**Contribution of the arbuscular mycorrhizal symbiosis to the regulation of radial root
water transport in maize plants under water deficit**

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Abstract

In roots, water flows radially through three parallel pathways: apoplastic, symplastic and transcellular (the last two referred as the cell-to-cell), with a different contribution depending on the environmental conditions. Thus, during drought, the cell-to-cell pathway, which is largely regulated by aquaporins, dominates. While it is accepted that water can flow across roots following the apoplastic, symplastic and transcellular pathways, the relative contribution of these pathways to whole root hydraulic conductivity is not well established. In addition, the symbiosis with arbuscular mycorrhizal (AM) fungi was reported to modify root water transport in host plants. This study aims to understand if the AM symbiosis alters radial root water transport in the host plant and whether this modification is due to alteration of plant aquaporins activity or amounts and/or changes in apoplastic barriers. Hence, the combined effect of mycorrhizal fungus, water deficit and application of the aquaporin inhibitor sodium azide (NaN_3) on radial root water transport of maize plants was analyzed. The development of Casparian bands in these roots was also assessed. NaN_3 clearly inhibited osmotic root hydraulic conductivity (L_o). However, the inhibitory effect of sodium azide on L_o was lower in AM plants than in non-AM plants, which together with their higher relative apoplastic water flow values suggests a compensatory mechanism for aquaporin activity inhibition in AM plants, leading to a higher hydrostatic root hydraulic conductivity (L_{pr}) compared to non-AM plants. This effect seems to be related to the mycorrhizal regulation of aquaporins activity through posttranslational modifications. The development of Casparian bands increased with drought and AM colonization, although this did not decrease water flow values in AM plants. The work provides new clues on the differential mycorrhizal regulation of root water transport.

Keywords: arbuscular mycorrhizal symbiosis; aquaporins; root hydraulic conductivity; sodium azide

1. Introduction

In plants, the water status of the shoot is determined by the root resistance to water flow, which is the highest within the soil-plant-atmosphere continuum (SPAC) (Steudle and Peterson, 1998). Within the SPAC, the balance between water uptake and water loss is finely tuned between root hydraulic properties and stomatal control in leaves. Thus, according to the demands of the shoot, root water supply can be adjusted (Kim et al., 2018). Roots are the first organs to sense drought in soil, as it starts with a decrease in soil water potential. Thus, they play a crucial role in the response to dehydration (Zingaretti et al., 2013). Drought is a major constraint in crop production at global scale, and expected to increase in coming years (Lesk et al., 2016). Therefore, studies on the effect of water deprivation in plant roots are extremely important. In the case of maize, a staple crop worldwide whose yield is heavily affected by this constraint (Daryanto et al., 2016), understanding the mechanisms of drought tolerance seems essential.

Water must flow radially across a series of concentric cell layers in the root to move from soil into the vascular tissues. These layers are the epidermis, the exodermis (not always present), one or several layers of cortex cells, the endodermis, the pericycle, the xylem parenchyma cells, and, finally, the vessels (Hachez et al., 2006a). In this radial movement, water and nutrients obtained from soil are translocated to the vascular tissues by three major routes, apoplastic, symplastic and transcellular (the last two referred to as cell-to-cell), following a hydrostatic (bulk) or osmotic gradient. This radial transport was best described by the composite transport model (Steudle and Peterson, 1998), although subsequent studies have shown new aspects to be considered, such as the contribution of the serial radial pathways (cortex, endodermis) alongside to the parallel components and the development and composition of apoplastic barriers in root tissues (Schreiber et al.,

2005; Meyer et al., 2011; Ranathunge et al., 2017; Kreszies et al., 2019; Wang et al., 2019). Depending on the environmental conditions, the relative contribution of each pathway to overall water uptake or hydraulic conductivity may change substantially (Steudle, 2000, 2001; Hachez et al., 2006a; Vandeleur et al., 2009). Moreover, under drought conditions, root hydraulics is adjusted by switching between the cell-to-cell and apoplastic pathways, depending on the driving forces (Ranathunge et al., 2004; Barberon, 2017). According to this, under transpiring conditions (i.e. in the day with normal water supply), the hydrostatic pressure gradient would dominate the transport of water and solutes, increasing the contribution of the apoplastic pathway. Apoplastic barriers in endodermal and exodermal cell walls can block this water transport pathway (Kreszies et al., 2018). In the absence of transpiration (i.e. in the case of drought stress), the osmotic gradient would govern water and solutes transport following the cell-to-cell pathway (Kim et al., 2018). It is currently known that all these pathways are interconnected and operate in combination along plant tissues, producing a system with series and parallel resistances, so that water moves by a combination of hydraulic and osmotic forces that explain the deviations from the original model of root water movement (Steudle and Peterson, 1998; Knipfer and Fricke, 2010; Fritz and Ehwald, 2011).

