$). Cell elastic properties are described by the cell wall volumetric elastic modulus (ε) that is a measure of the cell wall resistance to be deformed and produce changes in cell volume upon pressure application. It was calculated by inducing changes in turgor that resulted in modification of the cell volume ($\varepsilon = V \bullet \Delta P / \Delta V$). The cell hydraulic conductivity is given by $L_{pc} = \ln(2)V/(AT_{1/2}(\varepsilon+\pi_i)$, where V is the cell volume, A is the cell surface area and π_i is the cell osmotic pressure.

Root cell protoplast isolation and protoplast swelling assay

Measurements were done as described by Moshelion *et al.* (2004) with slight modifications. Root segments taken at 4 cm from the tips were cut into small fragments and placed in a plate containing 2.5 mL of ~ 660 mOsm isotonic buffer (10 mM KCl, 1 mM CaCl2,

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8 mM MES, pH 5.75), adjusted with the appropriate amount of D-sorbitol 2M, and containing 2% cellulose R-10, 0.5% BSA, 0.5% PVP K30, 0.1% pectolyase and 0.3% macerozyme R-10 in order to degrade the cell wall. Samples were submitted to vacuum for 15 min and transferred for digestion in the dark during 3 h on a shaker at 70 rpm. Subsequently, enzyme solution was removed, and 2.5 mL of new isotonic buffer without enzymes were added and put into the dark for 1 h on a shaker at 120 rpm. The protoplast-containing liquid was then filtrated through a 100 μm nylon mesh and centrifuged at 90*g* for 3 min, then washed once with isotonic buffer and centrifuged at 90*g* for 3 min. The protoplasts were suspended in 100 μL of isotonic buffer and measured as rapidly as possible.

The hypotonic challenge assay was performed as described in Shatil-Cohen *et al.* (2014).

Sub-cellular fractionation

Sub-cellular fractionation was performed according to Hachez *et al.* (2006). Pieces of intact roots were grinded with 600 µL of a protein extraction buffer containing 250 mM Sorbitol, 50 mM Tris-HCl (pH 8), 2 mM EDTA and protease inhibitors. All steps were performed at 4ºC. The homogenate was centrifuged during 10 min at 770*g* and the supernatant obtained was centrifuged 10 min at 10000*g*. The resulted supernatant was finally centrifuged during 30 min at 100000*g* and the final pellet (corresponding to the microsomal fraction) was resuspended in 30 µL of suspension buffer (5 mM KH_2PO_4, 330 mM sucrose, 3 mM KCl, pH 7.8) and sonicated twice for 5 s.

PIP aquaporins abundance and phosphorylation status

Ten micrograms of the microsomal fraction were solubilized for 10 min at 50ºC in loading buffer (27 mM Tris/HCl, 0.7% SDS, 3.3% glycerol, 0.0016% bromophenol blue, 1%

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DTT) and the proteins were separated by SDS-PAGE on a 10% polyacrylamide gel. After electrophoresis, the gel was incubated for 10 min in semi-dry buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS) before semi-dry transfer to a polyvinylidene fluoride (PVDF; Bio-Rad) membrane (30 min at 22 V).

Western blot analysis were performed using antisera against ZmPIP2;1/2;2, ZmPIP2;5, ZmPIP2;6 and ZmPIP1 as described in Hachez *et al.* (2006). The dilutions used were 1/3500 for the anti-ZmPIP2;1/2;2, 1/1000 for anti-ZmPIP2;5 and anti-ZmPIP2;6 and 1/350 for anti-ZmPIP1.

Ten micrograms of the same extract were used for Colloidal blue gel staining as a gel-loading control.

Signal quantification of the bands in the obtained images was performed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016).

Three antibodies recognizing the phosphorylation state of PIP2 proteins in the C-terminal region were used: PIP2A (which recognizes phosphorylation at Ser-280), PIP2B (which recognizes phosphorylation at Ser-283) and PIP2C (which recognizes phosphorylation at Ser-280 and Ser-283). The antibodies were described and used in previous experiments and the quantification of these proteins was done by ELISA technique (Calvo-Polanco *et al.* 2014; Quiroga *et al.* 2018).

Quantitative RT-PCR

Total RNA was extracted from three biological replicates of maize roots as described in Quiroga *et al.* (2017). First-strand cDNA was synthesized using 1 µg of purified RNA with the Maxima H Minus first strand cDNA synthesis kit (Thermo Scientific ^{TM}), according to the manufacters' instructions.

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The expression of previously selected maize aquaporins (Quiroga *et al.* 2017) together with *ZmPIP2;1*, *ZmPIP2;5* and *ZmPIP2;6* and the stress marker gene *ZmNCED1*, encoding for 9-cis-epoxycarotenoid dioxygenase (Capelle *et al.* 2010), was measured by qRT-PCR using 1 µL of diluted cDNA (1:9) with PowerUp™ SYBR™ Green Master Mix in a QuantStudio™ 3 system (Thermo Fisher Scientific). The reaction was repeated for 40 cycles at annealing temperature of 58ºC for all primers except for *ZmPIP2;6*, annealing at 60ºC. For normalization of gene expression values, four reference genes were measured in all the treatments. These genes were poliubiquitin (gi:248338), tubulin (gi:450292), GAPDH (gi:22237) and elongation factor 1 (gi:2282583) (Bárzana *et al.* 2014). Standardization was carried out based on the expression of the two best-performing reference genes under our specific conditions, which were chosen by using "NormFinder" algorithm (Andersen *et al.* 2004) (https://moma.dk/normfinder-software). Thus, expression levels were normalized according to *Zmtubulin* and *ZmGAPDH* genes. Fungal aquaporins (*GintAQP1*, *GintAQPF1* and *GintAQPF2*) were analysed as previously described (Aroca *et al.* 2009; Li *et al.* 2013) using fungal *elongation factor 1*α as reference gene for standardization. The relative abundance of transcripts was calculated using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen 2001). The threshold cycle (Ct) of each biological sample was determined in duplicate. Negative controls without cDNA were used in all PCR reactions.

