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Deciphering the contribution of maize aquaporins regulated by arbuscular mycorrhizae to the transport *in planta* of water and/or other solutes of physiological importance under drought

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ACRONYMS

6-FI	6-fluoroindol
ABA	Abscisic acid
AIP	2-aminoindan-2-phosphonic acid
AM	Arbuscular mycorrhizal
AN	Net photosynthesis
AQP	Aquaporin
ar/R	Aromatic/arginine
ARF7	Auxin response factor 7
CDPK	Calcium-dependent protein kinase
Ci	Internal CO_2 concentration
CK	Cytokinin
CMN	Common mycorrhizal network
CO	Chitin oligomer
CSSP	Common symbiosis signalling pathway
DS	Drought stress
DW	Dry weight
EL	Electrolyte leakage
Ɛ	Cell wall volumetric elastic modulus
ER	Endoplasmic reticulum
ERM	Extraradical mycelium
ET	Ethylene
FA	Fatty acid
FW	Fresh weight
GA	Gibberellin
GIP	GLpF-like intrinsic protein
GLP	Aquaglyceroporin
gs	Stomatal conductance
GWAS	Genome-wide association study
HIP	Hybrid intrinsic protein
HM	Heavy metal
IAA	Indol-3-acetic-acid
IRM	Intraradical mycelium
JA	Jasmonic acid

JA-Ile	Jasmonate isoleucine
Jv	Water flow rate
LCO	Lipochitooligosaccharide
LEA	Late embryogenesis abundant
Lo	Osmotic root hydraulic conductivity
Lpc	Cell hydraulic conductivity
Lpr	Root hydraulic conductivity
MDA	Malondialdehyde
MeJA	Metil-jasmonate
MIP	Major intrinsic protein
NIP	NOD26-like intrinsic protein
NPA	Asn-Pro-Ala
PAM	Periarbuscular membrane
PEPc	Phosphoenolpyruvate carboxylase
Pf	osmotic water permeability
PIP	Plasma membrane protein
PM	Plasma membrane
polyP	Polyphosphate
PPA	Pre-penetration apparatus
PSII	Photosystem II
PT	Phosphate transporter
RDW	Root dry weight
RG-II	Pectin polysaccharide rhamnogalacturonan-II
RH	Relative humidity
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTE	Rotten ear
SA	Salicylic acid
SDW	Shoot dry weight
SIP	Small basic intrinsic protein
SL	Strigolactone
SLA	Specific leaf area
SPAC	Soil-plant-atmosphere continuum
$T_{1/2}$	Half time of water exchange
TBAR	2-thiobarbituric acid-reactive substance
TIP	Tonoplast intrinsic protein

TSS	Total soluble sugars
Vmax	CO_2-saturated photosynthetic rate
Vpmax	Calculated maximum carboxylation capacity
WD	Water deficit
WUE	Water use efficiency
WW	Well-watered
XIP	Unknown intrinsic protein

ABSTRACT

Drought stress is one of the major abiotic factors affecting normal growth and development of plants from both natural and agroecosystems. Climate change is expected to intensify the periods of drought as well as to involve areas that were not threatened by this phenomenon in the past, which will consequently affect crop production and food security. The effect of drought in Mediterranean regions, which largely depend on agriculture, will have important social and economic consequences.

The natural-occurring symbiosis between arbuscular mycorrhizal fungi and roots of approximately 80% of land plants, including numerous crops, is able to enhance the uptake of water and nutrients in the soil, thanks to an extended hyphal network that allows the uptake of nutrients out of the root depletion zone. The benefits of the AM symbiosis also include the protection of the plants against a range of abiotic and biotic stresses. In fact, the association is well known for conferring drought stress tolerance in different plant species, including maize. The fungi colonize root cortex cells, forming the arbuscules, which are the exchange structures between the two partners. During this process, the plasma membranes of these cells suffer extensive morphological alterations to surround the arbuscules. Among these modifications, changes in location or abundance of membrane proteins are commonly produced.

Aquaporins (AQPs) are integral membrane proteins belonging to the major intrinsic protein (MIP) superfamily. These channels, present in all living organisms, facilitate the passive flux of water and a range of small solutes across cell membranes. AQPs have been mainly studied in relation to the hydraulic properties of plants. Nevertheless, the capacity of transporting different solutes has opened the possibility of their involvement in other physiological processes. In fact, AQPs participate in the symbiotic exchange at the plant-fungus interface, and several genes were modulated by the arbuscular mycorrhizal symbiosis under drought stress conditions. In maize, AM symbiosis has been shown to regulate mRNA abundance of a high number of aquaporins, including members of the different subfamilies. Additionally, it was demonstrated that they can transport water as well as other solutes of physiological importance (such as glycerol, ammonia, urea, boron, silicon, O_2, H_2O_2 or CO_2) under normal and drought stress conditions. Previous to this work, it was also shown that the AM symbiosis can modulate the switch between water transport pathways in the root of the host plant. This is understood as a way to provide higher flexibility in the response of AM plants to water deficit, according to the demands of the aerial part.

The present PhD thesis is mainly focused on the **identification of AM-regulated maize aquaporin isoforms key for drought tolerance, and the identification of their specific functions *in planta*. Moreover, it is a goal of this study to understand if these**

aquaporins have a key influence on root water transport capacity of the host plant and if they contribute to the higher flexibility of AM roots for switching between cell-to-cell and apoplastic water transport pathways. With this aim, the combination between *Zea mays* L. and *Rhizophagus irregularis* was used as a model in all the experiments carried out in this PhD thesis.

As a first approach to understand the differential regulation of maize aquaporins by the AM symbiosis, two maize cultivars with contrasting drought sensitivity were compared under normal and drought stress conditions: PR34G13 (drought-tolerant) and PR34B39 (drought-sensitive). Results showed that the AM symbiosis improved physiological parameters to a higher extent in the drought-sensitive cultivar. This effect was reflected in the higher membrane stability, efficiency of photosystem II, accumulation of soluble sugars and plant biomass production. The benefits of the AM inoculation were also related to a higher and broader regulation of root aquaporins in the drought-sensitive cultivar. From this initial study, eight maize aquaporins were selected for being regulated by the AM symbiosis or for being putative transporters of solutes with relevance in drought stress tolerance. These aquaporins were analysed in the subsequent experiments. This study is presented in the **first chapter** of this PhD thesis.

Subsequently, the **second chapter** had the objective of elucidating if the key effect of the regulation of maize aquaporins by the AM symbiosis was the enhancement of root cell water transport capacity. With this aim, pressure probe and protoplast swelling assays were performed using intact cortical cells and root cell protoplasts, respectively from AM and non-AM plants subjected or not to drought stress. The obtained results showed that cells from droughted-AM roots maintained cell hydraulic conductivity (Lpc) and water permeability coefficient (Pf) values of non-stressed plants, whereas in non-AM plants these values declined drastically as a consequence of water deficit. Under these conditions, phosphorylation status of plant PIP2 aquaporins was increased by the symbiosis, which may be related to a higher activity of their water channels. Additionally, AM symbiosis also enhanced photosynthetic capacity thanks to an increased PEPc activity and CO_2-saturated photosynthetic rate. In summary, this chapter demonstrated a better performance of AM root cells in water transport under water deficit, which is connected to a better performance of the shoot in terms of photosynthetic capacity.

The **third chapter** of this PhD thesis intended to elucidate the possible involvement of the AM-regulated aquaporins in the *in planta* transport of boron (B) under well-watered or drought stress conditions. With this objective, different B concentrations were applied in the nutrient solution to both non-AM and AM plants that were submitted or not to a water deficit treatment. It was shown that aquaporins and B efflux transports were generally

down-regulated in AM plants, suggesting that other mechanisms contribute to B homeostasis in these plants, probably more related to the enhancement of water transport which would concomitantly increase the passive transport of this micronutrient. In this study, different aquaporins (*ZmPIP2;2*, *ZmTIP2;3* and *ZmNIP1;1*) and B efflux transporters (*RTE*, *RTE2* and *RTE3*) were transcriptionally regulated by B levels *in planta*, which confirms their previously proposed role in B transport.

In the **fourth** and **fifth chapters** it is presented the research work corresponding to the elucidation of the fourth specific objective of this PhD thesis: Deciphering if the higher flexibility of AM plants to switch between water transport pathways is due to aquaporin regulation mediated by salicylic acid (SA) or indole-3-acetic-acid (IAA).

In chapter four, exogenous SA was applied to non-AM and AM plants subjected or not to drought stress treatment. Additionally, an inhibitor of SA biosynthesis (2-aminoindan-2-phosphonic acid, AIP) was also applied to half of the plants. It was demonstrated that exogenous SA application altered root hydraulic parameters decreasing root hydraulic conductivity (Lpr) and osmotic root hydraulic conductivity (Lo) under drought stress conditions. This effect could be related to the regulation of root aquaporins (as *ZmPIP2;4* and *ZmTIP1;1*), whose protein levels correlated with Lo under water deficit. Furthermore, SA differently modulated the percentage of water flowing by the apoplastic pathway, decreasing its contribution to total root water flow in AM plants and increasing it in non-AM plants.

In chapter five, IAA was applied, following the same experimental approach than with SA. Here, it was revealed that IAA affected root hydraulic parameters (mainly Lo) during water stress conditions, similarly to SA, which was decreased in both non-AM and AM plants. The regulation of the internal cell component of root water conductivity (Lo) suggested that aquaporins are involved in the IAA-dependent inhibition of this internal cell pathway. Interestingly, similarly to SA application, IAA regulated differently apoplastic water flow in AM and non-AM plants under water deficit, which confirms the previous hypothesis. In both experiments, exogenous application of the hormone altered endogenous levels of other phytohormones (such as ABA, SA, JA or JA-Ile), revealing the complex network that regulates water transport in roots.

The study described in **chapter six** intended 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. For that we measured osmotic (Lo) and hydrostatic (Lpr) root hydraulic conductivities and we used sodium azide (NaN_3) as inhibitor of aquaporins activity and of cell-to-cell water

transport. Additionally, the study constitutes a first approach to elucidate the role of the AM fungus on the modification of apoplastic barriers. Once more, it was confirmed that the AM fungus modifies water transport in roots, increasing all hydraulic parameters compared to non-AM plants. NaN_3 inhibition of Lo was lower in AM plants than in non-AM plants. The former plants also had higher relative apoplastic water flow values, suggesting a compensatory mechanism for aquaporin activity inhibition in these plants and leading to higher Lpr values as compared to non-AM plants. The lower inhibition of Lo in AM plants seems to be related to the regulation of aquaporins activity through posttranslational mechanisms. Casparian bands increased with drought but also in AM plants, although this did not decrease water flow values in these plants. There is the possibility that apoplastic barriers of AM roots have a different composition, which could explain the different water transport of these roots.

In summary, the study conducted in this PhD Thesis increases the general knowledge about the plant drought tolerance induced by the AM symbiosis. It is evidenced that the AM symbiosis has a role in the modulation of cell water conductivity in roots, which is probably related to aquaporins activity. Moreover, the higher flexibility of AM roots to modulate water transport is confirmed in independent experiments, which is translated into the better performance of these plants under water scarcity.

RESUMEN

La sequía es uno de los principales factores abióticos que afectan al crecimiento y desarrollo normal de las plantas, tanto en ecosistemas naturales como en agroecosistemas. Se espera que el cambio climático intensifique los periodos de sequía, además de implicar áreas que no estaban amenazadas por este fenómeno en el pasado, lo que, en consecuencia, afectará a la producción vegetal y la seguridad alimentaria. El efecto de la sequía en las regiones mediterráneas, que dependen en gran parte de la agricultura, tendrá importantes consecuencias sociales y económicas.

La simbiosis que ocurre naturalmente entre hongos micorrícico arbusculares y las raíces del 80% de las plantas terrestres, incluyendo numerosas plantas cultivadas, es capaz de mejorar la captación de agua y nutrientes en el suelo, gracias a una extensa red de hifas que permite la absorción de nutrientes más allá de la zona de agotamiento de la raíz. Los beneficios de la simbiosis micorrícico-arbuscular (MA) también incluyen la protección de las plantas frente a un amplio número de estreses abióticos y bióticos. De hecho, esta asociación es bien conocida por conferir tolerancia al estrés hídrico en diferentes especies de plantas, entre las que se incluye el maíz. El hongo coloniza las células corticales de la raíz, formando los arbúsculos, que son las estructuras de intercambio entre los dos organismos. Durante este proceso, las membranas plasmáticas de estas células sufren extensas alteraciones morfológicas para poder rodear los arbúsculos. Entre estas modificaciones, habitualmente se producen cambios en la localización o en la abundancia de proteínas de membrana.

Las acuaporinas (AQPs) son proteínas integrales de membrana que pertenecen a la superfamilia de las "*major intrinsic proteins"* (MIPs). Estos canales, que están presentes en todos los organismos vivos, facilitan el flujo pasivo de agua y una serie de pequeños solutos a través de las membranas celulares. Las AQPs han sido estudiadas principalmente en relación a las propiedades hidráulicas de las plantas. Sin embargo, la capacidad de transportar distintos solutos ha abierto la posibilidad de que estén implicadas en otros procesos fisiológicos. De hecho, las AQPs participan en el intercambio simbiótico en la interfaz planta-hongo, y diferentes genes fueron modulados por la simbiosis MA durante condiciones de estrés hídrico. En maíz, se ha visto que la simbiosis MA regula la abundancia de ARNm de un gran número de acuaporinas, incluyendo miembros de las diferentes subfamilias. Además, se ha demostrado que pueden transportar agua pero también otros solutos de importancia fisiológica (como glicerol, amonio, urea, boro, silicio, O_2, peróxido de hidrógeno o CO_2) tanto en condiciones normales como de sequía. En estudios previos se ha demostrado que la simbiosis MA puede modular el intercambio entre vías de transporte de agua en la raíz de la planta hospedadora. Este hecho fue entendido como una forma de proveer mayor flexibilidad en

la respuesta de las plantas MA al estrés hídrico, de acuerdo con las demandas de la parte aérea.

La presente Tesis Doctoral está enfocada principalmente en la **identificación de isoformas de acuaporina reguladas por MA que sean claves para la tolerancia a la sequía, y la identificación de sus funciones específicas *in planta*. Además, es un objetivo de este estudio comprender si estas acuaporinas tienen una influencia principal en la capacidad de transporte de agua en la raíz de la planta hospedadora y si contribuyen a la mayor flexibilidad de las raíces MA para cambiar entre las vías de transporte de agua célula a célula y apoplástica**. Con este objetivo, la combinación entre *Zea mays* L. y *Rhizophagus irregularis* fue empleada como modelo en todos los experimentos realizados en esta Tesis Doctoral.

Como primera aproximación para entender la regulación diferencial de las acuaporinas de maíz por la simbiosis MA, se compararon dos cultivares contrastantes de maíz con distinta sensibilidad a la sequía: PR34G13 (tolerante) y PR34B29 (sensible). Los resultados mostraron que la simbiosis MA mejoró los parámetros fisiológicos más ampliamente en el cultivar sensible a la sequía. Este efecto estuvo relacionado con la mayor estabilidad de la membrana, mayor eficiencia del fotosistema II, así como con la acumulación de azúcares solubles y una mayor producción de biomasa total. Los beneficios de la inoculación con MA se relacionaron también con una mayor regulación de las acuaporinas de la raíz en el cultivar sensible a la sequía. En este estudio inicial, se seleccionaron ocho acuaporinas por estar reguladas por la simbiosis MA o por tener la capacidad potencial de transportar solutos con relevancia en la tolerancia a la sequía. Estas acuaporinas fueron analizadas en los siguientes experimentos. Este estudio se presenta en el **primer capítulo** de la tesis doctoral.

Posteriormente, el **segundo capítulo** tuvo como objetivo comprender si el efecto principal de la regulación de las acuaporinas de maíz por la simbiosis MA era la mejora de la capacidad de transporte de agua en la raíz. Con este objetivo, se realizaron experimentos con sonda de presión celular y de hinchamiento de protoplastos en células corticales intactas y protoplastos de raíz, respectivamente, de plantas MA y no MA sometidas o no a condiciones de estrés hídrico. Los resultados obtenidos mostraron que las células de plantas MA sometidas a sequía mantuvieron niveles de conductividad hidráulica celular (Lpc) y coeficiente de permeabilidad al agua (Pf) similares a los de plantas no estresadas, mientras que en plantas no MA estos valores disminuyeron drásticamente como consecuencia del estrés. En estas condiciones, el estado de fosforilación de las acuaporinas PIP2 de la planta se incrementó con la simbiosis MA, lo que está relacionado con una mayor actividad de sus canales de agua. Adicionalmente,

la simbiosis MA también mejoró la capacidad fotosintética gracias al incremento de la actividad PEPc y la tasa fotosintética bajo saturación de CO_2. En resumen, este capítulo demuestra el mejor rendimiento de las células MA de la raíz en el transporte de agua en condiciones de estrés hídrico, que está relacionado con el mayor rendimiento de la parte aérea en términos de capacidad fotosintética.

El **tercer capítulo** de esta Tesis Doctoral tuvo como objetivo entender la posible implicación de las acuaporinas reguladas por MA en el transporte *in planta* de boro (B) en condiciones normales o de sequía. Con este fin, se aplicaron diferentes concentraciones de B en la solución nutritiva a plantas MA y no MA sometidas o no a estrés hídrico. Se observó una inhibición general de las acuaporinas y los transportadores de B, lo que sugiere que en estas plantas otros mecanismos contribuyen a la homeostasis de este micronutriente, probablemente más relacionado con el transporte pasivo a través del propio movimiento del agua. En este estudio, diferentes acuaporinas (*ZmPIP2;2*, *ZmTIP2;3* and *ZmNIP1;1*) y transportadores de B (*RTE*, *RTE2* and *RTE3*) fueron regulados transcripcionalmente por los niveles de B *in planta*, lo que confirma su papel en el transporte de B, propuesto previamente.

En los **capítulos cuarto** y **quinto** se presenta el trabajo correspondiente a la elucidación del cuarto objetivo de la Tesis Doctoral: Descifrar si la mayor flexibilidad de las plantas MA para conmutar entre vías de transporte de agua se debe a la regulación de acuaporinas mediada por ácido salicílico (SA) o por ácido indolacético (IAA).

En el capítulo cuatro, se aplicó exógenamente SA a plantas MA y no MA sometidas o no a estrés hídrico. Adicionalmente, se aplicó un inhibidor de la biosíntesis de SA (2-aminoindan-2-phosphonic acid, AIP) a mitad de las plantas. Se demostró que la aplicación exógena de SA altera las propiedades hidráulicas de la raíz, disminuyendo la conductividad hidráulica hidrostática de la raíz (Lpr) y la conductividad hidráulica osmótica (Lo) bajo condiciones de estrés hídrico. Este efecto podría estar relacionado con la regulación de acuaporinas como *ZmPIP2;4* y *ZmTIP1;1*, cuyos niveles proteicos se correlacionaron con Lo en condiciones de sequía. Además, el SA moduló de manera diferencial el porcentaje de agua que fluye por la vía apoplástica, disminuyendo su contribución al flujo de agua total en plantas MA y aumentándolo en plantas no MA.