The water transport capacity of the root system (root hydraulic conductivity; L_{pr}) is regulated in a large proportion by aquaporins (Tournaire-Roux et al., 2003; Vadez, 2014) that contribute to the transcellular water flux. These proteins are small channels that allow the passage of water and small molecules through the membranes of most living organisms. In vascular plants they constitute a large family (>30 members) subdivided in the following subfamilies: PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin 26-like intrinsic proteins) and SIPs (small basic intrinsic proteins) (Chaumont et al., 2001; Maurel et al., 2015). Some plants contains also the

94 uncharacterized XIPs (X intrinsic proteins) (Gupta and Sankararamakrishnan, 2009),
95 which are not present in maize. Under adverse environmental conditions, aquaporins
96 appear to have a key role in the regulation of plant water balance (Kaplan et al., 2018),
97 affecting important parameters such as the root hydraulic conductivity (Hachez et al.,
98 2006b). The use of aquaporin inhibitors may provide, thus, information on the relative
99 participation of cell-to-cell and apoplastic paths in the whole-root water uptake. Several
100 inhibitors of aquaporin activity have been used to this purpose, being mercurials the most
101 widely used. Hg causes conformational changes in the protein leading to aquaporin pore
102 blockage and inhibition of water transport (Niemietz and Tyerman, 2002). Nevertheless,
103 Hg has been reported to have a large number of collateral effects that causes indirect
104 inhibition of cellular metabolism (Kamaluddin and Zwiazek, 2001; Niemietz and Tyerman,
105 2002; Maurel et al., 2008). Sodium azide has also been commonly used to inhibit
106 aquaporin activity and Fitzpatrick and Reid (2009) compared sodium azide (0.5 mM) and
107 butyric acid (10 mM) and observed that sodium azide was more efficient than butyric acid
108 inhibiting the activity of aquaporins. Such an effect was related to the fact that azide has a
109 dual effect: it causes acidification of cytoplasm but also inhibits the phosphorylation
110 process, both effects contributing to close the aquaporin channel (Tournaire-Roux et al.,
111 2003).

112 Arbuscular mycorrhizal (AM) symbiosis occurring between soil fungi from the
113 subphylum Glomeromycotina and most plant roots enhance water and nutrient uptake
114 from soil due to a vast mycelial network that can access further than the root depletion
115 zone in the rhizosphere (Smith and Read, 2008). Inside the plant, they provide numerous
116 benefits to plant physiology, the most evident being the stimulation of plant growth and
117 improved mineral nutrition (Azcón-Aguilar and Barea, 2015). In addition, they have the
118 ability to improve plant performance under different abiotic stresses such as drought,

119 salinity, waterlogging or pollution (Lenoir et al., 2016). In the case of water deficit, the
120 enhancement of drought tolerance was reported in different plant species (Ortiz et al.,
121 2015; Chitarra et al., 2016; Ruiz-Lozano et al., 2016; Quiroga et al., 2017). Under these
122 conditions, but also when the plant is well irrigated, AM symbiosis was found to differently
123 regulate root water transport, generally inducing a rise in L_{pr} (Aroca et al., 2007; Bárzana
124 et al., 2012, 2014, 2015; Quiroga et al., 2017).

125 While it is accepted that water can flow across roots following the apoplastic,
126 symplastic and transcellular pathways, the relative contribution of these pathways to whole
127 root hydraulic conductivity is not well established. Moreover, results by Knipfer and Fricke
128 (2010) in barley emphasize that membranes (and aquaporins) are control points for radial
129 water transport in roots and question the well accepted idea that low-resistance apoplastic
130 pathway of water movement driven by hydrostatic gradients is required in roots to meet the
131 transpirational water demand of the shoot. Furthermore, AM fungi were suggested to
132 modulate the switching between water transport pathways in roots (Bárzana et al., 2012),
133 which would provide higher flexibility to these plants to cope with water stress. This
134 mycorrhizal water regulation could be in part mediated by the regulation of aquaporins, as
135 it was found in several species, including maize (Bárzana et al., 2014; Quiroga et al.,
136 2017). However, this aspect requires a more in deep investigation to elucidate the
137 mechanisms and the conditions under which this could occur.

138 Therefore, the aim of this investigation was to determine if the AM symbiosis alters
139 the routes of radial water movement in the root of the host plant. We hypothesize that this
140 may be achieved by the regulation of the direct water supply to the plant via fungal hyphae
141 and that this effect may be mediated by changes in the host plant aquaporins activity or
142 amounts, as well as, by changes in apoplastic barriers. Hence, the combined effect of the
143 presence of a mycorrhizal fungus, water deficit and application of the aquaporin inhibitor
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sodium azide (NaN_3) on radial root water transport and aquaporins accumulation and phosphorylation was studied in maize plants. We also analyzed the development of Casparian bands in these roots. Unravelling the mechanisms by which the mycorrhizal symbiosis governs water movements in roots is a step forward in the understanding of the AM-induced drought tolerance.

2. Materials and methods

2.1. Experimental design

The experiment consisted of a factorial design with three factors: (1) watering treatment, so that half of the plants were grown under well-watered (WW) conditions throughout the entire experiment and the other half was subjected to drought stress (DS) for 15 days before harvest; (2) inoculation treatment, with non-inoculated control plants (Non-AM) and plants inoculated with the AM fungus *Rhizophagus irregularis*, strain EEZ 58 (AM); (3) chemical treatment, so that sodium azide (NaN_3) was added 30 minutes before harvest to half of the plants, resulting in eight different treatments with fifteen replicates per treatment ($n=15$), giving a total of 120 plants.