Measurements in experiment 2

Gas exchange measurements

Net photosynthesis (An), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and instantaneous water use efficiency (iWUE = An/g_s) of fully expanded young leaves in five different plants were measured using portable photosystem system LI-6400 (LICOR

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Biosciences, Lincoln, NE, USA) after 8 weeks of growth. The response of An to C_i was recorded to examine the effects of mycorrhizal inoculation on the maximum apparent rate of phosphoenolpyruvate carboxylase (PEPc) activity (V_{pmax}) and CO_2-saturated photosynthetic rate (V_{max}). The gas exchange response to CO_2 was initiated at ambient intercellular [CO_2], then the reference [CO_2] was stepped down to 25 µmol mol^{-1} and afterwards it was increased stepwise to 1000 µmol mol^{-1} at 1500 µmol m^{-2} s^{-1} PPFD as described in Yendrek et al. (2017). According to Caemmerer (2000) the initial slope of the An/C_i curve (C_i < 60 µmol mol-1) was used to estimate V_{pmax}. Assessment of V_{max} as the horizontal asymptote of the An/C_i curve was performed using a four parameter nonrectangular hyperbolic function as described in Yendrek *et al.* (2017).

Specific leaf area

Specific leaf area (SLA) was determined by weighting a known area of six leaf discs per plant, five plants per treatment, after drying for seven days at 65ºC. SLA was calculated as the ratio between leaf area and leaf dry mass.

Shoot area

Shoot area was determined from 8-bit images of the detached leaves and shoots on six plants per treatment using ImageJ processing software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016.).

Photosynthetic efficiency

The efficiency of photosystem II of light adapted maize leaves was measured with Fluor-Pen FP100 (Photon Systems Instruments, Brno, Czech Republic) as previously

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described in Quiroga *et al.* (2017, 2018) in the second youngest leaf of 10 different plants of each treatment after 8 weeks of growth.

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RESULTS

Experiment 1

Biomass production and symbiotic development

Plant biomass production significantly decreased (by 41%) with water deficit. The decrease was unaffected by mycorrhization, being similar in AM and non-AM plants (Table 1).

The percentage of fungal root colonization was similar in well-watered and droughted plants inoculated with *R. irregularis*, being of 57% for well-watered plants and of 52% for the plants subjected to water deficit. Uninoculated control plants did not present fungal colonization (Table 1).

Stomatal conductance (gs)

We measured *gs* in order to characterize the magnitude of the water stress in the aerial part of the plants. Well-watered AM plants presented significantly higher *gs* compared to non-AM plants. Water deficit significantly decreased *gs* to similar values in both AM and non-AM plants (Table 1).

Water permeability of roots cells

To investigate the effect of the mycorrhizal colonization on root cell water permeability, we measured cell hydraulic parameters by means of the cell pressure probe. The cell turgor pressure (P) was similar for all the treatments regardless of AM inoculation or water regime (Table 2), as well as, the cell size (data not shown). The half time of water exchange ($T_{1/2}$) was similar in AM and non-AM plants under well-watered conditions, but under water deficit

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conditions it was 45% lower in AM plants than in non-AM ones (Table 2). The cell wall elastic modulus (ε) was 116% higher in AM plants than in non-AM ones under well-watered conditions, while under water deficit it was 40% lower in AM plants (Table 2). In any case, water deficit enhanced notably the ε values in non-AM plants to reach 8.16 MPa, while it did not affect these values in AM plants that remained at 4.86 MPa. Under well-watered conditions, AM plants showed significantly lower cell hydraulic conductivity (L_{pc}) values than non-AM plants (42% of decrease). However, under water deficit, L_{pc} values were maintained unaltered in AM plants, but diminished drastically in non-AM plants (68% of decrease). Thus, under water deficit conditions AM plants exhibited 110% higher L_{pc} values as compared to non-AM plants (Figure 1). The obtained L_{pc} values were converted into osmotic water permeability (P_f) values (Figure 1) for comparison with P_f data from the protoplast swelling assay (Volkov *et al.* 2007).

P_f values obtained by a swelling assay on isolated root protoplasts of AM and non-AM plants did not differ significantly under well-watered conditions (Figure 2a). When plants were subjected to water deprivation, P_f values decreased in non-AM plants, although the decrease was not significant. In contrast, in AM plants P_f values remained similar as under well-watered conditions, almost doubling the values of non-AM plants subjected to water deficit.

We analysed the distribution of P_f values among the different treatments (Figure 2b), establishing three categories (less than 6 µm/s, 6 to 12 µm/s and more than 12 µm/s). Data showed that non-AM plants did not present protoplasts with P_f values over 12 µm/s. These higher P_f values were observed only in protoplasts coming from AM treatments, either under well-watered or water deficit conditions. Most P_f values were under 6 µm/s in all treatments. However, AM plants exhibited a lower proportion of protoplast with P_f values under 6 µm/s than non-AM plants. Moreover, the highest proportion of P_f values within this category (92%) was found in droughted non-AM plants and the lowest proportion of P_f values within this

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category (65%) was found in well-watered AM plants. Altogether, the P_f values distribution could explain the differences in water permeability between the treatments.

Aquaporin accumulation and phosphorylation status of PIP2s

Since aquaporins could be responsible for changes in cell water permeability, we measured the accumulation of these proteins in root cell membranes by western blot (Figure 3). Protein accumulation showed the same pattern for PIP2;1/2;2 and PIP2;6 aquaporins. Water deficit slightly decreased the amounts of both proteins in non-inoculated plants. In AM plants, the protein amounts remained unchanged regardless of the water treatment, being in both cases significantly lower as compared to non-AM plants.

In the case of PIP1s, AM plants subjected to water deficit showed a significantly lower amount of protein compared to non-AM counterparts. However, no-significant differences were found between both treatments under well-watered conditions.

No significant differences were found among treatments when analysing PIP2;5 accumulation.

We also used three antibodies recognizing the phosphorylation of PIP2 aquaporins at two serine residues in the C-terminal region in order to estimate the phosphorylation status of such proteins (Figure 4). Interestingly, the three antibodies showed the same PIP2 aquaporins accumulation pattern, with lower levels of phosphorylation at Ser-280, Ser-283 or both Ser residues in well-watered AM plants compared to the corresponding non-AM plants. However, when plants were subjected to water deficit, AM plants increased significantly the phosphorylation levels of these Ser residues to reach values similar to non-AM plants.

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Root gene expression levels under well-watered and water deficit conditions

We analysed the mRNA levels of *ZmPIP1;1, ZmPIP1;3, ZmPIP2;2, ZmPIP2;4, ZmTIP1;1, ZmTIP2;3*, *ZmTIP4;1* and *ZmNIP2;1* genes, previously selected as being regulated by the AM symbiosis (Quiroga *et al.* 2017), as well as, other aquaporins recognized for their role in root water transport (*ZmPIP2;1*, *ZmPIP2;5* and *ZmPIP2;6*) (Figure 5a). Several of the analysed aquaporins did not show significant differences in their expression patterns. Nonetheless, the levels of *ZmPIP2;2* and *ZmPIP2;6* mRNA were indeed regulated by the AM symbiosis and/or by water deficit and exhibited a similar expression pattern. Under well-watered conditions the mRNA levels of both genes decreased significantly in AM plants. The expression of both genes in non-AM plants did not vary after the application of the water shortage treatment. In contrast, in AM plants the expression of both genes increased significantly to reach the same levels than well-watered non-AM plants.