En el capítulo cinco, se aplicó IAA, siguiendo el mismo diseño experimental que con la aplicación de SA. En este caso, se reveló que el IAA afecta a los parámetros hidráulicos de la raíz (principalmente Lo) durante condiciones de estrés hídrico, de manera similar a SA, que disminuyeron en plantas MA y no MA. La regulación del componente interno celular de la conductividad hidráulica de la raíz (Lo) sugiere que las

acuaporinas están implicadas en la inhibición dependiente de IAA de esta vía interna de transporte de agua. Es interesante resaltar que, de forma similar a la aplicación de SA, el IAA reguló de manera diferente el flujo de agua apoplástico en plantas MA y no MA durante el estrés hídrico, lo que confirma la hipótesis previa. En ambos experimentos, la aplicación exógena de la hormona alteró los niveles endógenos de otras fitohormonas (como ABA, SA, JA o JA-Ile), revelando el complejo sistema que regula el transporte de agua en la raíz.

El estudio descrito en el **capítulo seis** pretende determinar si la simbiosis MA altera el flujo radial de agua en la raíz de la planta hospedadora y si esta modificación se debe a una alteración de la actividad o la cantidad de acuaporinas y/o a cambios en las barreras apoplásticas. Para ello se midió Lo y Lpr y se utilizó azida sódica (NaN_3) como inhibidor de la actividad de las acuaporinas y del flujo de agua por la vía célula a célula. Asimismo, el estudio constituye una primera aproximación para comprender el papel del hongo MA en la modificación de las barreras apoplásticas. Una vez más, se confirmó que el hongo MA modifica el transporte de agua en las raíces, aumentando todos los parámetros hidráulicos en comparación con las plantas no MA. La inhibición de Lo mediada por NaN_3 fue menor en plantas MA que en plantas no MA. Las primeras presentaron también un mayor flujo de agua apoplástico, sugiriendo la existencia de un mecanismo compensatorio para la inhibición de la actividad de las acuaporinas en estas plantas, lo que conlleva a valores de Lpr más altos en comparación con plantas no MA. La menor inhibición de Lo en plantas MA parece estar relacionada con la regulación de la actividad de las acuaporinas mediante mecanismos postranscripcionales. Las Bandas de Caspary se incrementaron con la sequía, también en plantas MA, aunque esto no disminuyó el flujo de agua en estas plantas. Existe la posibilidad de que las barreras apoplásticas de las raíces MA tengan una composición diferente a las no MA, lo que explicaría la diferente capacidad de transporte de agua.

En resumen, los estudios llevados a cabo en esta Tesis Doctoral aumentan el conocimiento general sobre la tolerancia a la sequía inducida por la simbiosis MA. Se evidencia que la simbiosis MA tiene un papel en la modulación de la conductividad hidráulica celular en las raíces, lo que está probablemente relacionado a la actividad de las acuaporinas. Además, la mayor flexibilidad de las raíces MA para modular el transporte de agua se confirma en experimentos independientes, lo que se traduce en una mejora de la capacidad de estas plantas para enfrentarse al déficit hídrico.

INTEREST OF THE STUDY AND OBJECTIVES

1. Interest of the study

Drought stress is one of the major abiotic factors affecting plant development and growth, causing agricultural losses worldwide. Moreover, climate change is expected to spread the problem of water deficit to regions that were not affected in the past. In this context, Mediterranean regions, which largely depend on agricultural sector, would suffer a decline on water availability that would dramatically impact crop production, with important social and economic effects on these areas.

Plants have developed physiological and molecular mechanisms to tolerate drought stress and allow plant performance, and the knowledge of such mechanisms is crucial in order ensure ecosystems functioning and food production in the near future.

One of the mechanisms developed to tolerate drought stress and other abiotic and biotic factors is based on the association with beneficial rhizospheric microorganisms. In particular, arbuscular mycorrhizal (AM) fungi form symbiosis with most land plants and has been shown to be beneficial for the alleviation of drought stress in a number of crops. The establishment of this symbiosis originates extensive morphological alterations in order to accommodate the fungus within the root. These changes involve vacuolar and membrane systems, modifying membrane-associated proteins such as aquaporins.

Despite the intensive research on aquaporins, their relationship with drought plant responses is still unsolved and with contradictory results. Nevertheless, the importance of aquaporins for both water and solutes exchange during AM symbiosis has been recently recognized. Previous studies of the group where this thesis has been conducted have shown that AM symbiosis regulates the expression of a high number of aquaporins in maize (*Zea mays* L.), including members from the different aquaporin subfamilies. Some of those aquaporins were functionally characterized in heterologous systems (*Xenopus laevis* oocytes or yeast). Thus, it was demonstrated that they can transport water but also other molecules of physiological importance for plant performance under normal and drought conditions (such as glycerol, urea, ammonia, boric acid, silicon or hydrogen peroxide).

Additionally, this same group has also shown that the presence of arbuscular mycorrhizal fungi in the root of the host plant can modulate the switching between radial root water transport pathways. This fact was considered as a way to provide higher flexibility in the response of AM plants to water deficit, according to the demands of the shoot. Additional evidences suggested that auxin and/or salicylic acid levels in the host

plant might contribute to this switching between water pathways mediated by the AM fungus, through aquaporin regulation.

With the aim of understanding the mechanisms of the AM fungi providing plant drought tolerance and the specific role *in planta* of AM-regulated aquaporins, the present PhD thesis was carry out using *Zea mays* L. as plant species and *Rhizophagus irregularis* as a model fungal species. Nonetheless, the result obtained from this study may be extrapolated to other plant-fungal combinations.

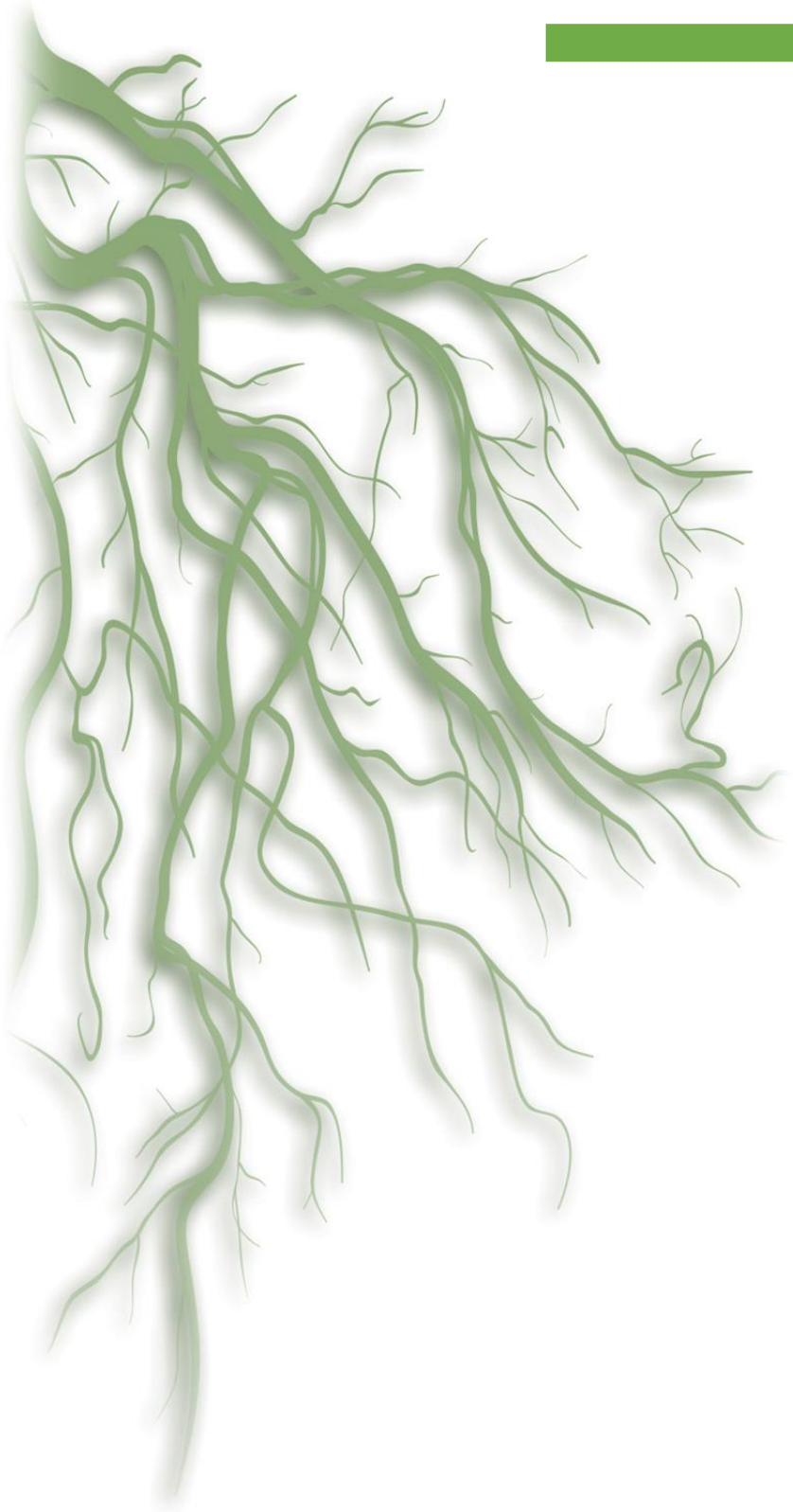
2. Objectives

The main objective of this doctoral thesis is the identification of those maize aquaporin isoforms induced by the AM symbiosis that are key for the drought tolerance, as well as the identification of their specific function *in planta*. Moreover, a goal of this study is to understand if these aquaporins have a key influence on the root water transport capacity of the host plant and if they contribute to the higher flexibility of AM roots for switching between cell-to-cell and apoplastic water transport pathways.

To achieve this general aim, the following **specific objectives** were established:

1. Identification, among the aquaporin isoforms found to be regulated by the AM symbiosis in maize, those with an essential role in the improved plant drought tolerance (Chapter I).
2. Understanding if the key effect of the AM regulation of aquaporins during drought is the enhancement of water transport capacity or if it would rather be affecting other solutes transport capacity (Chapter II).
3. Elucidating if the key aquaporins selected in objective 1 are involved in the *in planta* transport of physiologically important solutes such as nitrogen compounds or boron (Chapter III).
4. Deciphering if the higher flexibility of AM plants to switch between cell-to-cell and apoplastic water transport pathways is due to an aquaporin inhibition mediated by indol-3-acetic-acid (IAA) or salicylic acid (SA) (Chapter IV and V).
5. To determine the contribution of the AM symbiosis to the regulation of aquaporins and radial water transport in maize roots under water deficit (Chapter VI).

INTRODUCTION



1. Drought: Impact in agriculture with a focus in the Mediterranean area

As sessile organisms, plants encounter unavoidable abiotic stresses during their life cycles: salinity, drought, flooding, extreme temperatures, heavy metal toxicity or UV-B radiation, among others. Drought, however, is the more catastrophic worldwide, affecting the normal growth and development of plants from both natural and agroecosystems (Hasanuzzaman *et al.*, 2014). It is the result of low precipitation with the often combination of warm temperatures during long periods that can range from months to years. Despite being a natural climate feature, climate change is amplifying its severity and impact due to the decrease in precipitation and increase in the number of dry days. Moreover, drought events involve areas that were not affected in the past (Harrison *et al.*, 2014); hence, it is an issue of global concern. Agricultural production is dramatically affected by this phenomenon, and in this context food security may become more vulnerable (Lesk *et al.*, 2016). Furthermore, the impact of climate change in agriculture will probably intensify the disparities among regions. Thus, it seems essential to improve our knowledge in the mechanisms of plant drought tolerance.

The Mediterranean-climate regions (constituted by the Mediterranean Basin, but also other regions with similar climate: Central Chile, California, south of Australia and the Cape Region of South Africa) are characterized by winters with low or mild precipitations and warm and dry summers (del Pozo *et al.*, 2019). In the Mediterranean Basin almost one-third of the extension was classified as agricultural land (Underwood *et al.*, 2009), being one of the main economic sectors of the region. This sector is the largest water consumer, with consumption of nearly 68% of the total in Spain, similarly to other countries of the region (Barbero, 2006). Although Mediterranean countries are used to adapt to water scarcity, these regions are expected to suffer a critical decline in water availability due to the reduced precipitation and higher inter- and intra-annual rainfall variability. Drought events will be probably longer and more severe, which would negatively impact crop production (Peña-Gallardo *et al.*, 2019).

Maize (*Zea mays* L.), which is the object of this study, represents one of the main sources of calories for the majority of human population. According to FAOSTAT, its worldwide annual production represents more than one trillion tons, and only in Spain the production in 2017 was higher than three million tons. However, its production is highly affected by drought stress, especially during the reproductive stage (Daryanto *et al.*, 2016), generating declines in global production by 3.8% (Lobell *et al.*, 2008). In fact, some parts

of the Mediterranean region are considered to be 'vulnerability hotspots' for maize production under drought stress (Fraser *et al.*, 2013).

Plants have evolved different physiological strategies to cope with drought stress and preserve water supply even under reduced soil water potential (Ψ_w), prevent water loss and sustain long periods of low water availability. The responses of the plant occur at both cellular and whole plant levels, which require complex mechanisms of stress perception, signalling, regulation and organization. The physiological effects in plants and mechanisms of drought tolerance are explained in more detail below.

1.1. Impact of drought stress in plants

Drought stress is usually perceived on a systemic level in the plant, which undergoes a cascade of metabolic alterations to cope with it. Furthermore, recent evidences showed that plants can also respond locally to differences in water availability, positioning lateral branches toward regions of higher water content, a mechanism called hydropatterning (Robbins & Dinneny, 2018).

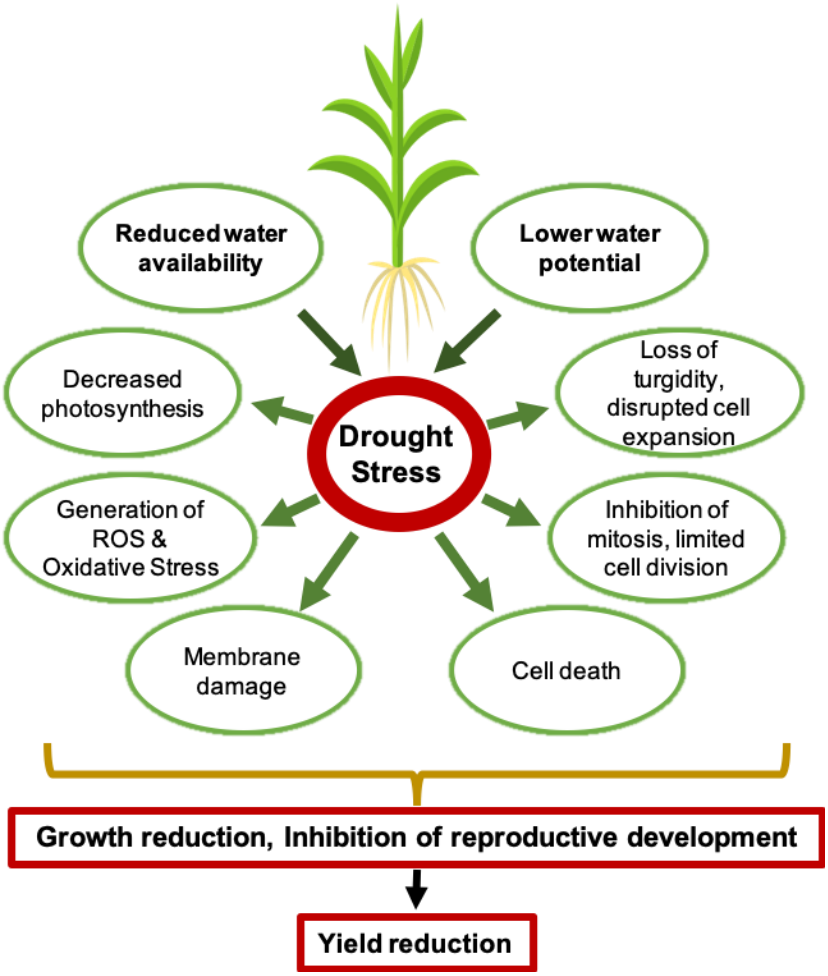


Figure 1. Physiological effects of drought stress in plants. Modified from Hasanuzzam *et al.* 2018

Generally, in the short-term, water deficit limits root water uptake, which concomitantly decreases water content in tissues, leading to reduced cell turgor pressure. A fast response starts with the production of abscisic acid (ABA), being a signal for stomatal closure. This is a measure for water saving, but also reduces the available carbon (C) for photosynthesis. After a sustained drought, the plant needs further acclimation reactions. The reduction of CO_2 assimilation leads also to an inhibition of photosystem II (PSII), which induces the production of reactive oxygen species (ROS). This effect is commonly accompanied by the production of reactive nitrogen species (RNS), causing cellular damage (Laxa *et al.*, 2019; Rodrigues *et al.*, 2019). Signalling is also mediated by hormonal changes, protein phosphorylation cascades, calcium ions and lipids, leading to changes in gene expression and metabolism (Beacham *et al.*, 2018). This situation drives to the inhibition of cell division, expansion of leaf surface, stem growth and proliferation of root cells, which dramatically reduces plant productivity and, in some cases, leads to death.

The responses of plants to water deficit are described below:

- Germination, growth and yield

The first effect of drought is the impairment of germination due to the lack of water imbibition, resulting in reduced seedling vigour (Farooq *et al.*, 2009). Water deficit disturbs osmotic balance, affects metabolic activity of the cell and induces ROS production, which produces changes in DNA, RNA and proteins, damaging membranes and reducing respiration (Hussain *et al.*, 2018).

The reduction of turgor pressure induced by drought negatively impacts cell division, cell enlargement and differentiation. Cell growth is one of the most drought-sensitive physiological processes (Farooq *et al.*, 2009). This effect is translated in reduced leaf area, plant height, stem diameter, and plant biomass in a range of crops (Hussain *et al.*, 2018). The reduction of leaf area and number decreases transpiration rates, reducing, thus, water loss. Moreover, plants generally develop more roots that allow them to access more water, which increases the ratio root/shoot. The increase of root area was related to increased ABA levels (Hussain *et al.*, 2018). Usually, the growth of the primary root is not affected by the stress, but it has a effect in the suppression of lateral root meristems (Basu *et al.*, 2016). Overall, these modifications usually increase drought tolerance.

Yield reduction upon drought stress has been reported in a number of crops, although is very dependent on the duration and severity of the stress period. In maize, water deficit delayed silking, thus increasing the anthesis-to-silking interval and reducing

yield (Cattivelli *et al.*, 2008). During pollination, water deficit also increased frequency of kernel abortion (Farooq *et al.*, 2009).

- Photosynthesis

Photosynthesis is one of the first processes affected by drought. The reduction of photosynthesis is produced by the inhibition of leaf area and decreased rate of photosynthesis per unit leaf area (Farooq *et al.*, 2009). The directs effects of drought on photosynthesis start with the limitation of CO_2 diffusion through the stomata due to the ABA-induced stomatal closure, that occurs in response to leaf turgor decline. Consequently, leaf mesophyll conductance to CO_2 is also reduced, limiting CO_2 fixation by Rubisco (in C_3 plants) or PEPc (in C_4 plants). During a mild stress, this reduction can improve water use efficiency (WUE) of the plant, which means a greater C gain relative to the amount of water used, allowing water saving. Under a long period of drought, the reduction of available C induces changes in leaf biochemistry, resulting in the down-regulation of the photosynthetic apparatus. Under severe stress conditions, the lack of CO_2 in leaves permits more electrons to form ROS, leading to oxidative stress, which can gravely modify photosynthetic machinery (Chaves *et al.*, 2009b).