2.2. Soil and biological materials

The growing substrate consisted of a mixture of soil and sand (v/v 1:9). 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). The original soil had a pH of 8.1 (water); 0.85% organic matter, nutrient concentrations (mg kg^{-1}

¹): N, 1; P, 10 (NaHCO₃-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. from the drought-sensitive cultivar PR34B39 were provided by Pioneer Hi-Bred (Spain) and used in previous studies (Quiroga et al., 2017; 2018). Two seeds were sown in 1.5 L pots containing 1250 g of the substrate described above and thinned to one seedling per pot after emergence. At the time of planting, half of the plants were inoculated with ten grams of AM inoculum. *Rhizophagus irregularis* (Schenck and Smith), strain EEZ 58 was used as AM fungal inoculum. The inoculum consisted of soil, spores, mycelia and infected root fragments. Non inoculated plants received a 10 mL aliquot of a filtrate (<20 µm) of the AM inoculum in order to provide the natural microbial population free of AM propagules.

2.3. Growing conditions

Plants were grown for eight weeks under greenhouse conditions (25/20°C, 16/8 light dark period, 50-60% RH and average photosynthetic photon flux density 800 µmol m⁻² s⁻¹). They were irrigated three times per week with 50 mL of Hoagland nutrient solution (Hoagland and Arnon, 1950) modified to contain 25% P in order to avoid AM symbiosis inhibition. The same amount of water was applied on alternate days. A drought stress treatment was applied for the last 2 weeks, by irrigating plants with half the water/Hoagland volume of well-watered ones (25 mL vs. 50 mL). To avoid a combination of drought stress plus nutrient deficiency, for droughted treatments a 2X Hoagland nutrient solution was used, so that 25 mL provided the same nutrient levels as 50 mL of a 1X Hoagland nutrient solution used to irrigate well-watered plants. This water stress was similar to previous studies (Quiroga et al., 2017; 2018) and is considered as a severe

stress. Sodium azide (NaN_3) 2 mM was added to the nutrient solution and applied to half of the plants 30 minutes before harvesting. The exposure time and the concentration of the compound were established in preliminary tests.

2.4. Parameters measured

2.4.1. Biomass production

At harvest (8 weeks after sowing) the shoot and root system of eight replicates per treatment were separated and the dry weight (DW) measured after drying in a forced hot-air oven at 70 °C for 2 days.

2.4.2. Symbiotic development

Roots of maize were stained according to Phillips and Hayman (1970), in order to differentiate fungal structures. The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980) in five replicates per treatment.

2.4.3. Stomatal conductance

Stomatal conductance (g_s) was measured two hours after the onset of photoperiod in the second fully expanded youngest leaf from at least seven plants per treatment with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the manufacturer's recommendations. Measurements were taken one day before harvest, thus, before the NaN_3 treatment.

2.4.4. Leaf chlorophyll content

Leaf chlorophyll content was estimated four hours after sunrise using a Chlorophyll Content Measurement System CL-01 (SPAD, Hansatech Instruments Ltd., Norfolk, UK) on the second fully expanded youngest leaf for each plant. This device determines relative chlorophyll content using dual wavelength optical absorbance (620 and 940 nm wavelengths) measurements from leaves samples. Relative chlorophyll content was measured in 10 different plants per treatment after 8 weeks of growth and before the NaN_3 treatment.

2.4.5. 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 described in Quiroga et al. (2017, 2018) in the second fully expanded youngest leaf of 10 different plants of each treatment after 8 weeks of growth and before the NaN_3 treatment.

2.4.6. Hydrostatic root hydraulic conductivity (L_{pr})

The L_{pr} was determined at noon in seven plants per treatment with a Scholander pressure chamber, 30 minutes after NaN_3 application and following the method described by Bárzana et al. (2012). A gradual increase of pressure (0.2, 0.3 and 0.4 MPa) was applied at 2-minutes intervals to the detached roots. Sap was collected at the three pressure points. Sap flow was plotted against pressure, with the slope being the root hydraulic conductance (L) value. L_{pr} was determined by dividing L by root dry weight (RDW) and expressed as $\text{mg H}_2\text{O g RDW}^{-1} \text{ MPa}^{-1} \text{ h}^{-1}$.

2.4.7. Osmotic root hydraulic conductivity (L_o)

L_o was measured at noon on detached roots exuding under atmospheric pressure by the free exudation method (Benabdellah et al., 2009) and using eight plants per treatment. Under these conditions, water is only moving following an osmotic gradient. Therefore, the water would be moving through the cell-to-cell path (Steudle and Peterson, 1998). The exuded sap was collected after 2 hours and weighed. The osmolarity of the exuded sap and the nutrient solution was determined using a cryoscopic osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany) and used for L_o calculation, according to Aroca et al. (2007). L_o was calculated as $L_o = J_v/\Delta\Psi$, where J_v is the exuded sap flow rate and $\Delta\Psi$ the osmotic potential difference between the exuded sap and the nutrient solution where the pots were immersed. Measurements were carried out 30 minutes after applying NaN_3 .