The expression of the three aquaporin genes of *R. irregularis* was also analysed (*GintAQP1*, *GintAQPF1* and *GintAQPF2*) (Figure 5b). *GintAQPF1* was found to be down-regulated when the AM plants were subjected to water deficit. On the contrary, *GintAQPF2* was up-regulated with water shortage. In the case of *GintAQP1* no significant differences in expression levels were found.

As a marker of the water stress level, we measured the expression of *ZmNCED1* gene, encoding for 9-cis-epoxycarotenoid dioxygenase, which is involved in the biosynthesis of the stress hormone ABA (Capelle *et al.* 2010). The expression of this gene was significantly up-regulated by the water deficit imposed in both experiments, regardless of mycorrhizal inoculation, with increases ranging 6 to 8 folds (Figure 6).

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Experiment 2

Plant growth, specific leaf area (SLA) and symbiotic development

Despite water deficit treatment decreased significantly plant fresh weight (PFW), AM plants showed 19% higher PFW than non-AM ones after 8 weeks of growth (Table 3). Similar result was observed for shoot area. It declined under water deficit conditions by 48% in non-AM plants and by 34% in AM plants, but was maintained 44% higher in AM plants.

No changes in specific leaf area (SLA) were observed due to watering conditions, but AM inoculation led to lower SLA values regardless of water regime (Table 3).

The extent of mycorrhizal root colonization was similar for both AM inoculated treatments, with an average of 58% of mycorrhizal root length for the well-watered plants and 52% of mycorrhizal root length for the plants subjected to water deficit. Uninoculated plants did not show fungal colonization (Table 3).

Gas exchange, photosynthetic capacity and efficiency of photosystem II

After 8 weeks of growth, at the end of the water deficit treatment, plants maintained under well-watered conditions showed no significant differences in gas exchange parameters (net photosynthetic activity, An; stomatal conductance, gs, or intercellular CO_2 concentration, Ci) due to AM fungal inoculation (Table 4). Nevertheless, plants under water deficit exhibited lower An, gs, as well as, Ci leading to greater intrinsic water-use efficiency (data not shown), regardless of the fungal treatment. Under such water limited conditions AM inoculated plants featured enhanced An (by 75%) and gs (90%) values. Similar result was obtained when analysing pnhotosynthetic capacity at this growth period (Figure 7). Well-watered plants presented no differences in the maximum apparent rate of phosphoenolpyruvate carboxylase

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activity (Vpmax) or CO_2-saturated photosynthetic rate (Vmax) due to AM inoculation, but water deficit treatment significantly dropped both photosynthetic parameters regardless of AM inoculation. In any case, droughted AM plants showed greater Vpmax (by 30%) and Vmax (by 32%) than non-AM ones.

Under well-watered conditions the light-adapted maximum quantum yield of photosystem II primary photochemistry (ΔFv/Fm') did not show significant differences due to AM inoculation after 8 weeks of growth (Table 4). However, when plants were subjected to water deficit, AM plants presented the highest ΔFv/Fm' value, being significantly greater than in non-AM plants.

DISCUSSION

In the last years, a number of experimental evidences have shown that the AM symbiosis alters several aquaporin isoforms in the host plant and that these isoforms can transport water and other solutes of physiological importance (Barzana *et al.* 2014, Quiroga *et al.* 2017). It has been also demonstrated that the AM symbiosis regulates root hydraulic conductivity in different plant species, including maize (Sánchez-Blanco *et al.* 2004; Aroca *et al.* 2008; Ruiz-Lozano *et al.* 2009; Bárzana *et al.* 2012, 2014; Quiroga *et al.* 2017, 2018). The osmotic root hydraulic conductivity (Lo) is considered as an estimation of water flow via the cell-to-cell pathway, and is highly related to the activity or density of water channels in the plasma membrane (Chaumont & Tyerman 2014; Fox *et al.* 2017). Some studies have suggested that increases in root hydraulic conductivity in mycorrhizal roots could be the result of increased cell-to-cell water flux mediated by aquaporins (Marjanović *et al.* 2005; Lee *et al.* 2010; Ruiz-Lozano & Aroca 2017). However, the intimate mechanisms of such an effect are not well-understood. Indeed, the described increase in Lo in AM plants may be due to positive regulation of abundance and activity of the host aquaporins, (Chaumont &

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Tyerman 2014; Fox *et al.* 2017) or to changes in size or density of plasmodesmata in AM roots, as described by Blee & Anderson (1998), since symplastic water movement via plasmodesmata may also significantly contribute to Lo (Galmés *et al.* 2007). Thus, in this study we aimed to determine whether the AM symbiosis could improve the root cell membrane water permeability, possibly mediated by modification of aquaporins activity/abundance and if the expected changes in root cell water permeability have a significant effect on plant physiology and performance under water deficit. For that, two independent experiments were conducted with the same design, maize cultivar, AM inoculum and water deficit level, although the growing conditions during the last three weeks varied slightly. In any case, the trend of plant biomass production in response to water deficit and the percentage of mycorrhizal root colonization in both experiments were similar. Thus, water deficit reduced plant biomass by about 40% in non-AM plants and by 30-40% in AM plants. The stomatal conductance (gs) showed a higher variation in both experiments. In experiment 1 gs declined by 38% and 49% in non-AM and AM plants, respectively, while in experiment 2 the declined was by 69% and 40%, respectively. However, these fluctuations of gs values were likely due to the differences in the growing conditions during the last three weeks in the greenhouse or in the controlled environmental chamber and can be considered within a similar range, indicating that the water deficit imposed was effective and of similar intensity in both experiments. As a probe of that the expression of the stress marker gene *ZmNCED1* was considerably up-regulated in both experiments due to the water deficit imposed, regardless of mycorrhizal inoculation (Figure 6).

Mycorrhization enhanced the cell water permeability of root cortex cells under water deficit conditions

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Previous studies demonstrated an improved whole root hydraulic conductivity under water deficit in presence of a mycorrhizal fungus (Ruiz-Lozano & Aroca 2017), but, to our knowledge, this is the first time that an enhancement of the root cell water permeability was measured both in intact cortex cells and protoplasts from AM plants. Droughted-AM maize plants maintained P_f levels observed in non-stressed plants, while these levels declined drastically in the absence of the AM fungus (Figures 1 and 2a). Interestingly, P_f values higher than 12 $\mu m\ s^{-1}$ were found only in protoplasts extracted from AM plants (Figure 2b), revealing the higher water permeability of AM root cells, as compared to non-AM ones.