Photosynthetic enzymes may be also directly inactivated or degraded due to the changes in viscosity of the cytoplasmic induced by drought (Farooq *et al.*, 2009). The plant has developed a range of adaptive responses to reduce the damage to photosynthesis induced by drought, such as the xanthophyll cycle, thermal dissipation of light energy or dissociation of the light-harvesting complexes from photosynthetic reaction centres. These photoprotective mechanisms lead to a down-regulation of photosynthesis (as the decrease in quantum yield of PSII), as they compete with photochemistry for the absorbed energy mechanisms (Chaves & Oliveira, 2004). Moreover, photosynthetic pigments like chlorophyll are also reduced under drought conditions. Thus, photochemical efficiency under drought can also be estimated by measuring chlorophyll fluorescence (Reddy *et al.*, 2004).

The C_4 pathway of carbon assimilation that possesses some crops such as maize, is considered to be the major adaptation to reduce photorespiration, concentrating CO_2 at the site of carboxylation due to the accumulation of oxaloacetic acid within the bundle sheath. This mechanism improves photosynthetic efficiency and limit water loss under drought stress (Basu *et al.*, 2016).

- Water relations

Water-stressed plants generally show lower leaf water potential, relative water content and transpiration rate, while water-use efficiency is increased (Farooq *et al.*, 2009).

During the early periods of drought stress the root hydraulic conductivity (Lpr) is often reduced, limiting water uptake. This effect may be a mechanism to inhibit water loss when soil water potential decreases. However, it is very dependent on the species and drought stress conditions. Lpr behaviour has been extensively reported to be related to aquaporins (AQPs) function, especially to PIPs, although their specific role is still not well established (Aroca *et al.*, 2012). This topic will be addressed in section 2. The changes of Lpr under these conditions are regulated by hormonal profile, where ABA seems to have a key role, together with jasmonic acid (JAs) salicylic acid (SA) or ethylene (ET). However, their contribution to the regulation of Lpr is not fully elucidated. In addition, different ROS levels may control Lpr under drought and other abiotic stresses, inhibiting or stimulating it depending on the intracellular concentrations (Aroca *et al.*, 2012).

- Nutrients

The reduced transpiration rate during drought stress also decreases the availability of both macro- and micronutrients for the plant, hampering their uptake and translocation to the roots and shoots (Hussain *et al.*, 2018). Furthermore, the difference in active transport and membrane permeability of cations (K^+, Ca^{2+}, and Mg^{2+}) induced by drought, results in decrease absorption of these ions. Water deficit can also impair the activities of enzymes involved in nutrient assimilation, affecting nutrient metabolism (Hussain *et al.*, 2018).

- Oxidative damage

Under drought conditions, downregulation of PSII activity leads to an increase in the production of ROS, such as single oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$); as well as RNS, such as NO; resulting in imbalance between production and scavenging (Reddy *et al.*, 2004). Under non-stressful conditions, these molecules participate in the transmission of signals to the nucleus and other compartments, reprogramming plant performance.

Chloroplasts may be an important source of ROS formation, as excited pigments in thylakoid membranes interact with O_2 at PSI or via the Mehler reaction to form strong oxidants (i.e. $^1O_2$ or $O_2^{\cdot-}$). Other ROS are produced in further downstream reactions, such as $OH^{\cdot}$ or H_2O_2. In mitochondria, the interaction of O_2 with reduced components of the electron transport chain may also lead to ROS formation (Farooq *et al.*, 2009).

Under drought stress, ROS originated in the apoplast induce lipid peroxidation, rising malondialdehyde (MDA) levels as an indicator of membrane damage. The role of RNS, mainly NO, is less understood. NO may play a significant role in delaying

germination during drought stress in some crops (Laxa *et al.*, 2019). ROS production also affects macromolecules as DNA. Nonetheless, plants possess effective antioxidant systems to control the excess of ROS (Hussain *et al.*, 2018).

- Hormonal regulation

Phytohormones such as ABA, cytokinins (CKs), gibberelins (GAs), auxins, jasmonates (JAs), strigolactones and ethylene (ET) play essential roles in the reaction to drought stress (Basu *et al.*, 2016). Normally, ABA and ET levels are enhanced, whereas endogenous contents of cytokinins, gibberellins and auxins decrease (Farooq *et al.*, 2009). ABA is the main hormone associated with water deficit. Under these conditions, endogenous ABA levels are increased in roots and shoots, leading to many changes in development, physiology and growth (Zingaretti *et al.*, 2013). It is ubiquitous in flowering plants and acts as a growth inhibitor, also changing relative growth rates (inhibition of leaf area development, increase in the root-to-shoot ratio, production of deeper roots, etc.). Moreover, it induces the expression of genes leading to the synthesis of metabolites that act as osmoprotectants (Basu & Rabara, 2017). As explained above, it also triggers stomatal closure, which is a measure for water saving and controlling transpiration rate. In addition, it acts as a signal inducing the expression of specific water stress-related genes (Farooq *et al.*, 2009).

A range of enzymes, cofactors and transporters regulate the formation, transport and activation of ABA. *De novo* biosynthesis occurs in plant roots, being translocated to the shoots. ABA is a relatively weak acid, easily crossing plasma membranes. However, different transporters, such as ABC proteins help in the translocation to foliar tissues. Under water deficit, the increased accumulation of ABA into guard cells activates the calcium permeable channels, which triggers Ca^{2+} import. Ca^{2+} influx finally upregulates calcium-dependent protein kinases (CDPKs) mediated signalling cascade (Basu & Rabara, 2017; Vishwakarma *et al.*, 2019).

Several other hormones and the crosstalk among them participate in the control of stomatal conductance during drought, although little is understood about the relationship among them. Generally, cytokinins, auxins and ET inhibit the effect of ABA in stomatal closure, while brassinosteroids, JAs and salicylic acids (SAs) support the effects of ABA. In particular, JAs contribute significantly to opening and closing of stomata under drought, interplaying with ABA. In contrast, ET is involved in the stimulation of stomata opening and leaf growth, inducing also senescence (Osmolovskaya *et al.*, 2018) in response to drought and preventing ABA accumulation (Basu & Rabara, 2017). ET may also affect yield by increasing embryo and grain abortion (Basu *et al.*, 2016).

CKs were demonstrated to delay premature leaf senescence and death under drought stress, which are useful adaptive traits for increasing yield (Basu *et al.*, 2016). In contrast, auxins have been shown to negatively impact drought adaptation. For instance, transcriptional levels of genes encoding late embryogenesis abundant (LEA) proteins, which are implicated in drought tolerance, were negatively related to indole-3-acetic acid (IAA) content. Although the role of GAs in drought tolerance needs further research, they have been suggested to positively regulate the adaptation to drought, resulting in the inhibition of plant growth (Basu *et al.*, 2016). SA was shown to be involved in the responses to drought stress (Miura *et al.*, 2013), also having a role in the increase of antioxidant defences (Nazar *et al.*, 2011) and regulating the synthesis of osmolytes (Li *et al.*, 2017).

1.2. Drought resistance strategies

The term drought resistance can be defined as the ability to maintain cell turgor and water balance during drought stress. The adaptive strategies to achieve this water deficit resistance are species specific and can be divided in three groups (Levitt, 1980):

- Escape

It consists in shortening plant life cycle or growing season, as a strategy to avoid the damages induced by drought stress, reproducing before the environment becomes dry. However, the decline in the length of crop duration usually negatively impacts yield. Another mechanism of drought escape is developmental plasticity. Plants that use this strategy experiment little grow during the dry period, developing few flowers and seeds, growing indeterminately during the wet season (Basu *et al.*, 2016). The strategy will be determined both by the environment and by the plant genotype (Farooq *et al.*, 2009).

- Avoidance

It is characterized by the ability of plants to maintain tissue water potential despite the water deficit; by increasing water uptake in anisohydric (water spenders) or restricting water loss in isohydric species (water savers). This mechanism ensures the maintenance of plant productivity during short periods of drought (Levitt, 1980; Basu *et al.*, 2016). Drought avoidance is often achieved through morphological changes in the plant. Thus, water savers adapt to drought by reducing transpiration, transpiration area, radiation absorption, among other modifications, and consequently reducing water loss. In contrast, water spenders increase root area and hydraulic conductance in order to maintain water uptake (Basu *et al.*, 2016).

- Tolerance

Tolerant species are those that can bear with low tissue water content through adaptive traits, minimizing the negative effect of drought (Levitt, 1980). The adaptive traits involve cell- and tissue-specific, biochemical, physiological and molecular mechanisms, including specific gene expression and accumulation of proteins. In the next section, the main mechanisms conferring plant drought tolerance are summarized.

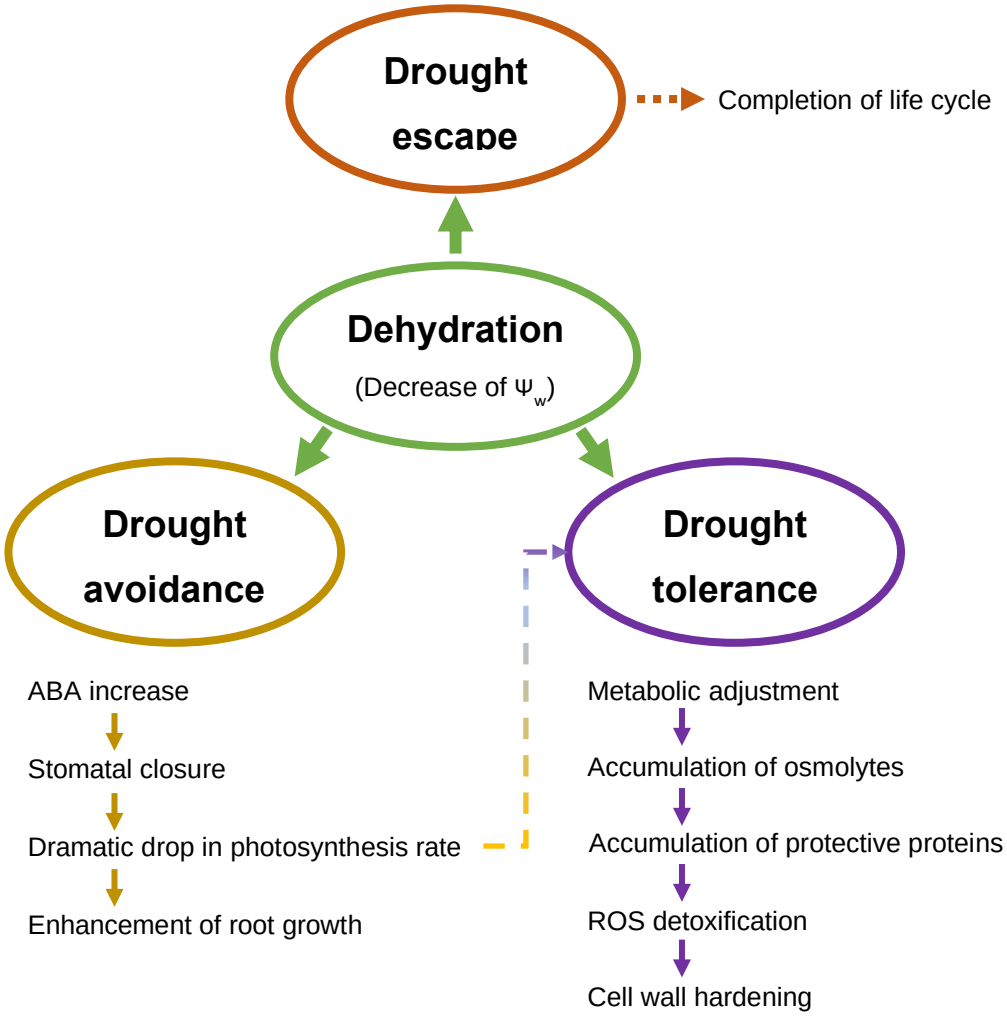


Figure 2. Plant strategies against dehydration. Modified from Osmolovskaya *et al.* 2018

1.3. Mechanisms conferring plant drought tolerance

- Antioxidant systems

Water deficit induces the overproduction of ROS, reacting with proteins, lipids and DNA and resulting in oxidative damage of plant cells (Zingaretti *et al.*, 2013). Generally, the production of ROS is linear with the severity of the stress imposed (Hussain *et al.*, 2018). In this context, the plant activates its antioxidant defence systems, which consists

in the concerted action of both enzymatic and non-enzymatic mechanisms. In fact, the up-regulation of antioxidant enzymes is considered an important marker for drought stress (Laxa *et al.*, 2019).

Antioxidant enzymes are one of the most efficient mechanisms mitigating the stress. They include catalase, superoxide dismutase, glutathione reductase, peroxidase or ascorbate peroxidase, among others. Non-enzymatic antioxidants comprise low molecular weight compounds such as ascorbic acid, cysteine, oxidized and reduced glutathione, α-tocopherol and carotenoids (Farooq *et al.*, 2009; Hussain *et al.*, 2018).

- Osmotic adjustment

Osmotic adjustment is the active over accumulation of different types of organic solutes and inorganic compounds in response to the reduction of water potential during drought stress, thereby helping to maintain cell turgor (Hussain *et al.*, 2018). Some of these osmotically active molecules/ions include soluble sugars, sugar alcohols, organic acids, glycinebetaine, proline, calcium, potassium and chloride ions, among others. Under drought stress, osmotic adjustment has been shown to maintain stomatal conductance, leaf water content, photosynthesis and consequently growth (Basu *et al.*, 2016). Moreover, greater accumulation of osmolytes may be related to a higher drought tolerance, although the effect depends on growth stage, plant type and stress severity (Hussain *et al.*, 2018).

Proline has been recognized as one of the main compatible solutes against abiotic stresses in higher plants, being also used in other organisms such as algae, bacteria and animals (Delauney & Verma, 1993). It is accumulated in younger leaves due to a combination of increase in biosynthesis and slow oxidation in mitochondria. A range of physiological functions have been assigned to this molecule, as stabilization of macromolecules or store of carbon and nitrogen for using after the water deficit period, although its functions are still under debate (Farooq *et al.*, 2009; Kavi Kishor & Sreenivasulu, 2014). However, its accumulation is a common marker of drought stress.

- Molecular mechanisms: Drought stress-related proteins

Water deficit induces the regulation of the expression of a number of genes in plants. Gene products of some genes induced under drought are thought to function in drought stress tolerance (Farooq *et al.*, 2009). Most of the proteins involved in sensing external stimuli like drought stress are receptors located in the plasma membrane, and aid to the regulation of plant-water relations (Priya *et al.*, 2019). Some transcription factors (TFs) play significant roles in coordination with receptors at the first steps of the drought period, controlling plant growth and development under water deficit. They include NAC, bZIP, WRKY, ZFs, APETALA and AP2/ERF, which link the *cis*-binding domain in the

promoter zone of genes encoding proteins with specific metabolic functions (Priya *et al.*, 2019).

Late embryogenesis abundant-related proteins (LEA), osmotins and dehydrins protect cells from dehydration.

LEA proteins, also termed 'hydrophillins', are involved in developmental activities such as shoot and root development or pollen grain formation. They protect other proteins from desiccation, aggregation or osmotic stress, although their specific functions are still not well established (Priya *et al.*, 2019).

Osmotins are activated during osmotic stress in plants, participating in osmotic adjustment under the regulation of ABA. They were suggested to have a role protecting chlorophyll molecules and photosynthetic apparatus during drought, and they also regulated ROS production and active antioxidant machinery (Priya *et al.*, 2019).

Dehydrins, a group of the LEA proteins family, represents another important group in plant abiotic tolerance. They are very abundant in plant embryos during embryo maturation and desiccation. However, during water deficit they accumulate in all vegetative tissues, enhanced by ABA contents. Thus, they can be used as molecular markers in drought stress responses (Priya *et al.*, 2019).

In addition, transporters are essential for maintaining cellular homeostasis under stress conditions. Drought stress tolerance is also related to the regulation of different ion channels, ABA-induced transporters and aquaporins, that regulate stomatal opening as well as hydraulic conductivity of the roots (Vishwakarma *et al.*, 2019). The role of aquaporins in the drought stress tolerance will be addressed in the next section.

2. Plant-water relations: The role of aquaporins

Aquaporins belong to a higher conserved super family of membrane proteins, the major intrinsic proteins (MIPs). They are ubiquitous channels of low molecular mass (around 26-35 kDa) that facilitate the passive flux of water and small solutes across cell membranes (in either direction) in all living organisms, which suggests their key role in basal life functions.

In plants, they constitute a large protein family and, apart from their functions in water and nutrient balance, they participate in important processes such as cell expansion, stomatal closure, long-distance signal transfer, pollen and seed development and tolerance to different abiotic stresses, including drought (Maurel *et al.*, 2015). They are present in almost all plant organs including seeds, roots, stems, leaves, flowers and fruits.

The high number of isoforms together with a complex regulation of their abundance, localization and gating difficult their study, and the understanding of their physiological roles *in planta* remains to be explored and further integrated with their functions. Besides, their central role in water relations has provided an improved understanding of the integrated mechanisms of water transport in roots (Chaumont & Tyerman, 2014).

2.1. Diversity and evolution of plant aquaporin isoforms

Phylogenetic analysis considered that the major split of MIPs was the division between water channel AQPs and glycerol transporters or aquaglyceroporins (GLPs) within archaea and bacteria (Groszmann *et al.*, 2017). Afterwards, the AQP family in plants underwent a great expansion, including among 30 and 70 isoforms in higher plants. This situation may be linked to subfunctionalization of paralogs in different tissues or neofunctionalization of the different isoforms (Abascal *et al.*, 2014). Horizontal gene transfer would also have contributed to the diversification of land plants, as the acquisition of the NIP subgroup (Fox *et al.*, 2017).

Plant AQPs are usually classified into seven subfamilies, based on sequence identity and/or putative sub-cellular localizations. In seed plants, there are five groups: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs) NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and unknown intrinsic proteins (XIPs) that have not been found in monocots and *Brassicaceae* so far. Two additional groups are found in mosses, hybrid intrinsic proteins (HIP) and GLpF-like intrinsic proteins (GIPs). The fact that these groups are only found in older lineages suggests a loss between the common vascular ancestor and seed plants. In ferns, however, the number of paralog groups is unknown so far (Abascal *et al.*, 2014; Laloux *et al.*, 2018).

Each subfamily could be further divided in different subgroups. PIPs diverged in two highly conserved groups (PIP1 and PIP2) prior to the emergence of terrestrial plants, and afterwards, a substantial proliferation occurred in both subgroups. TIPs present up to five paralog groups in seed plants, although an independent diversification in primitive plants gave rise to an additional group. Coinciding with their wide substrate specificities, NIPs are the most divergent subfamilies among the higher plant AQPs, which makes difficult to decipher the phylogenetic relationships with distantly related species. Four paralog groups were found in plants, whereas only NIP1 is exclusive of seed plants. SIPs split into two groups after the emergence of angiosperms, but compared to other subfamilies, suffered less diversification (Abascal *et al.*, 2014; Groszmann *et al.*, 2017).