2.4.8. Relative apoplastic water flow

Relative changes in apoplastic water flux among treatments were estimated using light green dye (light green SF yellowish; Sigma-Aldrich Chemical, Gillingham, Dorset; colour index 42095, molecular weight $792.85 \text{ g mol}^{-1}$), which has the ability to move apoplastically but not symplastically (López-Pérez et al., 2007). Fluorescent dyes may not precisely measure the total apoplastic water flux (Zimmerman and Steudle, 1998). However, they can be used to determine relative changes in the apoplastic water transport of plants from different treatments (Voicu and Zwiazek, 2004; Bárzana et al., 2012; Quiroga et al., 2018). Thus, the relative apoplastic water flow was calculated as explained in Quiroga et al. (2018), using eight plants per treatment. Briefly, 30 min after NaN_3 application, detopped root systems were immersed in $250 \text{ }\mu\text{M}$ light green solution

inside the pressure chamber and kept in this solution during measurement. Sap was collected after 2 min at 0.2, 0.3 and 0.4 MPa in a Scholander pressure chamber. At the end, the absorbance of the whole collected sap was determined immediately at 630 nm. The average baseline absorbance value in the nutrient solution before addition of the dye was subtracted to the values obtained after adding the dye and in the collected sap. The percentage of apoplastic pathway was calculated from the ratio between the absorbance in the sap flow and in the nutrient solution. The concentration of dye in the nutrient solution of each treatment was considered to be 100%.

2.4.9. Apoplastic barriers

To detect the development of Casparian bands, hand-cut sections from fresh root tissue were taken at 50 mm of root tips and stained for 1 h with 0.1% (w/v) berberine hemisulfate and for 45 min with 0.5% toluidine blue (w/v) (Brundrett et al., 1988; Hachez et al., 2006a; Kreszies et al., 2019), then mounted in 0.1% FeCl₃ in 50% glycerol. Sections were immediately examined under an epifluorescence microscope with A filter (excitation at 340-380 nm, emission at 425 nm). The same sections were also used to detect autofluorescence of lignified tissues, using the filter setup (UV illumination) as employed for berberine hemisulphate-stained sections.

2.4.10. Aquaporins abundance and PIP2s phosphorylation status

Sub-cellular fractionation was performed according to Hachez et al. (2006a) with slight modifications. Pieces of intact roots were grinded with 6 mL 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 4°C. The homogenate was centrifuged during 10 min at 770 g and the supernatant obtained was centrifuged 10 min at 10000g. The

resulted supernatant was finally centrifuged during 30 min at 100000g and the final pellet (corresponding to the microsomal fraction) was resuspended in 20 µL of suspension buffer (5 mM KH₂PO₄, 330 mM sucrose, 3 mM KCl, pH 7.8) and sonicated twice for 5 s. Total protein amounts were quantified by Bradford analysis and abundance of specific proteins was measured by ELISA. A 2 µg aliquot of microsomal fraction was incubated at 4°C overnight in carbonate/bicarbonate coating buffer at pH 9.6. The next day, proteins were cleaned by 3x 10 min washes with Tween Tris-buffered saline solution (TTBS), and blocked with 1% Bovine serum albumin (BSA) on TTBS 1 hour at room temperature. After three more washes with TTBS, proteins were incubated with 100 µL of the primary antibody (1:1000 in TTBS v/v) for 1 hour at room temperature.

We used ten different primary antibodies, two antibodies that recognize several PIP1s and PIP2s, three antibodies that recognize the phosphorylation of PIP2 proteins in the C-terminal region: PIP2A (Ser-280), PIP2B (Ser-283) and PIP2C (Ser-280/Ser-283) (Calvo-Polanco *et al.* 2014), as well as antibodies recognizing ZmPIP2;1/2;2, ZmPIP2;4, ZmPIP2;5, ZmPIP2;6 and ZmTIP1;1 (Hachez *et al.*, 2006a). A goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma-Aldrich Co.) was used as secondary antibody at 1:10000.

2.5. Statistical analysis

Statistical analyses were performed in SPSS Statistics (Version 23, IBM Analytics). Data were analyzed by one-way ANOVA. Duncan's or T-Test were used to find out differences between means at $\alpha=0.05$.

3. Results

3.1. Plant biomass and symbiotic development

The application of sodium azide did not affect plant biomass and symbiotic development due to its short time of application (only 30 minutes). In contrast, AM colonization enhanced plant dry weight both under well-watered conditions (by 50%) and under drought stress (by 18%) (Figure 1). Drought stress reduced plant dry weight over 30% both in AM and non-AM plants.

The AM root colonization was about 60% of mycorrhizal root length, with no significant differences under well-watered and drought stress conditions (Table 1).