It is noteworthy that the duration of the protoplast isolation process (4 h) could affect the gene and protein expression or the regulation of aquaporin activity in the membranes, leading to low P_f values (1-17 $\mu m\ s^{-1}$) compared to the pressure probe P_f values (10-100 $\mu m\ s^{-1}$) inferred from L_{pc} data. These discrepancies were previously observed using the two techniques in different cell types (Volkov *et al.* 2007; Moshelion *et al.* 2009). Furthermore, the pressure probe assay integrates the elastic properties of the cell wall (ε), while this information is lacking on isolated protoplasts. The higher accuracy of the pressure probe technique for measuring hydraulic parameters *in planta* may explain the greater differences observed by this method among treatments, compared to the protoplast swelling assay. In any case, it is remarkable that the enhanced L_{pc} and P_f values in AM plants as compared to non-AM plants are observed just under water deficit conditions, when root water mobilization is crucial for plant performance. Besides, the P_f reduction observed in AM plants compared to non-AM under well-watered conditions using the pressure probe could be related to the inactivation of aquaporins during the transpiring conditions (Figure 1), when the apoplastic flow is predominant, as proved in mesophyll cells (Morillon & Chrispeels 2001). In consequence, mycorrhizal plants could have higher apoplastic water flow compared to non-

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inoculated plants under these conditions, as it has been already reported for maize roots colonized by the same mycorrhizal fungus (Bárzana *et al.* 2012).

The $T_{1/2}$ parameter is considered to be a direct measure of hydraulic conductivity ($T_{1/2}$~ 1/ L_{pc}) at constant ε (Wan *et al.* 2004). In our study, only non-AM plants under water deficit presented significantly higher half-times of water exchange (~5 s compared to ~3 s in the other treatments, Table 2). The higher differences observed in L_{pc} values can be due to the differences in $T_{1/2}$ and ε among treatments, as cell volume (data not shown) and P (Table 2) were unaltered. AM plants had similar ε values regardless of the water treatment. However, in non-AM plants, the water deficit treatment increased ε compared to well-watered plants, enhancing the cell resistance to be deformed and produce changes in cell volume upon pressure application (Zimmermann 1989), evidencing a dynamic control of the cell elastic properties during the water stress (Tomos & Leigh 1999) that is modified by root AM fungal colonization.

Are aquaporins responsible for the P_f and L_{pc} changes in AM roots?

Several aquaporin isoforms were analyzed in this study, in order to determine if protein or mRNA levels correlated with the observed changes in cell water permeability. The genes *ZmPIP2;2* and *ZmPIP2;6* presented significant differences among treatments (Figure 5a) and were down-regulated by mycorrhization either under well-watered conditions, as previously evidenced by Bárzana *et al.* (2014). However, in AM plants both *ZmPIP2;2* and *ZmPIP2;6* had enhanced mRNA levels under water deficit conditions as compared to well-watered conditions. ZmPIP2;2 and ZmPIP2;6 have a high water transport capacity when expressed in *Xenopus* oocytes (Bárzana *et al.* 2014; Moshelion *et al.* 2009) and are therefore strong candidates for the reported changes in P_f. Although ZmPIP2;2 and ZmPIP2;6 showed similar trends compared to P_f and L_{pc} results, differences due to fungal

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inoculation after water deficit treatment were not significant, thus being not possible to explain the increased cell permeability of AM plants only by their mRNA expression levels. However, other aquaporin isoforms could be playing a prevalent role in the enhanced P_f of the AM plants during water deficit. In this line, AM fungal aquaporins need to be also considered, even if their contribution to the plant water transport is not well understood yet (Lehto & Zwiazek 2011). Among the three identified aquaporins in *R. irregularis*, only *GintAQPF2* showed a significant increased mRNA expression during water deficit (Figure 5b). This isoform has been described to feature high water transport capacity (Li *et al.*, 2013) and may have accounted for the enhanced L_{pc} values in AM root cells under water deficit. Using antisera raised against ZmPIP2;1/2;2, ZmPIP2;5, ZmPIP2;6 and ZmPIP1s we observed a decrease of protein levels (only significant for ZmPIP2;2 and ZmPIP2;6) in the membranes of AM plants compared to non-AM regardless of the water treatment (Figure 3). These results do not follow the same pattern described for mRNA expression levels or water permeability results. However, it is well known that changes in mRNA expression do not always translate into protein levels, as post-transcriptional and post-translational are common regulatory mechanisms (Baerenfaller *et al.* 2008). In fact, the mRNA level of a gene is not, necessarily strictly, related to the abundance and activity of a protein in a cell or tissue (Fox *et al.* 2017) and some previous studies did not found a correlation between aquaporin mRNA level and protein abundance (Aroca *et al.* 2005; Boursiac *et al.* 2005).

Gating of aquaporins through different mechanisms, one of them being the phosphorylation of some residues, could represent a fast way to respond to environmental stressful situations like water stress (Moshelion *et al.* 2009). We checked accumulation of phosphorylated PIP2s since aquaporin activity can be regulated by phosphorylation events and it has been previously shown that the phosphorylation of PIP2 aquaporins at Ser-280 and Ser-283 was linked to the regulation of hydraulic conductivity in plants (Prado *et al.*

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2013). Interestingly, during water deficit stress, AM plants increased phosphorylation levels of PIP2 at Ser-280, Ser-283 and Ser-280/283 (Figure 4), suggesting a higher activity of PIP2 aquaporins and overcoming their lower abundance in the AM membranes. Apart from phosphorylation, several other posttranscriptional regulation mechanisms not studied here have been demonstrated to affect the channel abundance and activity of aquaporins, such as heteromerization, interactions with syntaxin SYP121, protonation, pressure gradient, methylation, glycosylation, ubiquitination and Ca^{2+} concentration (Chaumont *et al.* 2005; Santoni 2017). These regulation mechanisms, together with the trafficking of each aquaporin to its target membrane, have high influence on the aquaporin water transport capacity and must be considered as they are continuously used by plants to regulated membrane permeability and are very often responsible of discrepancies between aquaporin expression data and biophysical measurements of water permeability (Chaumont & Tyerman 2014). For instance, Grondin *et al.* (2016), working with six rice varieties showed that drought stress decreased the aquaporin expression, while the contribution of aquaporins to root hydraulic conductivity increased. Prado *et al.* (2013) worked with the four most highly expressed PIP isoforms in Arabidopsis (PIP1;2, PIP2;1, PIP2;6, and PIP2;7), either in wildtype (Col-O) plants or in knockouts lines silenced individually for these PIP isoforms. They observed that, regardless of the gene expression level, light regime did not have any effect on their protein abundance, while phosphorylation of PIP2;1 critically enhanced leaf hydraulic conductivity in response to light.