- Diversity of maize aquaporins

The first study of maize aquaporins was based on expressed sequence tags (ESTs) and identified 36 expressed maize aquaporin genes comprising the four subfamilies present in monocot species: PIPs, TIPs, NIPs and SIPs (Chaumont *et al.*, 2001). Very recently, a genome-wide analysis identified 41 putative AQP genes in maize genome, containing 12 PIPs, 18 TIPs, 8 NIPs and 3 SIPs (Figure 3). PIP subfamily presented isoforms from the two subgroups conserved in plants, PIP1 and PIP2; TIP subfamily was divided in five subgroups (ZmTIP1-ZmTIP5); NIPs were divided in four subgroups (ZmNIP1, ZmNIP2, ZmNIP3 and ZmNIP7); and SIPs were distributed in ZmSIP1 and ZmSIP2 (Bari *et al.*, 2018).

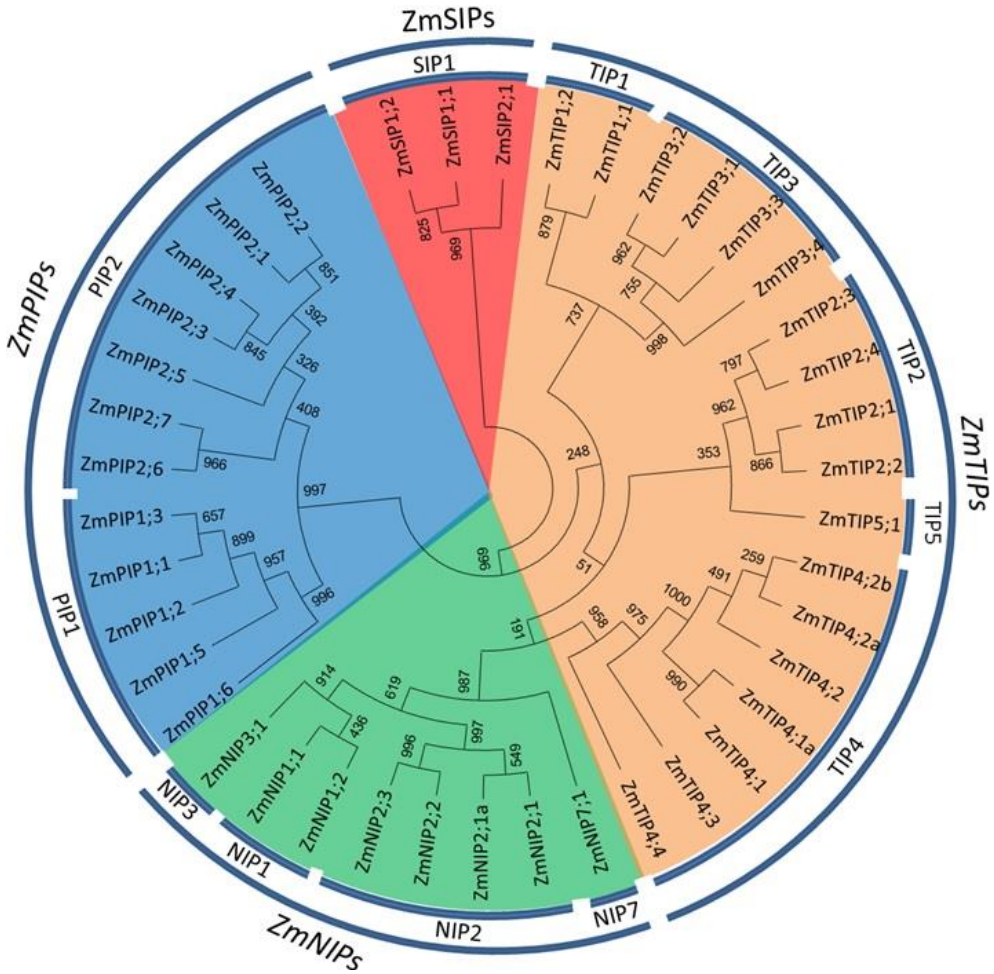


Figure 3. Phylogenetic tree of *Zea mays* L. aquaporins. From Bari *et al.* 2018

2.2. Subcellular localization of the different AQP subfamilies

Although the name of the subfamilies refers to the membrane where the group was firstly identified, they can be located in different cell compartments. In fact, some members exhibit a dual localization in distinct cell membranes, whereas other show polarized or

domain-specific expression (Luu & Maurel, 2013). Thus, PIPs are the most abundant in the plasma membrane (PM) but have been found in other membranes such as the chloroplast envelope (Uehlein *et al.*, 2008) or the ER of root elongating cells (Chaumont *et al.*, 2000). TIPs were initially thought to be exclusively vacuolar, but they can also exhibit multiple locations such as the plasma, chloroplast and thylakoid membranes (Groszmann *et al.*, 2017). NIPs were firstly localized to the peribacteroid membrane of nitrogen-fixing symbiotic nodules of legume roots. However, some members have been localized at the PM (Takano *et al.*, 2006) or the endoplasmic reticulum (ER, Mizutani *et al.*, 2006). SIPs have also been found in the ER (Ishikawa *et al.*, 2005). However, a transient localization on ER for most plant AQPs can be observed after transcription, and during the processes of translation and modification.

Moreover, polar distribution of some isoforms has been reported, although most AQPs show a uniform distribution at the cell surface. For instance, *At*NIP5;1, induced under boron limitation, is preferentially localized in the distal domain of the PM of root cells (Takano *et al.*, 2010). *Os*NIP2;1, considered a silicon channel, is also displaying a polar distribution (Ma *et al.*, 2006). *Zm*PIP2;5, *Os*PIP2;1 and *Os*PIP2;5 also showed a preferential polar distribution in maize and rice respectively (Hachez *et al.*, 2008; Sakurai-Ishikawa *et al.*, 2011).

In addition to this, most isoforms undergo a constitutive cycling, targeting to their membranes followed by the removal from the membrane and degradation or recycling in the endosome. This mechanism is highly regulated by different factors and abiotic stresses, which make the dynamics of sub-cellular localization a complex process (Chevalier & Chaumont, 2014).

2.3. Aquaporin structure and substrate specificity

- Structure

AQPs form tetramers in intracellular and plasma membranes, where each monomer constitutes an independent and functional channel and it is formed by six transmembrane helices (H1-H6) with N and C termini facing the cytosol (Chaumont & Tyerman, 2014). They also contain five loops (A-E) that connect the helices. Loop B and D are facing the cytosol and A, C and E are extracytoplasmic (Kapilan *et al.*, 2018). This structure delimits a pore with two selectivity filters: (1) One of the filters is formed by two NPA (Asn-Pro-Ala) motifs, located at the conserved loops B and E where it forms short helices. The pore forms hydrogen bonds with the water molecule and create an

electrostatic repulsion of protons, being among the most important features to maintain AQP function (Abascal *et al.*, 2014; Kapilan *et al.*, 2018).

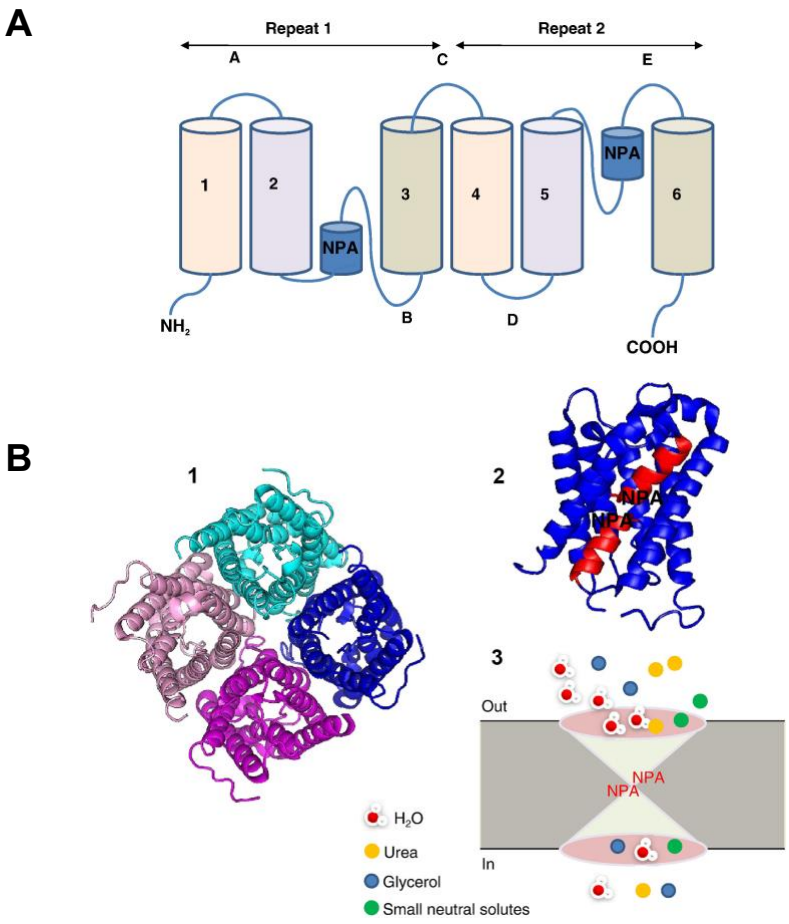


Figure 4. (A) Schematic diagram of an aquaporin monomer with six alpha helical domains (1-6) connected by five loops (A-E) with intracellular N- and C- termini. (B) Three-dimensional structure of spinach SoPIP2;1. 1) Lateral view of the tetramer, where each monomer functions as a single pore. 2) Loops B and E interact with each other through NPA motifs, participating to the pore selectivity. 3) Water and other small molecules can permeate some aquaporins. Modified from Gomes *et al.* 2009.

The high conservation of these NPA boxes serves for the identification of different members of the family. However, there have been found some exceptions to this NPA signature, where the alanine residue was replaced by other amino acids like leucine, valine, threonine, serine or cysteine in some maize and Arabidopsis NIPs and SIPs (Chaumont *et al.*, 2001; Johanson *et al.*, 2001). (2) The other filter is the so-called aromatic/arginine (ar/R) and it is formed by two aromatic amino acids and one Arg. This filter supposes the narrowest part of the pore, and is thought to be crucial for substrate specificity. Any substitution of amino acid in this filter can result in change of substrate specificity or loss of function (Mitani-Ueno *et al.*, 2011). The organization in tetramers

suggests the presence of a fifth pore in the centre of the structure, whose presence and role in solute transport is still under debate (Laloux *et al.*, 2018).

- Substrates

AQPs were first discovered as water channels in plants (Maurel *et al.*, 1993). However, they can facilitate the diffusion of a range of other small substrates. Until now it was described the diffusion of urea, ammonia, CO_2, H_2O_2, lactic acid, glycerol, metalloids, O_2, ions and Al-Malate (Fox *et al.*, 2017).

The channel substrate specificity is generally conserved within an AQP family. For instance, most of the plant PIPs facilitate water diffusion, TIPs allow the passive diffusion of water, glycerol, nitrogen compounds (urea, NH_3), and H_2O_2, and the NIPs the diffusion of metalloids (arsenite and boric acid), glycerol, lactic acid, urea and water (Maurel *et al.*, 2008). SIPs showed moderate water permeability and may also function in original pore conformation. XIPs are multifunctional channels with low water permeability, but permeable to metalloids and H_2O_2 (Bienert *et al.*, 2011; Afzal *et al.*, 2016; Groszmann *et al.*, 2017). CO_2 was demonstrated to be diffused by some PIPs with a function in stomatal opening and photosynthesis (Uehlein *et al.*, 2003; Groszmann *et al.*, 2017).

Recently, NtPIP1;3 was found to diffuse O_2 in yeast protoplasts and it increased its transcript levels in *N. tabacum* roots after hypoxia treatment (Zwiazek *et al.*, 2017). Additionally, the central pore has been suggested to permeate ions in human AQP-1 (Yu *et al.*, 2006), while the hydrophobic nature of this pore excluded the conductance of water and other neutral solutes (Murata *et al.*, 2000). Recently, plant AQPs were also shown to function as ion channels (Byrt *et al.*, 2017; Kourghi *et al.*, 2017). It is important to highlight that substrate specificity is usually tested in heterologous systems like *Xenopus laevis* oocytes, while the transport *in planta* may not correspond to the results obtained in those kinds of assays and its elucidation constitutes a major challenge to understand the physiological roles of aquaporins.

2.4. Mechanisms of aquaporins regulation

As membrane proteins, AQPs are synthesized in the endoplasmic reticulum (ER) and are transported across the secretory pathway (Golgi apparatus and different types of vesicles) to reach their target membrane. Once in membranes, their activity can be modulated by multiple mechanisms in order to control water and solutes homeostasis of cells. Gating of AQPs has been found to control the activity of the protein, and different factors affect the gating behaviour. Water permeability may also be modulated by membrane trafficking, which supposes a short-term regulation in response to external

stimuli. Both processes are subjected to different regulation mechanisms. Below, some of the main mechanisms of aquaporin regulation in plant membranes are explained.

- Gene expression

The function of AQPs is related to the abundance of the proteins. Although transcript level of a gene is not necessarily strictly related to the abundance and activity of a protein, these changes often reflect the protein abundance in a cell or tissue. Expression levels of AQPs may be altered by different abiotic factors such as drought, salinity, low temperatures or flooding. It can be also affected by different phytohormones. Due to the many signalling pathways than can affect gene expression and the complicated transcriptional, translational and posttranslational control of AQPs, it is difficult to distinguish specific expression patterns for each AQP isoform (Kapilan *et al.*, 2018). AQPs are also subjected to diurnal and circadian clock transcriptional regulation (Moshelion *et al.*, 2002; Lopez *et al.*, 2003).

- Posttranslational modifications

The response of the plant to changes in available water requires rapid regulation of membrane water permeability. Phosphorylation and dephosphorylation are considered key mechanisms regulating gating of aquaporins, and consequently their activity (Kapilan *et al.*, 2018). The open state is maintained by phosphorylation in different residues. However, it may also be a way to regulate protein trafficking (Chaumont *et al.*, 2005). Kinases and phosphatases are involved in this regulation. There have been found more than 70 different sites of phosphorylation in PIPs, TIPs and NIPs, where the loop B and the N- and C- terminal tails of AQPs are the important sites in water channel regulation, often involving serine residues (Santoni, 2017; Kapilan *et al.*, 2018).

Apart from phosphorylation, other posttranslational modifications have been found to modify AQP functioning, localization and degradation, such as N-terminal modification, deamidation, glycosylation, methylation or ubiquitination, although most of them are not fully understood and need additional research (Santoni, 2017).

- Heterotetramerization

Although monomers are considered the active unit, there are not evidences of free AQP monomers in the cell membranes (Fox *et al.*, 2017). Homotetrameric structures seem to be common in all AQPs. However, evidence of hetero-oligomers was only found in plants so far (Berny *et al.*, 2016). A synergistic effect of PIP1-PIP2 heterotetramers was first demonstrated in maize PIPs co-expressed in *Xenopus* oocytes (Fetter *et al.*, 2004). In this experiment, an increase in cell Pf was induced by the co-expression of inactive

PIP1s with active PIP2s, in comparison with cells expressing PIP2s alone. After that, the physical interaction of PIP1s and PIP2s was shown in maize protoplasts, revealing that it is required for *in planta* PIP1 trafficking (Zelazny *et al.*, 2007). Afterwards, other synergistic effects were found in several plant species (Berny *et al.*, 2016) and, in general, it seems to be a key regulatory process for the regulation of transport specificity and trafficking. However, heterotetramers may not be functional in all plant tissues or in some isoforms (Kapilan *et al.*, 2018).

- Plant hormones

The function of AQPs can be also regulated by plant hormones. In fact, expression of some AQPs was found to be regulated by gibberellins, ABA, cytokinins or auxins. ABA was shown to regulate AQP function and stomatal closure, as well as alter Lpr (Kapilan *et al.*, 2018). IAA acts through the auxin Response Factor 7 (ARF7) inhibiting the expression of most PIPs at both transcriptional and translational levels during lateral root formation (Péret *et al.*, 2012). Furthermore, salicylic acid induced PIPs internalization by a ROS-mediated mechanism in response to salt stress (Boursiac *et al.*, 2008).

- pH and cations

The inhibition by divalent cations (mainly Ca^{+2}) and/or protons could be a gating mechanism for adjusting channel functionality in different cell processes (Tournaire-Roux *et al.*, 2003; Kourghi *et al.*, 2017). For instance, during anoxia cytosolic acidosis is linked to a reduction of root cell water permeability, and His_{197} residue located in the loop D was related to the pH-mediated gating of AQPs (Tournaire-Roux *et al.*, 2003; Fischer & Kaldenhoff, 2008). Divalent cations are involved in gating by the anchorage of loop D onto the N-terminus, through ionic interactions and hydrogen bonds. This interaction is disrupted by the phosphorylation of Ser_{115}, which induces channel aperture (Laloux *et al.*, 2018).

- Chemical agents

Some chemical agents can act as AQP inhibitors blocking transport through the pore, being mercury one of the most common compounds. Mercurials (usually applied as $HgCl_2$) bind to the SH-groups of cysteine residues located at the NPA motif and block the pore, consequently inhibiting water flow. However, these compounds are highly toxic for the cells, inducing different collateral effects. Silver and gold can also be used as aquaporin inhibitors, and resulted to be more potent than mercurial ones (Niemietz & Tyerman, 2002). Sodium azide is also a common inhibitor of aquaporins, causing acidification of the cytoplasm and inhibiting phosphorylation, which leads to the closure of the channel (Tournaire-Roux *et al.*, 2003).

- Lipid environment

Lipid bilayer composition may affect AQP activity (Tong *et al.*, 2013; Kai & Kaldenhoff, 2014). The composition of the plasma membrane can change with environmental stresses such as drought or salinity. In this context, the modification of lipid membrane proteins may induce relocation or internalization of some AQP isoforms, although the effect on AQP activity is still unclear (Fox *et al.*, 2017).

2.5. Role of aquaporins in plant-water relations

A soil-plant-atmosphere continuum (SPAC) drives water flow by hydrostatic negative pressure maintaining the physiological functions of the plant under any stress imposed by a fluctuating environment. Axial water flow (from the root to the leaves) is mainly determined by vascular anatomy, without important mechanisms of membrane selectivity. However, radial water flow from root epidermal cells to xylem vessels or from these to the leaf cells and sub-stomatal cavity involves a higher resistance in order to control the water that enters the root or the water out flux through the leaf.