3.2. Stomatal conductance (gs)

The water stress imposed significantly decreased stomatal conductance in both non-mycorrhizal and mycorrhizal plants (in both cases more than 65% drop). However, inoculation with the mycorrhizal fungus caused a 1.8 and 1.6 fold increase respectively in stomatal conductance compared to non-inoculated plants regardless of the water treatment (Table 1).

3.3. Chlorophyll content and efficiency of photosystem II

Chlorophyll content was measured by SPAD and was reduced by drought stress in both AM and non-AM plants (Table 1). However, under drought stress conditions AM plants maintained higher values of chlorophyll content than non-AM plants (an increase of 24%).

The efficiency of photosystem II was significantly reduced by drought stress in non-AM plants only, while AM plants maintained similar values than under well-watered conditions (Table 1).

3.4. Hydrostatic root hydraulic conductivity (Lpr)

The AM symbiosis increased Lpr in maize plants, although the increase was statistically significant only under well-watered conditions (Figure 2). Moreover, under well-watered conditions, the application of sodium azide increased Lpr by 42% in *R. irregularis*-inoculated plants, but not in non-AM plants. Drought stress reduced Lpr in both treatments, regardless of sodium azide application. Under drought stress, the mycorrhization of maize roots tended to enhance Lpr, but the differences were not statistically significant.

3.5. Osmotic root hydraulic conductivity (Lo)

The mycorrhization increased considerably Lo values under well-watered conditions, both in absence and in presence of sodium azide (Figure 2). Under drought stress conditions the increase was statistically significant only in presence of sodium azide. Indeed, both under drought stress and under well-watered conditions, the application of sodium azide reduced significantly Lo in non-AM plants (t-student), but maintained similar values in AM plants. Thus, AM plants treated with sodium azide showed 4 to 5 fold higher Lo values compared to non-AM plants, regardless of the watering conditions.

The application of sodium azide affected not only the Lo values but also the number of plants exuding under these conditions. Indeed, under well-watered conditions, 100% of

the plants exuded spontaneously (both AM and non-AM), while sodium azide application reduced this percentage to 75% in non-AM plants but unaltered the percentage in AM plants. Under drought stress conditions, 38% of non-AM plants exuded spontaneously, while after sodium azide application only 13% of the plants got free exudation. In the case of AM plants, 100% of them exuded spontaneously under drought stress and this percentage was only reduced to 88% after application of sodium azide (data not shown).

3.6. Relative apoplastic water flow

Interestingly, the application of sodium azide increased the percentage of relative apoplastic water flow in AM and non-AM plants cultivated under well-watered conditions (Figure 2). However, under drought stress no significant differences were found. The mycorrhization itself also increased the apoplastic water flow both under well-watered (21% of increase) and under drought stress conditions (86% of increase). Drought stress reduced considerably this parameter in AM and non-AM plants, regardless of sodium azide application.

3.7. Apoplastic barriers

Structural changes in plants need longer time than other physiological or biochemical changes. As sodium azide was applied only for 30 min, the development of apoplastic barrier was unaffected by the chemical treatment. Figure 3 shows the development of Casparian bands in the exo- and endodermis of root sections taken at 50 mm from tips. Under well-watered conditions a weak signal was detected in the endodermis of maize roots, which was more intense in AM plants than in non-AM ones. In

the exodermis the formation of Casparian bands was visible, but weaker and more diffuse than in endodermis. Drought stress increased the development of Casparian bands of both exo- and endodermis. Again the intensity of the signal in endodermis was stronger in AM plants than in non-AM ones. In exodermis the signal was more diffuse in non-AM plants and more localized in AM plants.

3.8. Aquaporin protein accumulation and PIP2s phosphorylation status

In general, a drop in aquaporin protein levels was observed when plants were inoculated with the AM fungus, regardless of the watering conditions (Figure 4, 5). However, the decrease was not significant for all the analyzed aquaporins or treatments, being more evident when using specific antibodies for different PIP2 isoforms than when using the general antibodies for PIP1 or PIP2.

The application of NaN_3 positively regulated protein accumulation in non-AM plants under well-watered conditions in the case of ZmPIP2;4, ZmPIP2;5, ZmTIP1;1; PIP2A and PIP2C (Figure 4, 5). Interestingly, under drought stress NaN_3 had the opposite effect in non-AM plants in the case of ZmPIP2;5, PIP2A, PIP2B and PIP2C, which decreased their accumulation. In AM plants, the application of NaN_3 only affected negatively the root accumulation of PIP2B (PIP2 phosphorylated at Ser283) proteins.

In the absence of NaN_3 , drought stress increased the accumulation of PIP1 and PIP2 proteins in non-AM plants (Figure 4). In AM plants no effect was observed. In presence of NaN_3 , drought stress decreased the accumulation of ZmPIP2;4, ZmPIP2;5 and ZmTIP1;1 in non-AM plants and, again, no effect was observed in AM plants.