In any case, it must be also taken into account that L_{pc} and P_f values were measured on isolated cortical cells. Some of these cells may be colonized by the AM fungus and other not colonized. The lack of a clear correlation between L_{pc} and P_f values with aquaporin gene expression or protein accumulation patterns may be due to the fact that L_{pc} and P_f were measured on isolated cells, while gene and protein levels were measured on the whole root

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tissue. We do not know yet if the mycorrhizal effect on cell water transport is local or systemic. But, if the effect of the AM symbiosis is not systemic it may be diluted in the whole root system and this must be elucidated in future studies.

In summary, under well-watered conditions we observed a downregulation of certain aquaporins in AM plants (*ZmPIP2;2* and *ZmPIP2;6*) as well as PIP2 phosphorylation levels, whereas when AM plants were submitted to water deficit *ZmPIP2;2, ZmPIP2;6* and the fungal *GintAQPF2* genes were induced and there was a general increase in phosphorylation levels, which may translate into a higher water channel activity in these plants.

Mycorrhizal plants showed a higher photosynthetic efficiency under water deprivation

Plants need a constant water flow, starting from the absorption of water from soil by roots and its distribution throughout the plant body and evaporation in the atmosphere, in order to carry out all their physiological activities, especially the photosynthesis (Afzal *et al.* 2016). Thus, under water deficit conditions, the higher or lower capacity for water mobilization in roots in order to maintain transpiration must have an important influence on the shoot photosynthetic capacity. In this study we aimed to elucidate if the altered root cell water permeability in AM plants translate into altered gas exchange capacity and photosynthetic efficiency. Therefore we measured gas exchange parameters and contrasted them as both fixed CO_2 concentration and performing An/Ci curves in order to determine if the alteration of photosynthesis was due to the greater water availability, larger stomatal conductance, or to the higher enzymatic capacity.

Net photosynthetic rate (An) depends on internal CO_2 concentration (Ci) in the leaf, which is linked to the water evaporation rate. Hence, the reduction of stomatal conductance caused by water deficit may reduce CO_2 fixation (Chaves *et al.* 2009). However, in this study AM fungal inoculation increased An under water deficit conditions. This effect could be

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related to improved N and P nutrition in AM plants, as has been widely described for this symbiosis (Varma *et al.* 2008). However, AM plants also exhibited greater gs values (Table 4), suggesting an AM-enhanced photosynthetic efficiency due to higher water availability for transpiration. Moreover, the calculated maximum carboxylation capacity of PEPc (Vpmax) and CO_2-saturated photosynthetic rate (Vmax) values (Figure 7) revealed the higher photosynthetic capacity of AM plants. At the end, this is reflected in the bigger size of mycorrhizal-droughted plants (Table 3) compared to non-inoculated ones. The enhanced activity of enzymes involved in CO_2 fixation due to mycorrhizal inoculation was previously described. Chen *et al.* (2017) found a higher activity of key Calvin cycle enzymes in mycorrhizal cucumber plants, and a higher Rubisco activity was also found in grapevine for droughted-AM plants (Valentine *et al.* 2006) or in rice for AM plants subjected to salinity (Porcel *et al.* 2015). In our case, enhanced photosynthesis was due to both, decreasing stomatal limitation due to higher water availability and via the improvement of photosynthetic enzymatic apparatus.

SLA is a crucial leaf characteristic determining photosynthetic and hydraulic behaviour in plants (Zhu *et al.* 2013). Mycorrhizal plants from both treatments presented lower SLA values (Table 3), meaning thicker leaves. Barros *et al.* (2018) had similar results with mycorrhizal plants after few days of water stress. This can be interpreted as an hydraulic strategy to surpass the stress, as the decreased leaf area per leaf matter would be more efficient controlling water loss under water deficit conditions (Erice *et al.* 2010). Moreover, the lower SLA may be related to higher leaf protein concentration and linked to the enhancement of the photosynthetic capacity.

CONCLUSIONS

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The results of this study give a better understanding of the role of mycorrhizal symbiosis on root water conductivity, demonstrating that under water deprivation AM inoculated plants enhance root cells water permeability by increasing L_{pc} and P_f values, in order to overcome water deficit. Under these conditions, the AM symbiosis differentially regulates plant aquaporins, increasing the phosphorylation status of PIP2s during water deficit, which may mean a higher activity of their water channels. The better performance of root cells in water transport is connected to the physiological status of the shoot. AM plants displayed higher photosynthetic capacity thanks to an increased PEPc activity and CO_2-saturated photosynthetic rate. Altogether, we demonstrated the systemic benefits of the AM symbiosis for the tolerance of maize crop during water deficit episodes.

CONFLICT OF INTEREST STATEMENT:

Authors declare that they have no conflict of interest.

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Table 1. Percentage of mycorrhizal root length, plant fresh weight and stomatal conductance (gs) in maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD).

Experiment 1			
	Mycorrhization (%)	**Plant FW (g plant^{-1})**	**gs (mmol H_2O m^{-2} s^{-1})**
WW non-AM	**n.d.**	**59.5 ± 4.4 a**	**73.1 ± 8.4 b**
WW AM	**57.3 ± 5.4 a**	**57.6 ± 2.1 a**	**99.3 ± 5.4 a**
WD non-AM	**n.d.**	**35.1 ± 2.3 b**	**45.6 ± 4.9 c**
WD AM	**51.7 ± 6.6 a**	**34.0 ± 1.0 b**	**50.4 ± 3.4 c**
Mycorrhiza (M)		**ns**	**ns**
Water Regime (W)		*******	*******
M x W		**ns**	**ns**

Data represent the means of three values ± SE for mycorrhization, twelve values ± SE for plant FW and six values ± SE for gs. Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Different letter indicates significant differences between treatments ($p < 0.05$) based on t-test for mycorrhization and on Duncan's test for the other parameters. n.d. non detected.

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Table 2. Water relation parameters of intact root cortex cells of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD).