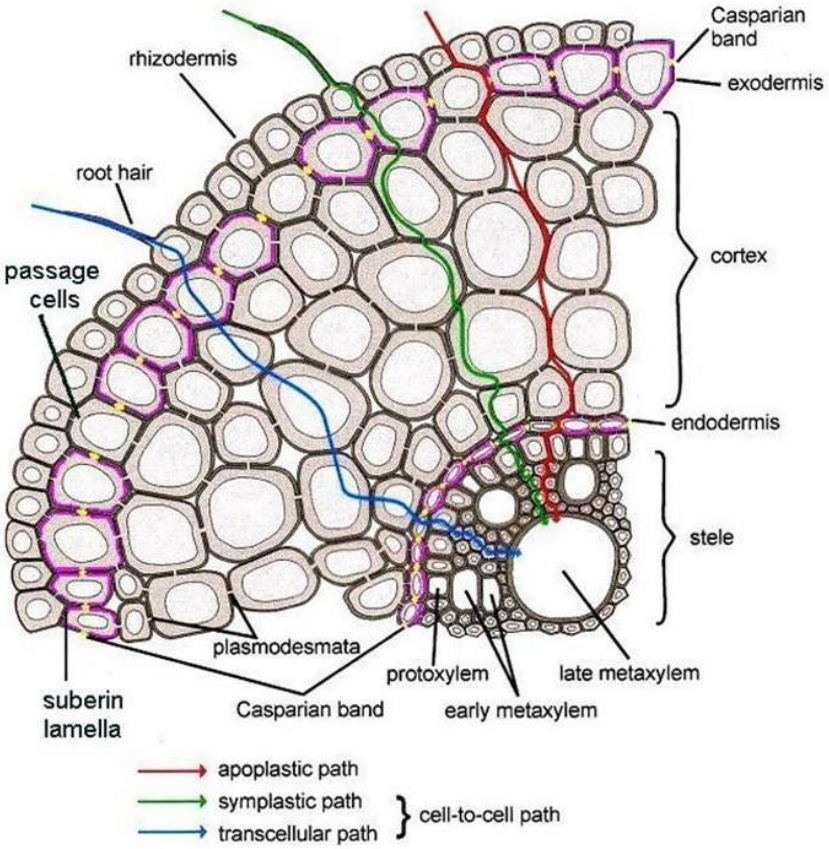


Figure 5. Diagram of a root cross-section showing the pathways of radial water and nutrient transport including the endodermal and exodermal barriers. Modified from Kim *et al.* 2018.

The radial flow of water through the plant can take three different parallel routes: (1) the apoplastic pathway, along the apoplast, (2) the symplastic route, crossing the cell through the plasmodesmata, and (3) the transcellular pathway, across the cell membranes (Steudle & Peterson, 1998). During transpiring conditions, long-distance bulk flow of water and sugars takes the apoplastic pathway through vascular bundles. Under these conditions, water is driven by the gradient of water potential ($\Delta\Psi$) and ascends through the xylem and phloem by capillarity.

Under water limiting conditions, when transpiration is restricted, short-distance non-vascular water movement across cellular membranes is crucial to maintain turgor pressure and cell water homeostasis (Vadez *et al.*, 2013a). In addition, the root is composed by different layers arranged in series (exodermis, cortex, endodermis and stele) that also affect the water transport capacity of the root (Meyer *et al.*, 2011; Ranathunge *et al.*, 2017; Kreszies *et al.*, 2018a). In particular, the endodermis is the main limiting boundary for water and ion flow within the root, as the hydrophobic Casparian strip restricts passive apoplastic diffusion (Figure 5).

In this context, AQPs play a key role in hydraulic regulation (Chaumont & Tyerman, 2014). The expression of root AQPs was reported to be regulated by the transpiratory demand of the shoot (Vandeleur *et al.*, 2014). In fact, AQPs have been shown to act as regulators of plant cell water relations in osmoregulation, root hydraulic conductivity (Lpr), leaf hydraulic conductivity, transpiration and cell elongation (Tournaire-Roux *et al.*, 2003; Hachez *et al.*, 2006b, 2012; Maurel *et al.*, 2009). According to this, it is widely accepted that PIPs and TIPs mainly mediate water uptake and transcellular water flow in roots of most plant species. In particular, PIPs have a major role in controlling changes in Lpr (Aroca *et al.*, 2012; Kapilan *et al.*, 2018) and TIPs in the regulation of cellular water homeostasis by fast water exchange between the vacuole and cytoplasms of plant cells (Sade & Moshelion, 2017).

2.6. Plant aquaporins in water stress

Due to the central role of aquaporins in water relations, the regulation of water transport during drought has been the object of many studies. The cell is very sensitive to the changes in cell turgor caused by water deficit. In consequence, the osmotic stress produced requires the regulation of membrane water permeability, a process in which AQPs are involved. In fact, numerous reports link AQP regulation with drought tolerance in plants (Deshmukh *et al.*, 2017).

PIPs seem to have a key role in the modulation of Lpr in response to different environmental stimuli (Aroca *et al.*, 2012; Qian *et al.*, 2015), being the most responsive to drought stress and usually undergoing down-regulation of their transcript levels (Afzal *et al.*, 2016). In addition, different studies also highlight the contribution of TIPs under water deficit conditions (Lin *et al.*, 2007; Sade *et al.*, 2009; Xu *et al.*, 2013). However, the involvement of NIPs, SIPs and XIPs in drought stress responses was less studied (Afzal *et al.*, 2016). Additionally to higher water retention, overexpression of some isoforms may induce drought tolerance through the reduction of ROS accumulation, membrane damage and an enhanced antioxidant activity (Zhou *et al.*, 2012).

Generally, when the drought stress imposed is short, plants reduce the expression/activity of AQPs as a measure to conserve their water content. Opposite to this, during a long-term stress a higher expression/activity of these proteins occur to reach water requirement of the plant (Chaumont & Tyerman, 2014). Stress can also affect the membrane trafficking of AQPs (Kapilan *et al.*, 2018). However, the regulation depends on the isoform, nature and duration of the stress, species and also specific organ and developmental stage (Qian *et al.*, 2015; Feng *et al.*, 2018), appearing to be highly complex. In fact, AQPs from different cultivars of a same species can behave differently under stress conditions (Lian *et al.*, 2006b; Grondin *et al.*, 2016).

It seems that phytohormones like ABA are also involved in the drought stress-induced aquaporin regulation. Under these conditions, ABA enhanced the expression of some AQP isoforms (Hose *et al.*, 2000; Parent *et al.*, 2009; Veselov *et al.*, 2016; Ding *et al.*, 2016; Li *et al.*, 2016) which consequently increases Lpr, although there is not much information regarding the integration of AQPs, Lpr and ABA across different time scales during drought stress (Gambetta *et al.*, 2017).

The role of aquaporins in the permeation of other physiologically important compounds such as N compounds, metalloids, glycerol or other signalling molecules has been suggested to be important for the drought stress tolerance (Bárzana *et al.*, 2014). For instance, transport of silicon by NIPs (Sonah *et al.*, 2017) or diffusion of H_2O_2 by some TIPs (Bienert *et al.*, 2007) may be related to drought stress tolerance. Nonetheless, the implication of different isoforms in the transport *in planta* of these compounds needs further research.

3. Use of beneficial microorganisms in agriculture: Arbuscular mycorrhizal symbiosis

Plants are in constant interaction with different microorganisms in the rhizosphere. These interactions can be deleterious for the plant or positively affect plant fitness. Mycorrhizas are mutualistic symbioses between certain soil fungi and roots of about 90% of land plant species, including grasses, forest trees and the majority of crops (Bonfante & Genre, 2010).

There are different types of mycorrhizal associations that involve different groups of fungi and plants. The main categories, ecto- and endomycorrhizas are classified depending on whether the fungus develops intercellularly in roots or colonizes the host cells (Bonfante & Desirò, 2015). Thus, in ectomycorrhizas, the fungus (belonging to Basidiomycota or Ascomycota) forms a mantle around the roots of about 3% of higher plants. The mycelium penetrates the root and develops between the epidermis and the cortex cells forming the so-called 'hartig-net', where the nutrient exchange between partners is produced. Endomycorrhizas are characterized by the colonization of root cortex cells and are further divided into ericoids, orchids and arbuscular mycorrhizas. A third class, the ectendomycorrhizas correspond to an intermediate group between the two already mentioned.

Arbuscular mycorrhizas (AM) are the most widespread and ancient symbioses in the plant kingdom, forming associations with the vast majority of terrestrial plants, including most crops (Smith & Read, 2008). Mycorrhizal roots are able to enhance the uptake of water and nutrients in the soil and to protect them against a range of biotic and abiotic stresses. Among them, these fungi are well known for conferring drought stress tolerance in different plants species. Thus, the use of AM inoculants supposes an environmentally friendly alternative to the use of agrochemical fertilizers, pesticides and herbicides in agriculture.

Key aspects about their biology, ecological function and physiological effects in plants are summarized in the coming sections.

3.1. Evolution, phylogeny and classification of AM fungi

AM fungi appeared in the Ordovician, around 480 million yr. ago with the emergence of early land plants, constituting the first wave of mycorrhizal evolution and probably the oldest symbiosis between fungi and land plants (Delaux, 2017; Brundrett & Tedersoo, 2018). The first known fossils, the Rhynie chert, dated to 407 million yr. ago

(Strullu-Derrien *et al.*, 2014) and additional data suggest that these fungi helped in the transition of plants to land ecosystems and in root development (Selosse *et al.*, 2015). Supporting this hypothesis, today mycorrhizal fungi are also found in basal plant lineages without a true radical system like hornworts, liverworts and ferns. All members of the clade require a photosynthetic partner to complete their life cycle, which suggests that the ancestral mycorrhizal fungi were already biotrophs (Parniske, 2008).

These fungi belong to the subphylum *Glomeromycotina* (previously classified in a monophyletic group, *Glomeromycota,* Schüßler, Schwarzott & Walker 2001) that belongs to the phylum *Mucoromycota*, which are classified as *zygomycete* fungi according to recent molecular evidence (Spatafora *et al.*, 2016). However, their phylogeny and taxonomy have been constantly discussed and they are still under debate due to their unique biological traits. According to the more recent consensus, the subphylum *Glomeromycotina* contains four orders: Archeosporales, Diversiporales, Glomerales and Paraglomerales. Altogether, these groups contain 11 families divided in 25 genera with about 250 described species, although new fungal species are frequently described (Redecker *et al.*, 2013; Bonfante & Desirò, 2015). These number of AM fungal taxa associate with around 200000 plant species (Brundrett, 2009), which means that host specificity must be very low, although some host preferences and selectivity have been reported (van der Heijden *et al.*, 2015).

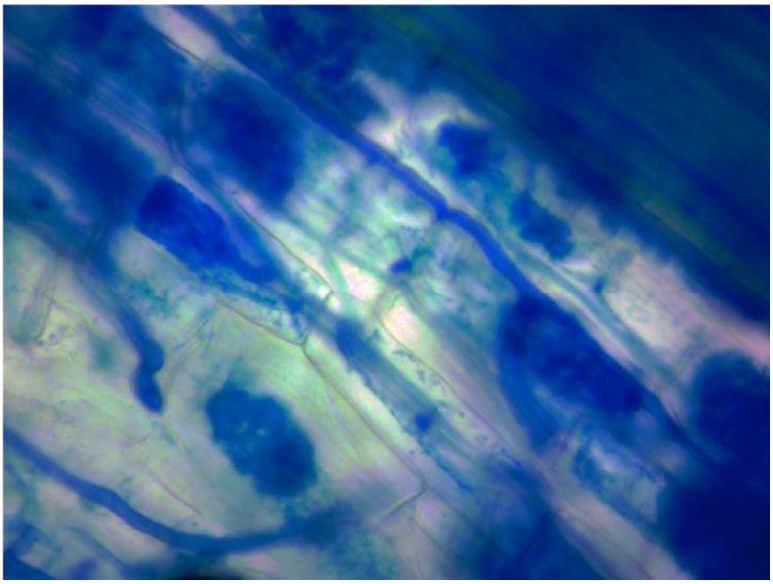


Figure 6. Maize cortex cells colonized by *R. intraradices* showing arbuscules and intraradical hyphae. Stained with Cotton Blue and visualized with bright-field microscopy at 40x.

AM fungi are aseptate and filamentous fungi (Spatafora *et al.*, 2016) with, as explained above, obligate biotrophy. The concept of species and individual are poorly defined in this group of fungi. They contain thousands of nuclei, which share a common

cytoplasm in their spores and coenocytic hyphae. Despite the indirect evidence of a sexual cycle through the finding of meiotic genes (Bruns *et al.*, 2017; Corradi & Brachmann, 2017), until date most of the studies indicate that they are asexual organisms with clonal reproduction, although anastomosis can occur between genetically distinct strains for genetic exchange (Croll *et al.*, 2009). Moreover, AM fungi contain endobacteria inside their cytoplasm, which increases more their genetic complexity (Bianciotto *et al.*, 2003; Bonfante & Desirò, 2017).

Depending on the morphological characteristics of the colonization process we can differentiate two classes: Arum and Paris types (Dickson, 2004). The former type forms intercellular hyphae, vesicles and the 'typical' arbuscules. The Paris type also forms intercellular hyphae and vesicles but also coils or arbusculated coils inside cells. Both morphological types depend on the combination of fungus and host plant, although it seems than Paris type is the most common in nature (Cosme *et al.*, 2018). Nonetheless, coexistence of both colonization types have been reported for some species (Kubota *et al.*, 2005).

3.2. Life cycle

As obligate biotrophs, AM fungi need the host plant to complete their life cycle, which provide physical support and metabolic machinery for the symbiotic establishment. The cycle is initiated in the rhizosphere, where a complex molecular dialog is established for the recognition of the fungus and the plant. Both symbiotic partners present a high degree of genetic and metabolic coordination.

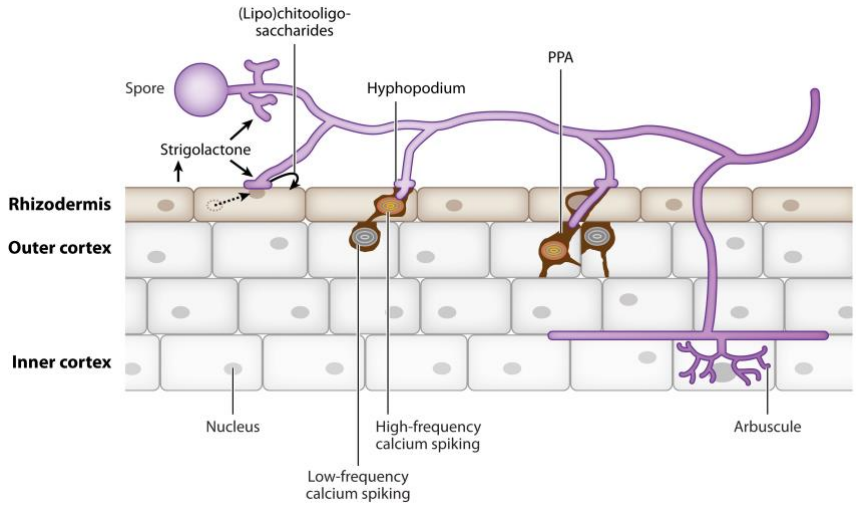


Figure 7. Steps of fungal root colonization. From Gutjahr & Parniske 2013.

The process of symbiotic colonization can be divided in three main steps: (1) asymbiotic hyphal growth, where the spores can germinate in the soil in the absence of a host root and develop hyphae during a short period, (2) presymbiotic growth, where host perception stimulates hyphal growth; and (3) symbiotic life, where the fungus colonizes the plant root and develops extraradical mycelium (ERM), to take up soil nutrients and water, and intraradical mycelium (IRM), to exchange nutrients between both partners (Figure 7).

- Asymbiotic phase

During this phase, the spores can germinate in the absence of the host plant, but as their reserve of lipids are few, they are only capable of a limited growth. Generally, the spores are dormant in the soil until environmental conditions are favourable to colonize new plants. The lack of plant signals retracts hyphal cytoplasm, which will be used in a future germination attempt.

- Presymbiotic phase

Mycorrhizal colonization starts when the fungal hyphae start to explore the soil in order to find a compatible host. By its part, the host plant exudates to the rizhosphere strigolactones, root-borne phytohormones produced by low Pi conditions that induce a set of fungal responses such as spore germination, hyphal growth and branching, and release of molecules that trigger the symbiotic response in the plant (Waters *et al.*, 2017). Other classes of AM-stimulating factors were recently identified to be exuded by the host, as 2-hydroxy fatty acids, that induce the elongation of lateral branches in the primary hyphal germ tube (Nagahashi & Douds, 2011) and cutin monomers, that were suggested to promote hyphopodia formation and intraradical hyphae elongation (Wang *et al.*, 2012; Gobbato *et al.*, 2013). At the same time, the fungus produces other diffusible elicitors, the 'Myc factors', which are essential for recognition of the fungal partner. These factors include lipochitooligosaccharides (LCOs) (Maillet *et al.*, 2011) and short-chain chitin oligomers (COs), which elicit nuclear Ca^{+2} spiking in roots (Genre *et al.*, 2013). However, the biological significance of producing these molecules remains elusive (Lanfranco *et al.*, 2018). Both factors are recognized by plant receptors and trigger the 'common symbiosis signalling pathway' (CSSP), that is common to the Rhizobium symbiosis with legumes, and prepares the root for the interaction (Schmitz & Harrison, 2014).

- Symbiotic phase

It starts with the physical contact of both partners. The hypha in contact with the root develops a hyphopodium or appressorium that serves to be attached to the root surface. Consequently, and after 4-5 hours, the epidermal cells suffer several

reorganization events resulting in the pre-penetration apparatus (PPA) that allows the entrance of the hypha through the plant cell (Genre *et al.*, 2005, 2008).

Previous to the formation of the PPA, the nucleus migrates towards the point of fungal entry, moving ahead with the PPA formation. This apparatus constitutes a cytoplasmic bridge formed by microtubules and microfilaments that crosses the vacuole of the root cell. When the 'tunnel' is completed, the fungal hypha can penetrate the host cell (Parniske, 2008).

Within root cortex cell, hyphae form the highly branched structures, called arbuscules due to their tree-shape, where the symbiotic exchange is produced. The fungus is always excluded from the cell cytoplasm by a plant-derived periarbuscular membrane (PAM). This PAM has a distinct protein composition to the plasma membrane that allows the nutrient exchange, and their transporters are key biotechnological targets. Arbuscules have a short lifespan (around 8.5 days), and a single cell can be colonized several times. These structures undergo a phase of growth until the maximum size, when they start to collapse until disappearing, a process that is partially controlled by the plant (Javot *et al.*, 2007).

3.3. Nutrient and water exchange

After symbiotic establishment, both partners benefit from nutrient supply by the other. This exchange has a nutritional aim, but it can also be a signal for AM development (Lanfranco *et al.*, 2018). Mycorrhizal symbioses are believed to play a key role in the global carbon (C) cycle, through the transfer of photosynthetically fixed plant C to the fungal symbiont. Plants transfer from 10-20% to 50% of the photosynthesized sugars to the fungus, mainly hexoses as glucose (Field & Pressel, 2018). However, it was recently found that sources of carbon for the AM fungi may include fatty acids from the host plant, as genes encoding cytosolic fatty acids (FA) synthase subunits are absent in AM fungal genomes, suggesting the fungal uptake of these compounds (Wewer *et al.*, 2014; Jiang *et al.*, 2017).

In exchange, the fungus provides the plant with up to 80% of the required nitrogen (N), 100% of the phosphorus (P), sulphur and micronutrients that are needed for correct growth and development of the host plant (Hoysted *et al.*, 2017). This successful nutrient transfer is due to the efficiency in exploring and acquiring these resources from the soil through the extraradical hyphae. These hyphae can be up to 100 times longer than root hairs, extending considerably the nutrient exploitation zone around roots, and they can

mobilize limiting nutrients in the soil. Once nutrients are taken up by the ERM, they are moved to the IRM in a package form, which is faster than diffusion (Parniske, 2008).

The symbiotic interface constituted by the arbuscules and the surrounding periarbuscular membrane of the host increases considerably the contact surface between the two partners. In addition, this area is acidified, allowing the active nutrient transport across the membranes (Krajinski *et al.*, 2014; Wang *et al.*, 2014). Therefore, arbusculated cortex cells are ideally suited for nutrient exchange.