399 4. Discussion

400 When a mycorrhizal fungus colonizes plant roots, structural changes are produced
401 in cells, affecting root water uptake and transport. The improvement of plant physiology by
402 the AM symbiosis during water-limiting conditions has been extensively studied in different
403 crop species (Ruiz-Lozano et al., 2012; Augé et al., 2015), although studies of their
404 differential effect on root water transport are still elusive. In previous studies, the AM
405 symbiosis was suggested to modulate the switching between apoplastic and cell-to-cell
406 water transport in roots when the water conditions were limiting (Bárzana et al., 2012). The
407 focus of the present study was to better understand how the arbuscular mycorrhizal
408 symbiosis regulates radial root water transport in maize plants. For that we measured
409 osmotic (Lo) and hydrostatic (Lpr) root hydraulic conductivities and we used sodium azide
410 as inhibitor of aquaporins activity and of cell-to-cell water transport (Tournaire-Roux et al.,
411 2003; Grondin et al., 2016). The development of Casparian bands was also assessed.

412 413 **The AM symbiosis positively affected plant development and physiology under** 414 **drought stress**

415 The beneficial effect of mycorrhizal inoculation during drought stress was confirmed
416 by plant growth and physiological data. AM plants exhibited higher dry weight under the
417 two watering conditions. Additionally, AM plants enhanced stomatal conductance (gs) both
418 under well-watered or drought stress conditions and the efficiency of photosystem II and
419 chlorophyll content were also significantly higher during water deprivation. The
420 maintenance of a high stomatal conductance allows the plant a better CO₂ uptake for
421 photosynthesis (Sheng et al., 2008). This combined with the improved chlorophyll content
422 and efficiency of photosystem II are surely related with the improved plant growth. These

changes have been linked to a higher capacity for CO₂ fixation in AM plants. For instance, a higher RuBisCo activity was found in droughted AM grapevine plants (Valentine et al., 2006) or in AM rice plants subjected to salinity (Porcel et al., 2015). More recently, Chen et al. (2017) found a higher activity of key Calvin cycle enzymes in AM cucumber plants and Quiroga et al. (2019) have found that the maximum carboxylation capacity of PEPc (V_{pmax}) and CO₂-saturated photosynthetic rate (V_{max}) were higher in AM maize plants subjected to drought, revealing the higher photosynthetic capacity of AM maize plants, which translated into improved growth of mycorrhizal droughted plants.

The inhibition of aquaporins activity negatively affected root water transport

NaN₃ is a metabolic inhibitor that mimics O₂-deficient conditions by blocking the cytochrome pathway respiration, and consequently induces intracellular acidosis. Although not all aquaporins were found to be regulated by pH, the group of PIPs was commonly characterized as pH-dependent (Vitali et al., 2019). In consequence, NaN₃ application leads to H⁺-dependent gating of PIPs, due to a conserved structural basis for cytosolic pH sensing, where a histidine residue (His193 in spinach PIP2;1) of cytosolic loop D is involved (Tournaire-Roux et al., 2003; Törnroth-Horsefield et al., 2006; Fischer and Kaldenhoff, 2008; Frick et al., 2013b). Protonation of this residue would interact with the N-terminal divalent cation-binding site, stabilizing the closed conformation of the aquaporin (Frick et al., 2013b). Recently, it was found that the pH-dependence is also regulated by tetramer stoichiometry in PIPs (Jozefkiewicz et al., 2016). Besides, sodium azide is more efficient than other aquaporin inhibitors since it has a dual effect, causing cytoplasm acidification and inhibition of phosphorylation (Tournaire-Roux et al., 2003; Fitzpatrick and Reid, 2009).

The effect of sodium azide on root water flow rate (J_v) was previously studied (Kamaluddin and Zwiazek, 2001; Tournaire-Roux et al., 2003; Postaire et al., 2010; Sutka et al., 2011). A decrease in J_v similar to that of HgCl_2 (another aquaporin inhibitor) after treatment with sodium azide was observed in *Arabidopsis* plants and it was reversed upon washout of the inhibitor (Postaire et al., 2010). Kamaluddin and Zwiazek (2001) also observed an increase in apoplastic flow of water with the NaN_3 -induced decrease in root water flow rates. These results are in agreement with the present study. Indeed, NaN_3 application increased the apoplastic water flow both in AM and in non-AM plants. For instance, L_o was decreased by azide in non-AM plants, suggesting an inhibition of root aquaporins activity, but compensated by the increase of apoplastic water flow in these plants. In AM plants the apoplastic water flow and L_o values were already higher than in non-AM plants. Thus, in AM plants the inhibitory effect of sodium azide on L_o was lower than in non-AM plants, which together with the higher apoplastic water flow values suggests a compensatory mechanism for aquaporin activity inhibition in these plants, leading to a higher L_{pr} compared to non-AM plants. The general decrease of L_{pr} with water stress, that was slightly compensated with the mycorrhizal presence, could be related to the minimization of water loss from roots (Kaneko et al., 2015). Some differences in L_{pr} and L_o values between AM and non-AM plants in response to sodium azide or drought were not statistically significant when all treatments were analyzed jointly, but the use of a higher number of plants (here, $n = 7-8$ biological replicates) may have led to significant differences. In fact, pairwise comparison with t-student test showed indeed significant differences.