Experiment 1			
	Cell turgor pressure	**Cell wall elastic modulus**	**Half-time of water exchange**
	P (Mpa)	**ε (Mpa)**	**$T_{1/2}$ (s)**
WW non-AM	**0.29 ± 0.03 a**	**2.44 ± 0.5 c**	**3.03 ± 0.5 b**
WW AM	**0.36 ± 0.02 a**	**5.26 ± 0.6 b**	**3.34 ± 0.2 b**
DS non-AM	**0.33 ± 0.04 a**	**8.16 ± 1.8 a**	**5.44 ± 0.7 a**
DS AM	**0,29 ± 0.03 a**	**4.86 ± 0.9 bc**	**3.17 ± 0.3 b**
Mycorrhiza (M)	**ns**	**ns**	**ns**
Water Regime (W)	**ns**	******	*****
M x W	**ns**	*******	******

Data represent the means of ten to fifteen values ± SE (three different plants). Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test.

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Table 3. Percentage of mycorrhizal root length, plant fresh weight (FW), shoot area, specific leaf area (SLA) and in maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD).

Experiment 2				
	Mycorrhization (%)	**Plant FW (g plant^{-1})**	**Shoot area (cm^2)**	**SLA (cm^2 g^{-1})**
WW non-AM	**n.d.**	**33.2 ± 2.0 a**	**302.8 ± 7.9 b**	**408.8 ± 5.4 a**
WW AM	**58.0 ± 4.5**	**34.1 ± 1.2 a**	**343.5 ± 16.7 a**	**330.3 ± 6.1 b**
WD non-AM	**n.d.**	**20.1 ± 0.9 c**	**157.7 ± 8.9 d**	**401.5 ± 14.0 a**
WD AM	**52.3 ± 3.3**	**24.0 ± 0.8 b**	**227.1 ± 14.9 c**	**316.5 ± 10.4 b**
Mycorrhiza (M)		**ns**	*****	******
Water Regime (W)		*******	*******	**ns**
M x W		**ns**	**ns**	**ns**

Data represent the means of three values ± SE for mycorrhization, twelve values ± SE for SFW, six values ± SE for EL and five values ± SE for shoot area and SLA. Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Different letter indicates significant differences between treatments ($p < 0.05$) based on t-test for mycorrhization and on Duncan's test for the other parameters. n.d. non detected.

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Table 4. Net photosynthesis (An), stomatal conductance (gs), intercellular CO_2 concentration (Ci) and photosystem II efficiency in the light-adapted state (ΔFv/Fm') of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD).

	Experiment 2			
	An ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	**gs ($mmol\ H_2O\ m^{-2}\ s^{-1}$)**	**Ci $\mu mol\ mol^{-1}$**	**ΔFv/Fm'**
WW non-AM	**15.9 ± 0.7 a**	**123.5 ± 10.2 a**	**153.2 ± 8.4 a**	**0.43 ± 0.03 bc**
WW AM	**15.5 ± 0.8 a**	**121.7 ± 7.7 a**	**157.4 ± 6.2 a**	**0.50 ± 0.03 ab**
WD non-AM	**6.5 ± 0.8 c**	**37.8 ± 3.5 c**	**105.4 ± 18.4 b**	**0.40 ± 0.04 c**
WD AM	**11.4 ± 1.3 b**	**72.1 ± 12.5 b**	**107.4 ± 16.9 b**	**0.56 ± 0.02 a**
Mycorrhiza (M)	**ns**	**ns**	*******	*******
Water Regime (W)	******	*******	*******	**ns**
M x W	**ns**	**ns**	*******	**ns**

Data represent the means of five values ± SE. Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test for the other parameters.

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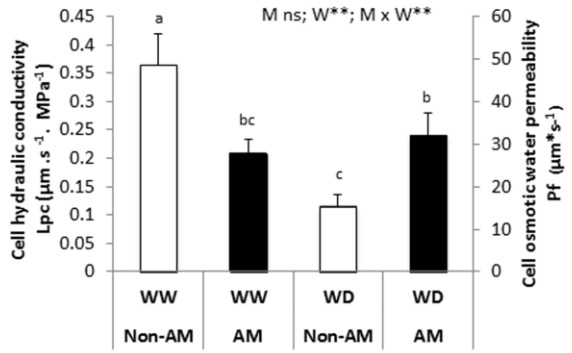

Figure 1. Experiment 1. Cell hydraulic conductivity (L_{pc}) and inferred P_f values of intact root cell of plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD), determined with the cell pressure probe. Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Values (mean ± SE for >15 cells) are given. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test.

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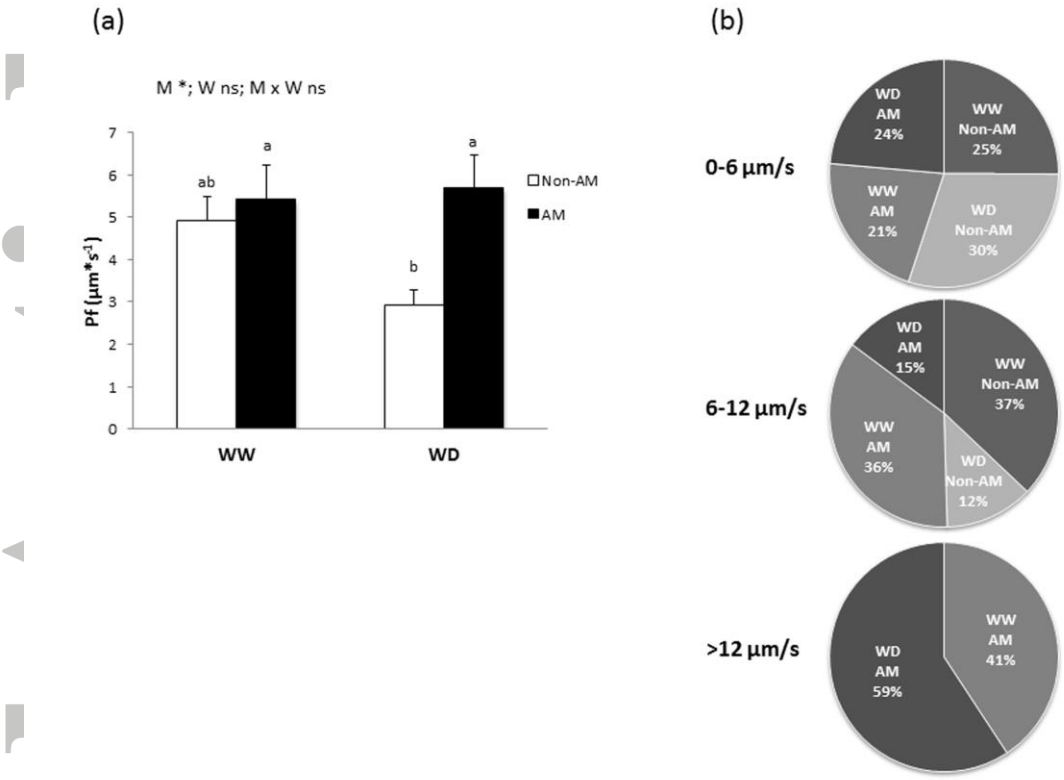

Figure 2. Experiment 1. **(a)** Osmotic water permeability coefficient (Pf) of isolated protoplasts from roots of plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data show the mean ± SE for 30 cells (at least three different plants). Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Different letter indicates significant differences between treatments (p<0.05) based on Duncan's test. **(b)** Percentage of protoplast of plants from the different treatments classified per range of Pf.