- Phosphorus nutrition

Phosphorus is the second macronutrient after nitrogen; however, mineable phosphorus (dihydrogen phosphate ion, $H_2PO_4^-$) is considered a non-renewable resource with low mobility, that rapidly forms a depletion zone in the root, being the most limiting macronutrient in agroecosystems (Peñuelas *et al.*, 2013). However, ERM greatly increases the absorbing surface area beyond the Pi depletion zone. In addition, AM fungi were also suggested to mineralized organic forms of P present in the soil (Lanfranco *et al.*, 2018). For this reason, the use of AM fungi is an interesting alternative for increasing the use of P available in the soil, improving nutritional status of crops.

Once absorbed by the ERM, Pi is transformed to ATP in the mitochondria and rapidly converted inside vacuoles to polyphosphate (polyP) chains, which contain hundreds of Pi molecules. Then, polyP is translocated to the IRM through the cytoplasmic streaming and/or along a motile tubular vacuolar network. Once in the arbuscules, polyP is hydrolysed to Pi, transported to the cytosol and released to the apoplast. Nonetheless, the mechanisms involved in Pi transport from the arbuscules remain unclear (Ferrol *et al.*, 2019).

Some plant Pi transporters in the periarbuscular membrane are responsible for the exchange of Pi between the two partners (Javot *et al.*, 2007) and were shown to be AM-inducible (Berruti *et al.*, 2016). The role of these transporters in the establishment of a successful colonization has been commonly suggested, as the lack of function of AM-inducible transporters in different plants impairs arbuscules formation (Salvioli & Novero, 2019). These PTs may act as transceptors, sensing the phosphate status apart from transporting it.

- Nitrogen nutrition

Nitrogen is an essential macronutrient, as it is part of a number of macromolecules such as nucleic acids, proteins, some polysaccharides and a range of secondary metabolites. Even if N nutrition is not as well studied as P nutrition, it is known that AM

fungi can transport the main mineral sources of N present in the soil, nitrate and ammonium (NO_3^- and NH_4^+). Although it seems that its direct acquisition is normally not limiting for the plant, as in the case of phosphate (Jansa *et al.*, 2019), recent studies suggest a five times higher affinity of the fungal uptake system compared to the plant system for NH_4^+ acquisition (Pérez-Tienda *et al.*, 2012). Moreover, these fungi can also accelerate decomposition and directly acquire nitrogen from organic material (small peptides and amino acids) as manure, compost or organic wastes (Lanfranco *et al.*, 2018).

Uptake from the soil by the ERM is followed of long-distance transport until the arbuscules. When NO_3^- is taken up by the ERM it is reduced to nitrite and then transformed into NH_4^+ by a nitrite reductase. The latter reduction or the NH_4^+ directly taken up by the fungus is then assimilated into amino acids following two pathways: (1) the NAD(P)-glutamate dehydrogenase or (2) the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway. The latter generates arginine, the most abundant amino acid in the ERM (Jin *et al.*, 2005). This molecule is transferred then to the IRM where it is split into urea and ornithine. Furthermore, NH_4^+ is produced from urea by the urease activity, and then released to the symbiotic interface to be incorporated into other free amino acids (Jin *et al.*, 2005; Salvioli & Novero, 2019). Similar to Pi transporters involved in the uptake from the arbuscules, AM-inducible ammonium transporters have also been identified (Guether *et al.*, 2009b), and a role in the maintenance of the arbuscules has been suggested (Breuillin-Sessoms *et al.*, 2015).

- Carbon metabolism

As obligate biotrophs AM fungi obtain most of their carbon from the host plant. Despite its importance for the symbiosis, the transport of C in the arbuscules is not as clear as P transport (Parniske, 2008). AM roots are considered strong C sinks, obtaining sucrose from photosynthetic tissues that is cleaved in the vicinity of colonized cortex cells by cytoplasmic invertases or sucrose synthases (Schaarschmidt & Hause, 2008). As a result, glucose and fructose are released and taken up by the IRM. However, recent studies suggest the role of SWEETs (a novel type of sugar transporters) in the direct export of sucrose, that would be latter converted to glucose and fructose by cell wall-bound invertases (Manck-Götzenberger & Requena, 2016).

Glucose has been established as a main carbohydrate substrate for AM fungi (Bago *et al.*, 2000), nonetheless, the discovery of the lack of *de novo* fatty acid biosynthesis enzyme (the type I FAS) in AM fungi opened the question of the C fungal uptake in form of fatty acids together with sugars (Wewer *et al.*, 2014; Jiang *et al.*, 2017). This is consistent with the fact that lipids are the main storage form of C in AM fungi (Rich

et al., 2017). However, currently there is no direct evidence of fatty acid uptake by the fungus.

- Micronutrient nutrition

AM fungi also contribute to the uptake of micronutrients that have low mobility in the soil, like Zn, Cu, Fe and Mn. The improved nutrition is mainly achieved by the higher capability of the external mycelium to explore the soil. Although AM fungi probably help in the transport of other micronutrients, their role is not well established so far (Ferrol *et al.*, 2016).

- Water acquisition

Enhancement of plant water content due to the transport along fungal hyphae is one of the main features of the AM symbiosis. One of the most common explanations for the improved water status and physiology in mycorrhizal plants is the strong increased absorbing surface caused by soil growing hyphae combined with the fungal capability to take up water from soils with low water potential (Lehto & Zwiazek, 2011; Ruiz-Lozano *et al.*, 2012c). Thus, the hyphal contribution to the total plant water uptake has been estimated to be at least 20% (Ruth *et al.*, 2011). However, although water transport through fungal hyphae has been hypothesized many times and inferred from indirect measures, water movement was not directly demonstrated.

It seems that water moves through fungal and root cells along potential gradients created by transpiration. Recently, a new model for polyP translocation through fungal hyphae towards the plant has been proposed (Kikuchi *et al.*, 2016). This model highlights the importance of water flow to passively direct P towards the plant by the transpiration stream, not being energy-driven by the fungus. A fungal aquaglyceroporin (*RcAQP3*, ortholog of *RiAQPF2*) localized in the fungal plasma membrane and probably upregulated in arbuscules would mediate the water flow across the membrane.

3.4. Other benefits of the AM fungus in the plant-soil system

Apart from nutrient acquisition, there are numerous benefits of the AM symbiosis for the host plant (Figure 8). Here, we summarize the most important, although probably other remains still unknown:

- Protection against abiotic stresses

AM fungi are well studied for their role in drought and salt stress tolerance, as well as, against heavy metal (HM) toxicity. However, they may be also important for alleviation of other abiotic stresses such as extreme temperatures or flooding. The mechanisms of

salt stress tolerance by AMF can be due to an improved water use efficiency (WUE) and nutrient uptake of these plants, better ion balance, higher production of osmolytes, enhanced photosynthesis and antioxidant production, among others (Ruiz-Lozano *et al.*, 2012a; Saxena *et al.*, 2017). In the case of heat stress, the AM protection was related to the photo-protection of the photosynthetic apparatus, better WUE and higher chlorophyll content and decreased stomatal resistance, increasing CO_2 assimilation and transpiration fluxes (Latef *et al.*, 2016). The AMF-mediated plant cold tolerance can be explained by an enhanced uptake of nutrients and water status, higher antioxidant activity and enhanced osmotic adjustment and gas exchange capacity (Latef *et al.*, 2016) in a similar way to other abiotic stresses. The effect of AMF in waterlogging was not as extensively studied as other environmental stresses, however, AM plants enhanced Lpr on flooded tomato plants, affecting also to the aquaporin expression, phosphorylation state and hormonal status (Calvo-Polanco *et al.*, 2014). Lastly, the (HM) toxicity alleviation is achieved by the development of strategies for the homeostasis of these components like chelation, storage in organelles, efflux, long-distance transport or changes in rhizosphere pH (Garrido *et al.*, 2010; Ferrol *et al.*, 2016).

The mechanisms of drought stress tolerance will be addressed in the next section.

- Protection against biotic stresses

AM fungi can also act as effective biocontrol agents (Whipps, 2004), mainly against root pathogens (Sikes, 2010) above- and belowground herbivores (Pozo & Azcón-Aguilar, 2007) and nematodes (Schouteden *et al.*, 2015; Wani *et al.*, 2018). There are different mechanisms that play a role in the AM fungi-plant protection: better plant nutrition of AM plants, fungal competition for photosynthates or ecological niche, changes in the architecture, morphology or exudates of the root, reprogramming of plant gene expression (Liu *et al.*, 2007) or priming of the plant defences (Pozo & Azcón-Aguilar, 2007).

- Improvement of soil structure

The huge hyphal network of the AM extraradical mycelium creates a three-dimensional matrix that crosslinks soil particles without compacting the soil. In addition, a soil glycoprotein, glomalin, that is thought to be produce by AM fungi, helps in the stabilization of soil aggregates (Bedini *et al.*, 2009). These direct and indirect effects of AM presence also result in higher water retention capacity (Augé, 2004), protection from erosion and consequently, reduced nutrient leaching (Chen *et al.*, 2018).

- Ecosystem biodiversity and functioning

In natural ecosystems, AM fungi play important roles as the uptake and transfer of nutrients, modification of soil environment, alteration of plant interactions with other biota, plant community structure and homeostasis and ecosystem functioning (Wang, 2017; Powell & Rillig, 2018) The symbiosis is normally advantageous for plants, as these ecosystems are often characterized by different environmental stress factors. Thus, AM symbiosis could be conceived as an evolutionary engine promoting intraspecific competition. However, the common mycorrhizal networks (CMNs) that connect plants and AM fungi with different partners at the same time add complexity to the analysis (Chen *et al.*, 2018). The effect of this network cannot be generalized, however in some cases it serves to attenuate the differences among individuals in the plant community, where stronger individuals can benefit weaker plants. This phenomenon is known as facilitation (Van Der Heijden & Horton, 2009). These AM networks participate in the internal cycling of nutrients and facilitate bacterial dispersion, increasing biodiversity of the plant community. Furthermore, AM fungi may play potential roles in ecosystem restoration of degraded soils and desertified areas (Wang, 2017).

3.5. AM symbiosis and drought stress tolerance

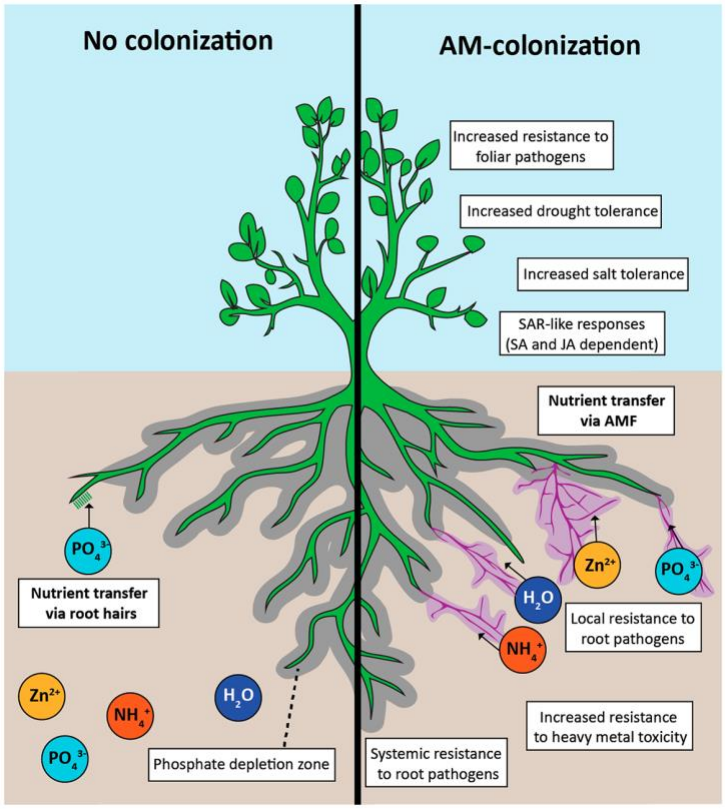


Figure 8. Potential benefits of AM colonization. From Jacott *et al*. 2017

Arbuscular mycorrhizal fungi have a crucial role in overcoming osmotic stresses (water and salinity), as explained above, but here we will focus on their effect under water deprivation.

The increased plant drought tolerance by AMF was demonstrated in numerous studies with different host plants and fungal species (Aroca *et al.*, 2008b; Chitarra *et al.*, 2016; Yooyongwech *et al.*, 2016; Essahibi *et al.*, 2017; He *et al.*, 2019). The positives effects of the symbiosis will also depend on the duration and the severity of the stress imposed.

Below, we briefly explain the best-known mechanisms of the AM-enhanced plant drought tolerance:

- Increase of the uptake surface

The formation of extensive hyphal networks together with the improved moisture retention properties of the soil (mainly due to the secretion of glomalin) are direct effects of AM fungi increasing water and nutrient uptake (especially P) of plants under water deficit. Indeed, studies have demonstrated that as the soil dries and water is retained only in smaller pores where fungal hyphae can grow, but roots cannot, the water uptake function of hyphae becomes more significant for plant survival and development (Allen, 2007).

- Growth promotion and enhanced plant gas exchange

As a consequence of the better plant hydration, water use efficiency (WUE) and cell turgor are increased, which in most cases results in a promotion of the photosynthetic capacity and plant growth (Andreo-Jimenez *et al.*, 2015). Transpiration rates and stomatal conductance are often increased in AM plants, although the effects on stomatal behaviour are very variable depending on the plant and fungal species involved. Moreover, it was observed that the AM symbiosis alters stomatal conductance of host plants more under drought that under well-watered conditions (Augé *et al.*, 2015). In addition, the symbiosis may also affect stomatal density, which consequently increases the capacity to absorb CO_2 and enhances photosynthesis, representing an advantage in drought stress conditions (Chitarra *et al.*, 2016).

- Better osmotic adjustment

As explained above, osmotic adjustment allows cells to maintain turgor and the processes that depend on it, such as cellular expansion and growth, stomatal opening and photosynthesis, as well as keeping a gradient of water potential favourable to water entrance in the plant. Thus, one of the main mechanisms to maintain cell hydration and

water uptake is the decrease of the plant osmotic potential by the accumulation of organic ions and compatible solutes.

The accumulation of osmolytes in AM plants is complex and has shown contradictory results. Some studies show an increase in proline, sugars or starch accumulation under drought, although in others opposite results were observed, depending on the plant tissue analysed and the AM fungus involved. Generally, despite the different responses, AM plants show a better osmotic adjustment and consequently enhanced water status when submitted to water deficit than non-AM plants (Ruiz-Lozano *et al.*, 2012c).

- Induction of antioxidant systems

AM plants usually exhibit reduced ROS production under drought, which in turns stabilize proteins and enzymes and reduces damage to lipids. This effect can be achieved by the improved production of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), glutathione reductase (GR), catalase (CAT) or ascorbate peroxidase (APX). SOD activity was the most studied in relation to the AM symbiosis and numerous studies reported an enhanced activity of this enzyme by the fungus (Ruiz-Lozano *et al.*, 2012c). In fact, fungal CuZn-SOD activity was found in *G. mosseae* and mycorrhizal roots exhibit two additional SOD isoforms compared to non-mycorrhizal ones (Palma *et al.*, 1993). Moreover, mycorrhization increased SOD activity in mycorrhizal lettuce plants under water deficit (Ruiz-Lozano *et al.*, 1996, 2001). In *C. glauca* seedlings subjected to drought, AM fungi significantly increased SOD and peroxide enzymes activities (Zhang *et al.*, 2014).

Enhanced production of non-enzymatic antioxidant compounds has also been reported in different plant species (Filho *et al.*, 2017; Santander *et al.*, 2017). Thus, higher levels of ascorbic acid and reduced glutathione were found in different AM plants, which were related to minor oxidative damage to lipids (Ruiz-Lozano *et al.*, 2012c). Results suggest that activation of antioxidant systems may be a key mechanism for the induced drought tolerance by the AM symbiosis.

- Alteration of plant hormonal profile

There are a number of publications showing that the levels of plant hormones such as cytokinins, jasmonates, auxins and abscisic acid (ABA) actually change upon the establishment of the AM symbiosis (Hause *et al.*, 2007; Pozo *et al.*, 2015).

The alteration of the hormonal profile in AM plants has also been proposed to have a role in alleviation of drought stress (Liu *et al.*, 2016a; Ruiz-Lozano *et al.*, 2016). ABA, as

the ‘abiotic stress hormone’, is the best studied in relation with the symbiosis. The increased ABA levels reported in some studies would promote drought tolerance, while also maintaining the establishment and functioning of the symbiosis (López-Raez, 2016).

The role of other hormones in the AM-induced drought tolerance was less studied. However, some studies reported increased levels of some hormones such as IAA, MeJA, ZR or brassinosteroids in AM plants under drought, suggesting a relationship with the drought tolerance (Liu *et al.*, 2016a). JAs may have a role in the symbiosis, but different results under drought have been found. Thus, it has been shown that both MeJA and AM colonization prevent the decrease in root hydraulic conductivity produced by drought stress in common bean. This protection could be related to the crosstalk between JA and SA (Sánchez-Romera *et al.*, 2016).

Under drought, increased levels of SLs were also found after AM colonization, demonstrating a regulation of this hormone under stress. It seems that the plant could sense the fungus and produce SLs to promote the symbiosis in order to tolerate better the adverse conditions (Ruiz-Lozano *et al.*, 2016).

- Modulation of root hydraulic properties

It has been demonstrated that AM symbiosis regulates root hydraulic properties, including root hydraulic conductivity (Lpr), modulating water flow under drought stress conditions (Aroca *et al.*, 2007, 2008b). This modulation was also related to the switch among water transport pathways in roots, as an increase in the relative apoplastic water flow was found in AM plants under both well-watered and drought stress conditions (Bárzana *et al.*, 2012). The changes in Lpr by AM were found to be largely mediated by changes in plant aquaporins (Ruiz-Lozano & Aroca, 2010, 2017).

3.6. Role of aquaporins in AM interactions during drought stress

The responses of AM plants to drought can be regulated by the expression of drought-related plant genes. Moreover, during the formation of the symbiotic relationship between the AM fungus and the root, extensive morphological alterations occur in the plasma membranes of cortex cells, which increase their surfaces to surround the arbuscules. Among the changes produced by these structures, alterations in the abundance and location of membrane proteins like AQPs are commonly reported.