Apoplastic barriers developed under drought stress conditions

The root system has a high plasticity for modulating the development of apoplastic barriers in order to adapt to different environmental stresses (Pauluzzi and Bailey-Serres, 2016). In our study the development of Casparian bands increased due to drought stress, as evidenced by other authors with different plant species (Shen et al., 2014; Kreszies et al., 2018; 2019). This was in parallel with a significant decrease of L_{pr} , as well as, of the percentage of apoplastic water flow. L_o was not significantly decreased by drought stress, except in AM plants untreated with sodium azide. In general, apoplastic barriers decrease L_{pr} although this effect may vary, and there are reports of no correlation between increased suberin deposition and decreased water permeability (Ranathunge and Schreiber, 2011). In any case, it has been shown that apoplastic barriers have a more pronounced effect on L_{pr} and lower or no effect on L_o . This has been proposed as a mechanism to avoid water loss toward soil via the nonselective apoplastic pathway, while favouring the water passage through the highly regulated cell-to-cell pathway (Kreszies et al., 2019). In this study, AM plants exhibited enhanced development of Casparian bands compared to non-AM plants. In contrast, AM plants maintained enhanced L_{pr} and L_o values and percentage of apoplastic water flow as compared to non-AM plants. An explanation for these contradictory effects may be related with a different composition of Casparian bands and suberin deposits in AM and non-AM plants. Indeed, it has been proposed that the effect of apoplastic barriers on radial water movement may depend on their composition and of the microstructure of their deposits (Schreiber et al., 2005). Thus, a different composition of the apoplastic barriers in AM and non-AM plants may explain also the different effect on root water flow in these plants, even if Casparian bands seem more developed in AM plants. More studies are required to analyze the composition of these barriers and to elucidate this hypothesis.

Aquaporins accumulation and posttranslational modifications are affected by sodium azide application and mycorrhization

The time of application and concentration of the sodium azide (30 minutes, 2 mM) was in the same range of previous studies (Tournaire-Roux et al., 2003; Postaire et al., 2010; Grondin et al., 2016) and enough for inhibiting osmotic root water conductivity, although no clear effect was observed on protein abundance of aquaporins. It should be taken into account that the effect of NaN_3 is mainly at posttranslational level affecting the water transport activity, not necessarily affecting gene expression or protein abundance. On the other hand, aquaporin regulation may be cell-specific, being masked in whole-organ extractions. Furthermore, many aspects of the aquaporin regulation have to be considered, as gating, cycling or internalization due to environmental stresses (Chu et al., 2018). Indeed, the activity of aquaporins must be controlled by regulation mechanisms allowing a rapid response to the frequent environmental changes that plants undergo. Posttranslational modifications are key to achieve such a rapid and reversible regulation (Chaumont and Tyerman, 2014; Vandeleur et al., 2014), and they control protein catalytic activity, stability, subcellular localization and interaction with other proteins (Prak et al., 2008).

Phosphorylation is the most widespread protein modification, affecting basic cellular processes. Phosphorylation/de-phosphorylation of specific serine residues in plant aquaporins generates conformational changes controlling the aquaporin gating (Santoni, 2017) or modifying its subcellular localization under stress conditions (Luu and Maurel, 2013). For instance, the phosphorylation of Ser283 is required for targeting AtPIP2;1 to the plasma membrane (Prak et al., 2008). Maize PIP1s and PIP2s aquaporins were also shown to phosphorylate *in vivo* (Van Wilder et al., 2008). In addition, the phosphorylation of Ser274 in the C-terminal region or of Ser115 in loop B of a PIP2 in spinach open the

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pore and enhances the water transport (Törnroth-Horsefield et al., 2006). Interestingly, we found that NaN_3 treatment significantly decreased phosphorylation levels (PIP2A, PIP2B and PIP2C) under drought stress in non-AM plants, while in AM plants it was significant only in the case of PIP2s phosphorylated at Ser283 (PIP2B). This result suggests a more intense closing of these channels in non-AM plants in response to the sodium azide application. Aquaporins gating by pH is another common phenomenon in plants that naturally occurs in response to flooding (Tournaire-Roux et al., 2003; Frick et al., 2013b) and that is also a consequence of NaN_3 application, as explained above. A conserved histidine residue in loop D is considered a major pH sensor regulating channel gating. The drop in cytosolic pH can be accompanied with an increase in cytosolic Ca^{+2} concentration, being possible its interaction with the divalent cation binding site, and maintaining loop D in a closed conformation at low pH (Frick *et al.* 2013b). However, divalent cations can directly inhibit aquaporins in some cases (Verdoucq et al., 2008). Altogether, this reveals how complex the gating of aquaporin proteins is, and the interplay among phosphorylation, pH and cation binding in the regulation of the pore opening.