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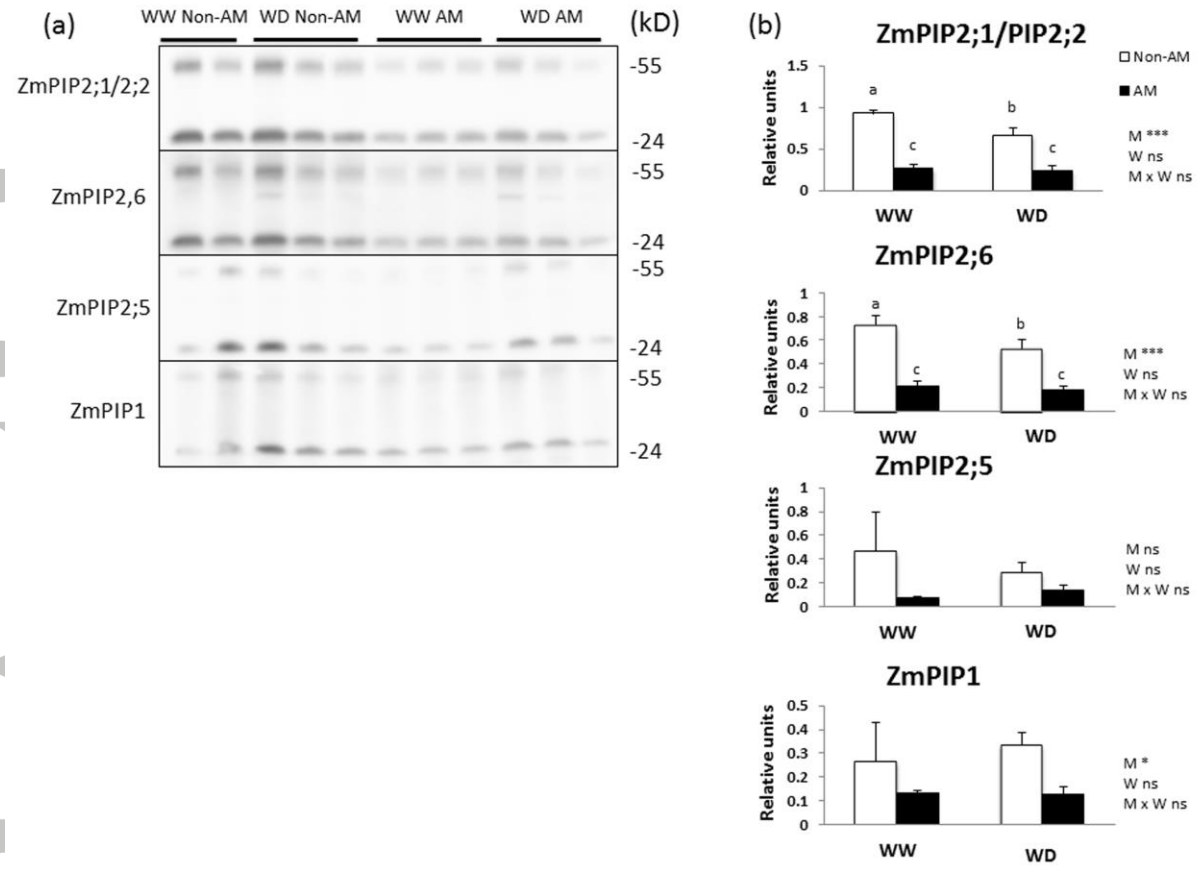

Figure 3. Experiment 1. **(a)** Accumulation of ZmPIP proteins in the microsomal fraction of roots from plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). The position of the molecular mass markers is indicated. **(b)** Quantification of signals on membranes (relative units, normalized to Commassie blue gel signal). Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Data show the mean ± SE for three biological replicates. Different letter indicates significant differences between treatments (p<0.05) based on Duncan's test.

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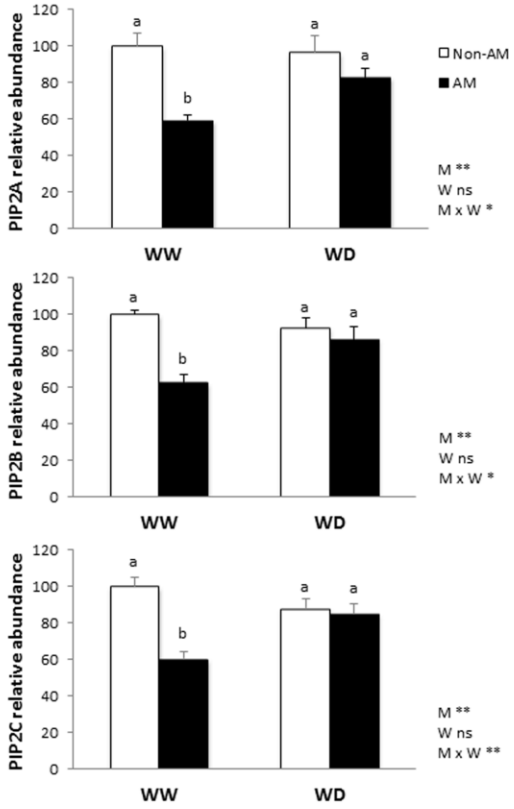

Figure 4. Experiment 1. PIP2A (Ph-Ser280), PIP2B (Ph-Ser283) and PIP2C (Ph-Ser280/Ser283) relative protein abundance in the microsomal fraction of roots from plants inoculated (black bars) or not (white bars) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Data were obtained by ELISA and show the mean ± SE for three biological replicates. Different letter indicates significant differences between treatments (p<0.05) based on Duncan's test.