The function and regulation of AQPs has been intensively studied to understand the hydraulic properties of plants. However, the identification of a variety of aquaporin substrates others than water, has opened the possibility for their involvement in many different processes of physiological significance for plants (Chaumont & Tyerman, 2014;

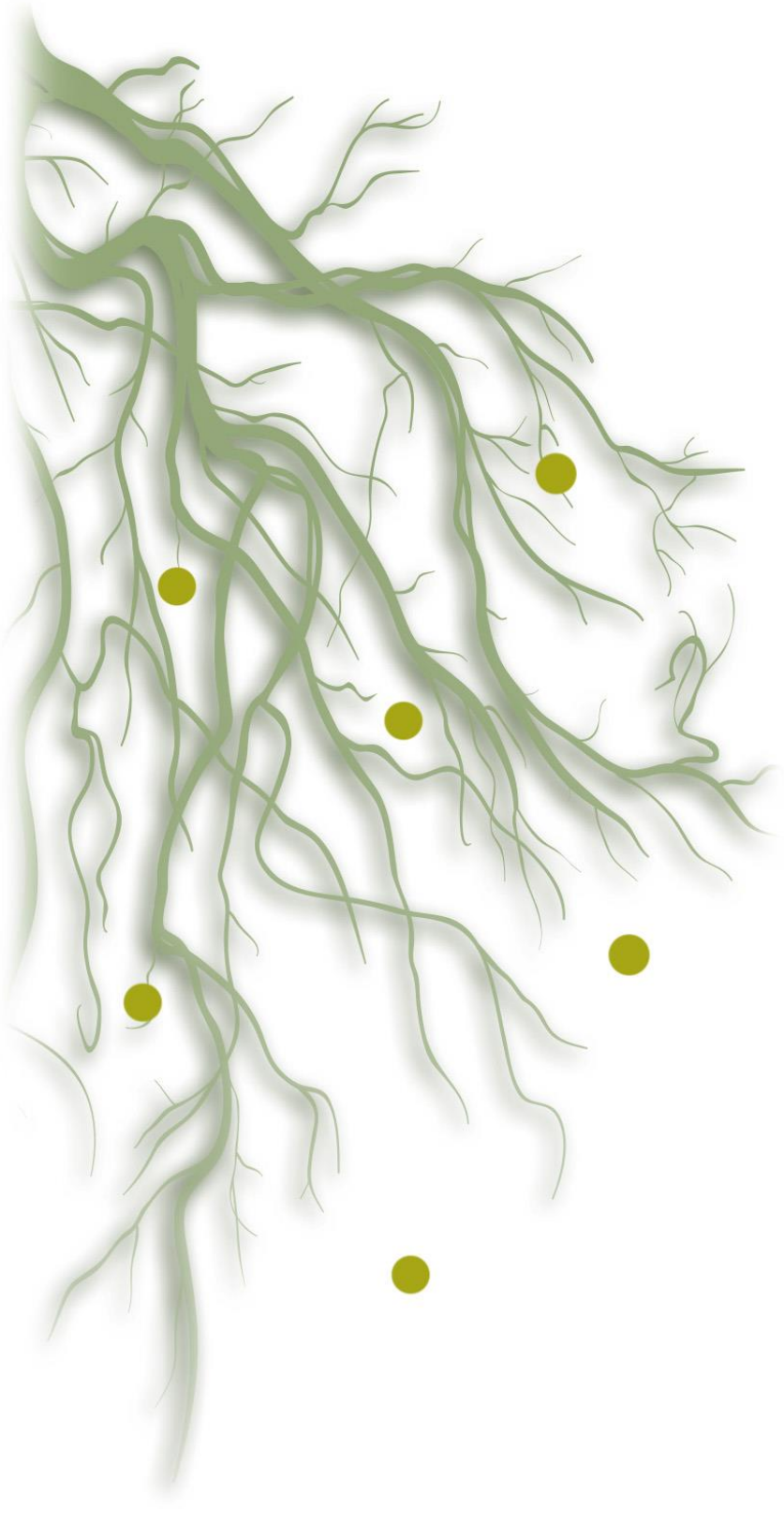
Li *et al.*, 2014) In this sense, AQPs are generally involved in the symbiotic exchange at the plant-fungus interface, which suggests a fine regulation of both water and nutrient exchange during the symbiosis (Maurel & Plassard, 2011; Bárzana *et al.*, 2014). In accordance with this, several plant aquaporin genes were modulated in different species under drought stress conditions when inoculated with an AM fungus (Aroca *et al.*, 2007; Uehlein *et al.*, 2007; Bárzana *et al.*, 2014).

Generally, the observation is that AQPs influence water permeability and nutrient exchange more efficiently in AM under water stress (Wang *et al.*, 2018). In this context, Bárzana *et al.* (2014) investigated in which way the AM symbiosis modulates the expression of the whole AQP gene family in maize under different drought stress scenarios. Results showed that the AM symbiosis regulated the expression of a wide number of AQP genes in the host plant, comprising members of the different AQP subfamilies. Several of these AM-regulated AQPs were functionally characterized in *Xenopus laevis* oocytes and by yeast complementation. It was shown that they can transport water, but also different molecules with physiological importance for plant performance under both normal and stress conditions (urea, ammonia, glycerol, silicon, boric acid and hydrogen peroxide). The regulation of these genes depended on the severity of the drought stress imposed, suggesting that under short-term drought conditions, the AM symbiosis may further stimulate the physiological processes in which these AQPs participate, but when the drought becomes sustained and severe, the AM symbiosis restricts most of the processes in which these AQPs are involved.

Three aquaporins were identified in the model fungal species *R. irregularis* (Aroca *et al.*, 2009; Li *et al.*, 2013). Two of them, *GintAQPF1* and *GintAQPF2*, were characterized to transport water in transformed protoplasts. The expression of these genes was also significantly enhanced during drought stress (Li *et al.*, 2013). However, their specific role during the symbiosis and in the conditions of water deficit is not well established yet.

In summary, it is generally observed that AM plants exhibit improved root hydraulic properties under drought stress conditions and also that they grow more than non-AM plants under drought conditions. The literature on AQP regulation by the AM symbiosis suggests that these effects are likely the result of the combined action of the different AQPs regulated by the AM symbioses, influencing both the transport of water and, most probably, also of the signalling molecules and other solutes of physiological importance for the plant under drought stress conditions. Thus, research in this field should focus on the identification of those AQP isoforms regulated by the AM symbiosis having a key role in plant tolerance to drought stress and to decipher their role *in planta* in the transport of water and other solutes with physiological importance (Ruiz-Lozano & Aroca, 2017).

RESULTS



CHAPTER I

Enhanced drought stress tolerance by the arbuscular mycorrhizal symbiosis in a drought-sensitive maize cultivar is related to a broader and differential regulation of host plant aquaporins than in a drought-tolerant cultivar

Adapted from:

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Enhanced drought stress tolerance by the arbuscular mycorrhizal symbiosis in a drought-sensitive maize cultivar is related to a broader and differential regulation of host plant aquaporins than in a drought-tolerant cultivar.

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Abstract

The arbuscular mycorrhizal symbiosis (AM) has been shown to improve maize tolerance to different drought stress scenarios by regulating a wide range of host plants aquaporins. The objective of this study was to highlight the differences in aquaporin regulation by comparing the effects of the AM symbiosis on root aquaporin gene expression and plant physiology in two maize cultivars with contrasting drought sensitivity. This information would help to identify key aquaporin genes involved in the enhanced drought tolerance by the AM symbiosis. Results showed that when plants were subjected to drought stress the AM symbiosis induced a higher improvement of physiological parameters in drought-sensitive plants than in drought-tolerant plants. These include efficiency of photosystem II, membrane stability, accumulation of soluble sugars and plant biomass production. Thus, drought-sensitive plants obtained higher physiological benefit from the AM symbiosis. In addition, the genes *ZmPIP1;1, ZmPIP1;3, ZmPIP1;4, ZmPIP1;6, ZmPIP2;2, ZmPIP2;4, ZmTIP1;1, and ZmTIP2;3* were down-regulated by the AM symbiosis in the drought-sensitive cultivar and only *ZmTIP4;1* was up-regulated. In contrast, in the drought-tolerant cultivar only three of the studied aquaporin genes (*ZmPIP1;6, ZmPIP2;2* and *ZmTIP4;1*) were regulated by the AM symbiosis, resulting induced. Results in the drought-sensitive cultivar are in line with the hypothesis that downregulation of aquaporins under water deprivation could be a way to minimize water loss, and the AM symbiosis could be helping the plant in this regulation. Indeed, during drought stress episodes, water conservation is critical for plant survival and productivity, and is achieved by an efficient uptake and stringently regulated water loss, in which aquaporins participate. Moreover, the broader and contrasting regulation of these aquaporins by the AM symbiosis in the drought-sensitive than the drought-tolerant cultivar suggests a role of these aquaporins in water homeostasis or in the transport of other solutes of physiological importance in both cultivars under drought stress conditions, which may be important for the AM-induced tolerance to drought stress.

Keywords: Aquaporins, arbuscular mycorrhizal symbiosis, drought, maize, tolerance.

Introduction

Crop adaptation to new environments is of crucial importance, especially in a climate change scenario. In order to secure food production in the future, efforts need to be directed to understand the mechanisms of plant adaptation and tolerance to abiotic stresses like water shortage, as these events are expected to intensify in coming years (Elliott *et al.*, 2014).

Plants cope with drought stress by recruiting drought avoidance and/or drought tolerance mechanisms, which include osmotic adjustment, regulation of stomatal conductance and photosynthesis, production of antioxidant and scavenger compounds or regulation of water uptake and flow in their tissues (Ruiz-Lozano *et al*., 2012b); Candar-Cakir *et al*., 2016). Maize is a primary food crop, even more important than other cereals such as rice or wheat since 2012 (Min *et al.*, 2016). The impact of drought on productivity of rice, wheat and maize will become of capital importance, as these crops represent the 50% of total consumed calories in most populated regions (Lobell *et al*., 2008).

Maize is fairly susceptible to drought stress, especially in the reproductive phase, experiencing important decreases in yields under drought stress in different world regions (Daryanto *et al*., 2016). Indeed, maize requires more water at the later vegetative and reproductive stages that at seedlings stage, but at the early crop establishment phase, water stress also influences seedlings adaptation and their grain yield potential, because of premature flowering and a longer anthesis-silk interval (Zhuang *et al.*, 2004; Min *et al.*, 2016) Despite the amount of information about crop responses to water deficit, our knowledge about the mechanisms originating drought tolerance in maize seedlings is still restricted (Min *et al.*, 2016). Previous studies of drought tolerance in maize have shown that tolerant cultivars enhanced antioxidant activity, presented lower lipid peroxidation, improved accumulation of osmolytes and turgor adjustment, maintained photosynthetic activity and regulated aquaporin genes (Min *et al.*, 2016; Anjum *et al.*, 2016).

In this context, the symbiosis of arbuscular mycorrhizal (AM) fungi with plant roots has been shown to be helpful to tolerate and overcome water stress episodes in different plant species (Chitarra *et al.*, 2016; Gholamhoseini *et al.*, 2013), including maize (Boomsma and Vyn, 2008; Bárzana *et al*., 2014, 2015). Authors have previously reported that AM-plant association leads to better plant antioxidant activity, osmotic regulation and root hydraulic properties (Ruiz-Lozano *et al*., 2012a,b). Also, AM inoculated plants generally present a higher level of photosynthetic pigments, enhanced chlorophyll fluorescence parameters and net photosynthetic rate (Yooyongwech *et al*., 2016), as well as, a different hormone regulation compared to control plants (Aroca *et al*., 2008a, b).

In maize, the improvement of physiological plant status of AM inoculated plants when subjected to drought stress has been related to a better uptake of soil nutrients and water, reduced oxidative damage, enhanced root water transport capacity, or facilitated switching between apoplastic and cell-to-cell water transport pathways (Boomsma and Vyn, 2008; Bárzana *et al*., 2012; 2015). Furthermore, the establishment of the AM symbiosis originates extensive morphological alterations in plant root cells, in order to accommodate the presence of an endophytic symbiont, with most of these changes concerning cytoplasmic or vacuolar membranes (Krajinski *et al.*, 2000). Thus, it is not surprising that AM plants may present different pattern of membrane proteins such as aquaporins, candidate proteins to be involved in the exchange of nutrients and water between both organisms (Uehlein *et al*., 2007; Maurel and Plassard, 2011; Bárzana *et al*., 2014).

Aquaporins are small membrane intrinsic proteins located in different cell membranes and constitute a highly diverse protein family in plants, with at least 30 isoforms in most higher plants. They transport water but some of them can also facilitate the membrane diffusion of other relevant molecules for the plant such as CO_2, silicon, boron, urea or ammonia (Li *et al*., 2014). Recently, oxygen has also been shown to be transported by several *Nicotiana tabacum* aquaporins, with NtPIP1;3 as the most promising one, which points to the significance of pore-mediated O_2 transport for respiration and opens new perspectives for aquaporins roles in plant physiology (Zwiazek *et al*., 2017).

Each aquaporin isoform often contributes, in concert with other isoforms, to several physiological functions. Thus, their numerous functions in plant growth and development seem to be essential but not well understood yet (Chaumont and Tyerman, 2014; Li *et al*., 2014; Afzal *et al*., 2016). However, their role in the maintenance of water homeostasis in the whole plant and in stress responses has been well established (Chaumont & Tyerman, 2014; Afzal *et al.*, 2016), affecting the radial water flow through the cell-to-cell pathway, which is predominant under conditions of low transpiration such as under drought stress (Steudle and Peterson, 1998). To this regard, it is also remarkable that several aquaporin genes have been found to be AM-responsive in plant species (Krajinski *et al*., 2000; Aroca *et al*., 2007; Guether *et al*., 2009; Bárzana *et al*., 2014; Chitarra *et al*., 2016; He *et al*., 2016; Liu *et al*., 2016).

There are 36 different aquaporin isoforms in maize (Chaumont *et al.*, 2001). In a recent study, 16 out of these 36 maize aquaporins, belonging to the four maize aquaporin subfamilies (PIPs, TIPs, NIPs and SIPs), were found to be regulated by the AM fungus *R. irregularis* (Bárzana *et al*., 2014). The expression of these proteins varies according to the

severity of the stress and depends on the duration of the water shortage period (Bárzana *et al.*, 2014). Essentially, these results highlight the complex regulation of these proteins in the presence of AM symbiosis and their putative role in drought alleviation (Bárzana *et al.*, 2014).

Previous studies have provided evidences that the beneficial effects of the AM symbiosis on plant stress tolerance are generally larger in plants sensitive to the imposed stress than in tolerant ones, or under more limiting growing conditions (Subramanian *et al.*, 1995; Subramanian & Charest, 1997; Gianinazzi *et al.*, 2010; Bonneau *et al.*, 2013; Yooyongwech *et al.*, 2016). This has been emphasized also for maize plants (Boomsma & Vyn, 2008). Thus, the above approach can be combined with the use of drought-sensitive and drought-tolerant cultivars for comparative analyses (Rigano *et al.*, 2014; Zhang *et al.*, 2016) and for identification of key aquaporins whose expression is altered by the AM symbiosis in the sensitive cultivar to render it more tolerant (Subramanian and Charest, 1997; Yooyongwech *et al*., 2016).

The present study deals with the hypothesis that aquaporin regulation by the AM symbiosis plays a significant role in the improvement of host plant tolerance to drought stress. Under such situation, aquaporin modulation mediated by the AM symbiosis could lead to improvements of the use of soil water and mineral resources, resulting in higher drought tolerance. The objective was to highlight the differences in aquaporin regulation by comparing the effects of AM symbiosis on root aquaporin gene expression and plant physiology in two maize cultivars with contrasting drought sensitivity.

This information would help to identify key aquaporin genes involved in the enhanced drought tolerance by the AM symbiosis. A similar approach has been followed to study aquaporins involved in stomatal gating in rice plants (Vinnakota *et al*., 2016). Moreover, the present work deeps on the role of aquaporins in drought tolerance and their regulation by AM fungi.

Materials and Methods

Experimental design and statistical analysis

The experiment consisted of a factorial design with two factors: (1) inoculation treatment, with non-inoculated control plants (C) and plants inoculated with the AM fungus *Rhizophagus irregularis*, strain EEZ 58 (Ri); (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 drought stress for 12 days before harvest

(DS). In addition, two maize cultivars with contrasting tolerance to drought stress were used. One cultivar was sensitive to drought (PR34B39) and the second was tolerant to drought (PR34G13). The different combinations of these factors gave a total of 4 treatments for the sensitive cultivar and 4 treatments for the tolerant cultivar. Ten replicates were used for each treatment, giving a total of 80 plants.

Within each maize cultivar, data were subjected to analysis of variance (ANOVA) with inoculation treatment, water regime and inoculation treatment-water regime interaction as sources of variation. Post Hoc comparisons with the Duncan's test were used to find out differences between groups. Within each water regime, drought-sensitive and drought-tolerant cultivars were also compared by means of Student's T test. The expression of the AM fungal aquaporins was analysed by means of Student's T test.

Soil and biological materials

A loamy soil was collected at the grounds of IFAPA (Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100°C for 1 h on 3 consecutive days). The soil had a pH of 8.1 (water); 0.85% organic matter, nutrient concentrations (mg kg^{-1}): N, 1; P, 10 (NaHCO3-extractable P); K, 110. The soil texture comprised 38.3% sand, 47.1% silt and 14.6% clay.

Maize (*Zea mays* L.) seeds from a drought-sensitive (PR34B39) and a drought-tolerant (PR34G13) cultivar were provided by Pioneer Hi-Bred, Spain (DuPont Pioneer Corporation). Seeds were pre-germinated on moist sand for 5 days and then transferred to pots filled with 1250 g of the soil/sand mixture 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 3 ml aliquot of a filtrate (<20 µm) of the AM inoculum in order to provide a general microbial population free of AM propagules.

Growth conditions

The experiments were carried out under greenhouse conditions with temperatures ranging from 19 to 25ºC, 16/8 light/dark period, a relative humidity of 50-60% and an average photosynthetic photon flux density of 800 µmol m^{-2} s^{-1}, as measured with a light

meter (LICOR, Lincoln, NE, USA, model LI-188B). Plants were cultivated for a total of 9 weeks.

Soil moisture was measured with the ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK). Water was supplied daily to maintain soil at 100% of field capacity during the first 6 weeks after sowing. The 100% soil water holding capacity corresponds to 22% volumetric soil moisture measured with the ThetaProbe, as determined experimentally in a previous experiment using a pressure plate apparatus. Then, half of the plants were allowed to dry until soil water content reached 60% of field capacity (one day needed), while the other half were maintained at field capacity. At this stage AM and non-AM plants of both genotypes had comparable size. The 60% of soil water holding capacity corresponds to 7% volumetric soil moisture measured with the ThetaProbe (also determined experimentally with a pressure plate apparatus in a previous assay).

The soil water content was daily measured with the ThetaProbe ML2 before rewatering (at the end of the afternoon), reaching a minimum soil water content around 55% of field capacity in the drought-stressed treatments. This water deficit treatment resulted in severe drought stress for maize plants, as evidenced by the decrease in stomatal conductance and efficiency of the photosystem II. The amount of water lost was added to each pot in order to keep the soil water content at the desired levels of 7% of volumetric soil moisture (Porcel & Ruiz-Lozano, 2004). Plants were maintained under such conditions for 12 additional days before harvesting.

Measurements

- Biomass production and symbiotic development

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

The percentage of mycorrhizal fungal colonization in maize plants was estimated by visual observation according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980) in five replicates per treatment.

- Stomatal conductance

Stomatal conductance was measured two hours after the onset of photoperiod with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from four different plants of each treatment.

- Photosynthetic efficiency

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

- Membrane electrolyte leakage

Leaf electrolyte leakage was determined in six plants per treatment. Leaf samples were washed with deionized water to remove surface-adhered electrolytes. The samples were placed in closed vials containing 10 mL of deionized water and incubated at 25 $_{o}$C on a rotary shaker (at 100 rpm) during 3 hours, and the electrical conductivity of the solution (L_0) was determined using a conductivity meter (Metler Toledo AG 8603, Switzerland). Samples were then placed at -80ºC for 2 hours. Subsequently, tubes were incubated again at room temperature under smoothly agitation and the final electrical conductivity (L_f) was obtained after 3 hours under these conditions. The electrolyte leakage was defined as follows: $[(L_0 - L_{water})/(L_f - L_{water})] \times 100$, where L_{water} is the conductivity of the deionized water used to incubate the samples.