Surprisingly, in this study we found that some aquaporins increased their protein levels when treated with NaN_3 (ZmPIP2;4, ZmPIP2;5, ZmTIP1;1) under normal irrigation. These aquaporins may be insensitive to the inhibitor and the protein increase could be a compensation mechanism to the decrease in root water conductivity. SoPIP2;1 was demonstrated to increase its water permeability when treated with mercury (Frick et al., 2013a). It is noteworthy that protein levels in membranes do not give information about their location, as they can be located in the secretory pathway such as endoplasmic reticulum, Golgi, or different vesicles, apart from plasma membrane, which affect their functionality as water channels (Chevalier and Chaumont, 2015). On the other hand, we observed a general drop in aquaporin protein levels when plants were inoculated with the

AM fungus, a result that is consistent with previous studies (Bárzana et al., 2014; Quiroga et al., 2019), being that more evident when using specific antibodies for different PIP2 isoforms than when using the general antibodies for PIP1s or PIP2s. This may suggest that some specific aquaporins may decrease in presence of the AM fungus, while other PIP isoforms not tested here should be more abundant in AM plants. This should be checked in future studies with a larger set of isoform-specific antibodies.

5. Conclusion

This study provides some clues on the differential mycorrhizal regulation of root water transport. Indeed, the presence of a mycorrhizal fungus significantly modified the radial transport of water within the root system. Thus, in AM plants without sodium azide application, L_{pr} , L_o and the percentage of apoplastic water flow raised as compared to non-AM plants. When sodium azide was applied, there was a clear inhibition of L_o in non-AM plants, both under well-watered conditions and under drought stress. In AM plants the inhibition was weaker and not significant. This was particularly important under drought stress, since 88% of AM plants treated with sodium azide got free sap exudation and had 4 fold higher L_o values than non-AM plants, where only 13% of the plants got free sap exudation. This seems to be related to the regulation of aquaporins activity through posttranslational mechanisms rather than with the regulation of aquaporin protein accumulation, probably due to the short time of sodium azide exposure. However, this should be addressed in future studies in order to understand the specific mechanisms involved.

Author statement

JMR-L conceived the study and participated in the analysis of data and manuscript writing. GQ and GE performed the experiments and the statistical analysis. RA and FC participated in the design of the experiments and interpretation of data. All authors read and approved the final manuscript.

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Table 1. Percentage of mycorrhizal root length, stomatal conductance (gs), SPAD values and photosystem II efficiency in the light-adapted state ($\Delta F_v/F_m'$) of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered-WW- or drought stress DS).

	Mycorrhization (%)	gs (mmol H ₂ O m ⁻² s ⁻¹)	SPAD	$\Delta F_v/F_m'$
WW non-AM	n.d.	49.8 ± 7.6 b	12.1 ± 0.7 a	0.65 ± 0.01 a
WW AM	64.5 ± 6.7	138.8 ± 10.4 a	11.5 ± 0.5 a	0.63 ± 0.01 a
DS non-AM	n.d.	16.0 ± 1.7 c	7.8 ± 0.4 c	0.58 ± 0.02 b
DS AM	57.8 ± 4.0	42.1 ± 4.1 b	9.7 ± 0.3 b	0.62 ± 0.01 a

Data represent the means of five values ± SE for mycorrhization, seven values ± SE for gs and ten values for SPAD and $\Delta F_v/F_m'$. 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.

826 Figure 1

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828 **Figure 1.** Total dry weight of maize plants inoculated or not with the AM fungus
829 *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought
830 stress, DS). Data show the mean $\pm$ SE for fifteen plants per treatment. Different letter
831 indicates significant differences between treatments ($p < 0.05$) based on Duncan's test.

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Figure 2. Hydrostatic root hydraulic conductivity (*Lpr*), osmotic root hydraulic conductivity (*Lo*) and relative apoplastic water flow of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). A group of plants from each treatment was treated with NaN₃ for 30 min before measurements or kept untreated (-). Data show the mean ± SE for seven plants per treatment for *Lpr* and apoplastic water flow and for eight plants for *Lo*. Different letter indicates significant differences between treatments (p < 0.05) based on Duncan's test. Asterisk denotes significant differences between AM and non-AM plants based on t-test.

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849 **Figure 3.** Development of Casparian bands in root sections taken at 50 mm from the root
850 tip. **A-B**, transverse control root sections not stained with berberine hemisulphate. **C-F**,
851 transverse root sections stained with berberine hemisulphate and toluidine blue as
852 described in the materials and methods. **(C)**, Non-AM plants under well-watered
853 conditions. **(D)**, AM plants under well-watered conditions. **(E)**, Non-AM plants under
854 drought stress. **(F)**, AM plants under drought stress. The presence of Casparian bands
855 was indicated by green-yellow fluorescence (see white arrows in C, D, E, F).

Figure 4. Relative protein abundance 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 drought stress, DS). A group of plants from each treatment was treated with NaN₃ for 30 min before harvest or kept untreated (-). Data indicate the mean ± SE for three biological replicates per treatment. Different letter indicates significant differences between treatments (p<0.05) based on Duncan's test.

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Figure 5. PIP2A (Ph-Ser280), PIP2B (Ph-Ser283) and PIP2C (Ph-Ser280/Ser283) relative protein abundance 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 drought stress, DS). A group of plants from each treatment was treated with NaN₃ for 30 min before harvest or kept untreated (-). Data indicate the mean $\pm$ SE for three biological replicates per treatment. Different letter indicates significant differences between treatments (p < 0.05) based on Duncan's test.