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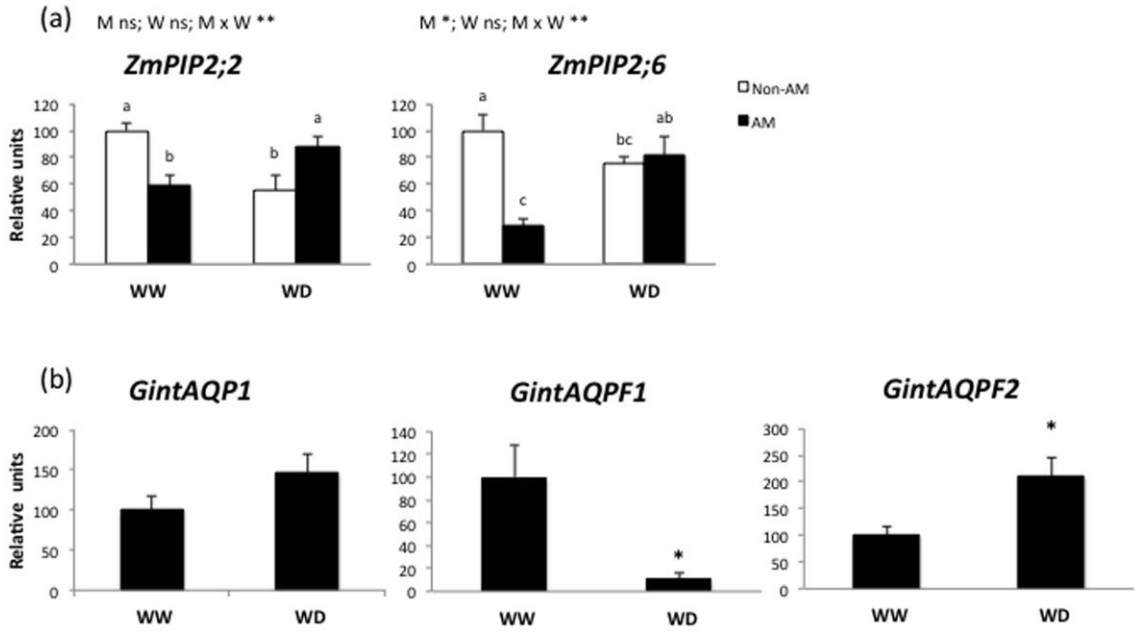

Figure 5. Experiment 1. **(a)** Relative mRNA levels of *ZmPIP2;2* and *ZmPIP2;6* normalized to *Zmtubulin* and *ZmGAPDH* genes. Plants were inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Data indicates the mean ± SE for three biological replicates. Different letter indicates significant differences between treatments (p<0.05) based on Duncan's test. **(b)** Relative mRNA levels of *GintAQP1*, *GintAQPF1* and *GintAQPF2* normalized to fungal EF 1α gene. Data indicates the mean ± SE for three biological replicates. Different letter indicates significant differences between treatments (p<0.05) based on t-test.

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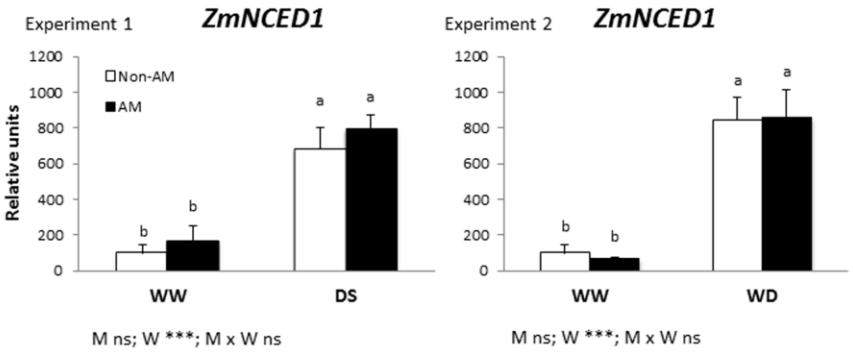

Figure 6. Relative mRNA levels of *ZmNCED1* gene normalized to *Zmtubulin* and *ZmGAPDH* genes. Plants were inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Data indicates the mean ± SE for three biological replicates. Different letter indicates significant differences between treatments (p<0.05) based on Duncan's test.

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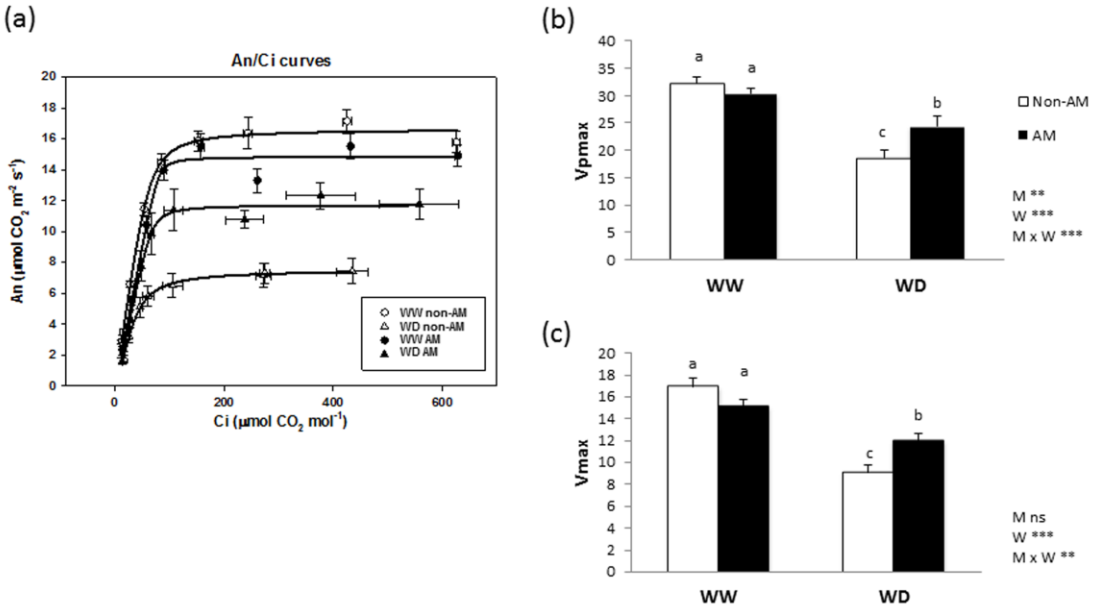

Figure 7. Experiment 2. **(a)** An/Ci curves, **(b)** Maximum carboxylation capacity of PEPC (Vpmax) and **(c)** CO_2-saturated photosynthetic rate (Vmax) of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analyzed by two-way ANOVA with mycorrhiza (M), water regime (W) and their interaction (M x W) as sources of variation. Significance of sources of variation were evaluated by P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001. Data show the mean ± SE for five biological replicates. Different letter indicates significant differences between treatments (p<0.05) based on Duncan's test.

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