- Oxidative damage to lipids

Lipid peroxides were extracted by grinding 500 mg of fresh leaf tissues with and ice-cold mortar and 6 ml of 100 mM potassium phosphate buffer (pH 7). Homogenates were filtered through one Miracloth layer and centrifuged at 15,000 g for 20 min. The chromogen was formed by mixing 200 ml of supernatants with 1 ml of a reaction mixture containing 15% (w/v) Trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl hydroxytoluene, 0.25 N HCl and by incubating the mixture at 100 ºC for 30 min (Minotti and Aust, 1987). After cooling at room temperature, tubes were centrifuged at 800 g for 5 min and the supernatant was used for spectrophotometric reading at 532 nm.

Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1985). The calibration curve was made using MDA in the range of 0.1-10 nmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25 N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction

mixtures to prevent artefactual formation of 2-thiobarbituric acid-reactive substances (TBARS) during the acid-heating step of the assay.

- Total soluble sugars accumulation

At harvest, total soluble sugars were extracted from 1 g fresh leaf tissues in 100 mM potassium phosphate buffer for total soluble sugars. Soluble sugars were analysed by 0.025 mL of plant extract reacting with 3 ml freshly prepared anthrone (200 mg anthrone + 100 ml 72% (w:w) H_2SO_4) and placed in a boiling water bath for 10 min according to Irigoyen *et al*. (1992). After cooling, the absorbance at 620 nm was determined in a spectrophotometer Hitachi U-1900 (Hitachi Corporation, Japan). The calibration curve was made using glucose in the range of 0.2 to 0.4 mg/ml.

- Hydrogen peroxide content

Hydrogen peroxide content was determined by Patterson's method (Patterson *et al*., 1984; Aroca *et al*., 2003), with slight modifications as described previously by Aroca *et al*. (2003). Five hundred milligrams of fresh leaf tissues were homogenized in a cold mortar with 5 ml 5% (w/v) TCA containing 0.1 g of activated charcoal and 1% (w/v) PVPP. The homogenate was centrifuged at 18,000g for 10 min. The supernatant was filtered through a Millipore filter (0.22 µm). A volume of 1.2 ml of 100 mM potassium phosphate buffer (pH = 8.4) and 0.6 ml of the colorimetric reagent were added to 130 µl of the supernatant. The colorimetric reagent was freshly made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-2 (2-pyridylazo) resorcinol (disodium salt). The samples were incubated at 45 ^{o}C for 1 h and the absorbance at 508 nm was recorded. The blanks were made by replacing leaf extract by 5% TCA.

- Root hydraulic conductivity (Lo)

Eight weeks after sowing the sap flow rate (*Jv*) and Lo were measured on detached roots exuding under atmospheric pressure for two hours (Aroca *et al.*, 2007). Osmotic root hydraulic conductivity (Lo) was calculated as Lo = $Jv/\Delta\Psi$, where *Jv* 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. These measurements were carried out 3 h after the onset of light.

- RT-qPCR

Total RNA was isolated from maize roots harvested at noon 8 weeks after sowing and kept at -80 ^{o}C, by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay *et al.*, 1987). The RNA was subjected to DNase treatment and reverse-

transcription using the QuantiTect Reverse Transcription Kit (Qiagen), following the instructions provided by manufacturer. To rule out the possibility of a genomic DNA contamination, all the cDNA sets were checked by running control PCR reactions with aliquots of the same RNA that have been subjected to the DNase treatment but not to the reverse transcription step.

The expression of the group of maize aquaporins previously selected as regulated by the AM symbiosis (Bárzana *et al.*, 2014) was studied by quantitative real-time PCR by using iCycler system (Bio-Rad, Hercules, CA, USA) adjusting protocols to optimize the PCR reaction to each gene. The primer sets used to amplify each aquaporin gene were designed in the 3' and 5' untranslated regions of each gene in order to avoid unspecific amplification of the different aquaporin genes (Hachez *et al.*, 2006a; Bárzana *et al.*, 2014). The specificity of amplicons was checked with a heat dissociation protocol (from 70 to 100°C), after the final PCR cycle. The efficiency of the primer sets was evaluated with the software Bio-Rad iQ5 (version 2.1.97.1001) by analysing the ratio Ct/fluorescence at four-six independent points of PCR curves (Ramakers *et al.*, 2003), giving values between 90 and 98%. The sequences of primers used for the aquaporin and reference genes are those described in Bárzana *et al*. (2014). Standardization was carried out based on the expression of the best-performing reference gene under our growing conditions. Thus, aquaporin expression levels were normalized according to the elongation factor 1 (gi:2282583).

The fungal aquaporin genes *GintAQP1*, *GintAQPF1* and *GintAQPF2* were also analysed using the primers and conditions described previously (Aroca *et al*., 2009; Li *et al*., 2013). Standardization was carried out based on the expression of the fungal *elongation factor 1a* gene in each sample (Aroca et al. 2009).

The relative abundance of transcripts was calculated by using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001a). RT-qPCR measurements were carried out in at least three independent RNA samples per treatment, with the threshold cycle (Ct) determined in duplicate. Negative controls without cDNA were used in all PCR reactions.

Results

AM root colonization and plant biomass

AM inoculated plants from drought-sensitive cultivar, PR34B39, had an average of 54% of mycorrhizal root length, with no significant differences due to the water treatment (Figure 1A). In the case of the drought-tolerant cultivar, PR34G13, mycorrhizal root length

was 50%, also with no significant differences due to the water treatment. Uninoculated maize plants did not exhibit AM root colonization (Figure 1A).

Shoot dry weight (SDW) in the sensitive line was similar for both AM and non-AM plants when cultivated under well-watered conditions. Drought stress decreased shoot dry weight by 37% in non-AM plants but only by 17% in AM plants (Figure 1B). When subjected to drought stress AM plants produced 35% more shoot dry weight than non-AM plants (Figure 1B). In the drought-tolerant cultivar, no effect of the AM symbiosis on SDW was observed either under well-watered conditions or under drought stress. Drought stress decreased SDW by 17% and 22% in non-AM and AM plants, respectively (Figure 1B). In any case, under drought stress conditions, significant differences in SDW between drought-sensitive and drought-tolerant non-AM plants were observed, with the latter growing 41% more than the former (Figure 1B).

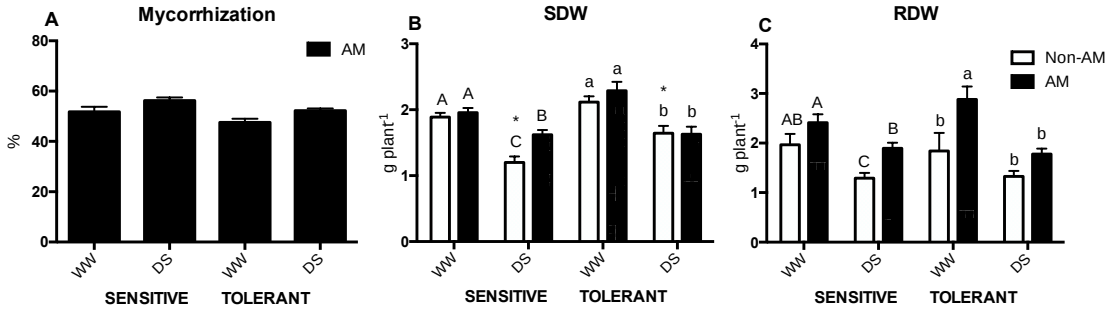

Figure 1. (A) Percentage of mycorrhizal root length, (B) shoot dry weight (SDW) and (C) root dry weight (RDW) in two maize genotypes differing in drought tolerance and inoculated or not with an arbuscular mycorrhizal (AM) fungus. Data represents the means of 5 values ± S.E for Mycorrhization and RDW and 10 values ± S.E for SDW. Different letter indicates significant differences between treatments ($p<0.05$) based on Duncan's test for sensitive (uppercase) and tolerant (lowercase) genotypes. Asterisks indicate significant differences between drought-sensitive and drought-tolerant genotypes within each watering regime, according to Student's T test.

Drought and AM inoculation had a similar effect on root dry weight (RDW) as in SDW for both PR34B39 and PR34G13 lines (Figure 1C). In non-AM plants drought decreased significantly RDW in the sensitive genotype, and AM plants produced higher root biomass under drought stress conditions only in the sensitive cultivar (Figure 1C). In contrast, under well-watered conditions, AM plants enhanced RDW only in the tolerant cultivar.

Stomatal conductance (*gs*) and efficiency of photosystem II

The stomatal conductance (*gs*) of drought-sensitive cultivar was enhanced by the AM symbiosis under well-watered conditions (36% of increase) but not under water deficit. In the drought-sensitive cultivar drought did not significantly affect this parameter (Figure

2A). The drought-tolerant cultivar showed enhanced *gs* by the AM symbiosis both under well-watered conditions (27%) and under drought stress conditions (143%) (Figure 2A). However, drought decreased this parameter as compared to well-watered conditions. This decrease was 69% in non-AM plants and 41% in AM plants (Figure 2A). Under well-watered conditions both AM and non-AM plants exhibited higher *gs* values in the drought-tolerant cultivar than in the drought-sensitive one (Figure 2A). In contrast, under drought stress conditions, non-AM plants from the drought-tolerant cultivar had lower *gs* values than the corresponding drought-sensitive ones.

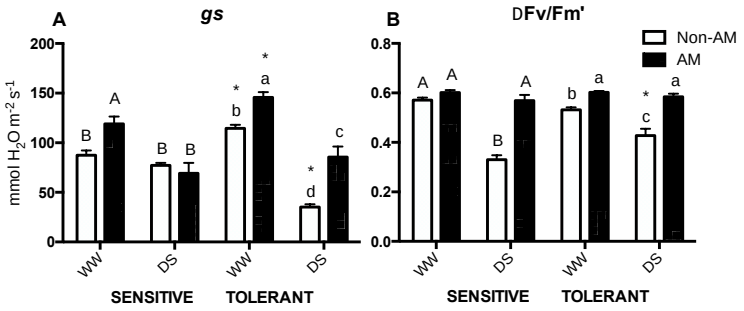

Figure 2. (A) Stomatal conductance (*gs*) and (B) photosystem II efficiency in the light-adapted state (∆Fv/Fm') in two maize genotypes differing in drought tolerance and inoculated or not with an arbuscular mycorrhizal (AM) fungus. Data represents the means of 8 values ± S.E. Different letter indicates significant differences between treatments ($p<0.05$) based on Duncan's test for sensitive (uppercase) and tolerant (lowercase) genotypes. Asterisks indicate significant differences between drought-sensitive and drought-tolerant genotypes within each watering regime, according to Student's T test.

The light-adapted maximum quantum yield of PSII primary photochemistry (∆F/Fm') in plants from drought-sensitive cultivar was affected by drought stress in the non-AM plants only, which reduced this parameter by 42% (Figure 2B). In contrast, in the AM plants, no significant effect was observed. In the case of the drought-tolerant cultivar, the ∆F/Fm' was enhanced by the AM symbiosis both under well-watered conditions (13% of increase) and under drought stress conditions (36% of increase) (Figure 2B). In this cultivar, drought stress also reduced this parameter (by 19%) in non-AM plants only (Figure 2B). Under drought stress conditions, significant differences in ∆F/Fm' between drought-sensitive and drought-tolerant non-AM plants were observed, with the latter having values 30% higher than the former (Figure 2B).

Membrane electrolyte leakage

The membrane electrolyte leakage was reduced by the AM symbiosis in drought-sensitive plants, both under well-watered conditions (50% of decrease) and under drought stress conditions (67% of decrease) (Figure 3A). The imposed drought stress increased this parameter by 58% but only in non-AM plants. In the drought-tolerant cultivar the

membrane electrolyte leakage increased by drought stress only in non-AM plants (by 279%), while AM plants did not increase this parameter as a consequence of drought (Figure 3A). The EL values were higher in non-AM drought-sensitive plants than in non-AM drought-tolerant ones, both under well-watered and under drought stress conditions (Figure 3A).

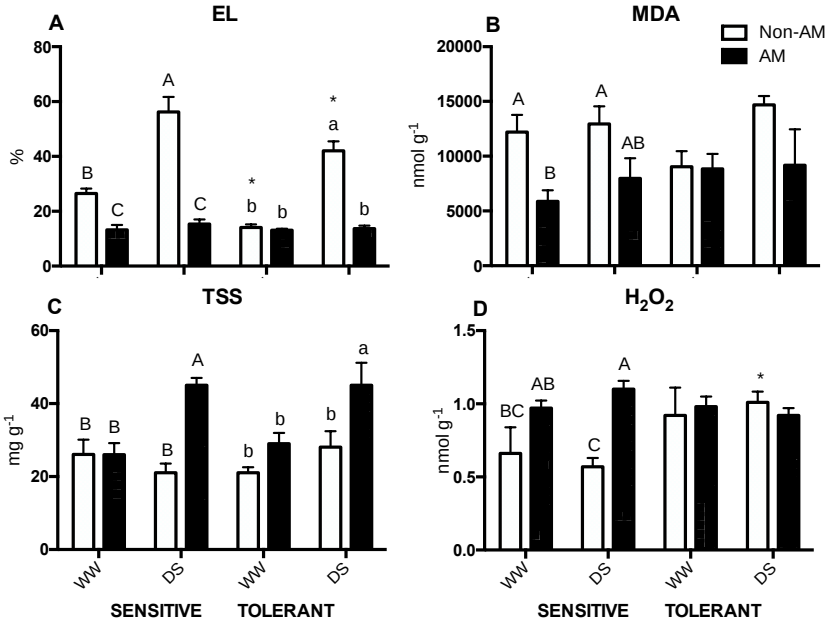

Figure 3. (A) Leaf electrolyte leakage (EL), (B) oxidative damage to lipids (as malondialdehyde MDA, equivalents), (C) total soluble sugars (TSS) and (D) hydrogen peroxide (H_2O_2) concentration in two maize genotypes differing in drought tolerance and inoculated or not with an arbuscular mycorrhizal (AM) fungus. Data represents the means of 6 values ± S.E. for EL and 3 values ± S.E. for MDA, TSS and H_20_2. Different letter indicates significant differences between treatments ($p<0.05$) based on Duncan's test for sensitive (uppercase) and tolerant (lowercase) genotypes. Asterisks indicate significant differences between drought-sensitive and drought-tolerant genotypes within each watering regime, according to Student's T test.

Oxidative damage to lipids (MDA)

The AM symbiosis reduced the oxidative damage to lipids measured as MDA equivalents in the drought-sensitive cultivar regardless of the water regime (Figure 3B). In contrast, drought stress did not significantly affect this parameter either in the AM or in the non-AM plants (Figure 3B). In the drought-tolerant cultivar, the oxidative damage to lipids was not significantly affected by the AM symbiosis or by the drought stress imposed (Figure 3B).

Total soluble sugars

The leaf total soluble sugars (TSS) concentration was significantly increased by the AM symbiosis in both maize cultivars, but only under drought stress conditions (Figure

3C). Plants cultivated under well-watered conditions did not alter their TSS content as consequence of the AM symbiosis (Figure 3C).

Accumulation of hydrogen peroxide

The accumulation of hydrogen peroxide was significantly affected by the AM symbiosis only in the drought-sensitive cultivar, increasing the values in AM plants under drought stress conditions (Figure 3D). Under drought stress conditions, hydrogen peroxide accumulation was higher in non-AM drought-tolerant plants than in non-AM drought-sensitive ones (Figure 3D).

Root hydraulic conductivity (Lo)

In the drought-sensitive cultivar root hydraulic conductivity (Lo) was strongly reduced by drought, but this reduction reached 95% in non-AM plants and 73% in AM plants (Figure 4). Thus, under drought stress conditions AM plants exhibited enhanced Lo values by 5-fold when compared to non-AM plants (Figure 4).

In the drought-tolerant cultivar, AM symbiosis reduced Lo by 33% under well-watered conditions but increased it by 82% under drought stress conditions. In this cultivar, drought stress also reduced significantly this parameter (Figure 4). Thus, non-AM plants decreased Lo by 81% as consequence of drought. The decrease was by 49% in AM plants (Figure 4). Under drought stress conditions, Lo values were significantly higher (by 360%) in non-AM drought-tolerant plants than in non-AM drought-sensitive ones (Figure 4).

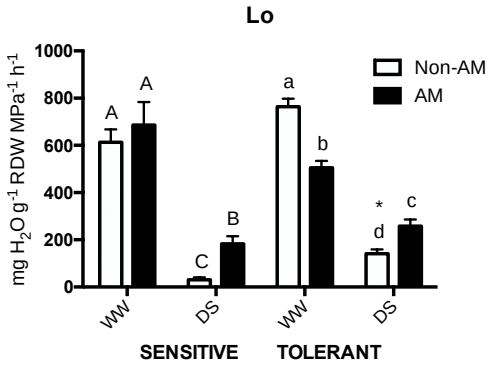

Figure 4. Osmotic root hydraulic conductivity (Lo) in two maize genotypes differing in drought tolerance and inoculated or not with an arbuscular mycorrhizal (AM) fungus. Data represents the means of 4 values ± S.E. Different letter indicates significant differences between treatments ($p<0.05$) based on Duncan's test for sensitive (uppercase) and tolerant (lowercase) genotypes. Asterisks indicate significant differences between drought-sensitive and drought-tolerant genotypes within each watering regime, according to Student's T test.

Expression of maize and fungal aquaporins

We analysed the expression of 16 maize aquaporins previously shown to be regulated by the AM symbiosis under drought stress conditions (Bárzana *et al.*, 2014). One of these genes, *ZmTIP4;2*, was not detected in any of the two genotypes, likely because of its low expression level. Besides, *ZmNIP1;1* was only detected in the sensitive genotype, but its expression was also very low, and it was not possible to detect any modification due to mycorrhization (Figure S1). When analysing the expression patterns in both maize cultivars some of these aquaporin genes were not affected by the AM symbiosis or by the drought stress in the drought-tolerant cultivar (*ZmPIP1;2, ZmPIP1;4, ZmTIP1;2, ZmNIP2;2 and ZmSIP2;1*) (Figure S1). The Figure 5 shows the expression data of the aquaporin genes that are affected by the AM symbiosis and/or drought stress in both maize cultivars or at least in the drought-sensitive cultivar.

The expression of *ZmPIP1;1* in the drought-sensitive cultivar was unaltered by the AM symbiosis under well-watered conditions. However, its expression was enhanced by drought stress by 108% in non-AM plants, while in AM plants its expression did not change as consequence of drought. Thus, under drought stress conditions, the expression of *ZmPIP1;1* gene was 77% lower in AM than in non-AM plants (Figure 5A). On the contrary, in the drought-tolerant cultivar drought induced *ZmPIP1;1* expression by 60% in AM plants only (Figure 5A). Under drought stress conditions, the expression of *ZmPIP1;1* was significantly higher in non-AM drought-sensitive plants than in non-AM drought-tolerant ones (Figure 5A).

In the drought-sensitive cultivar the expression of *ZmPIP1;3* gene was reduced under well-watered conditions by 70% due to mycorrhization (Figure 5B). In the same way, the exposition to drought stress reduced the expression of this gene by 65% in non-AM plants, reaching expression values similar to those in AM plants. AM plants showed unaltered expression levels under well-watered and drought stress conditions (Figure 5B). In the drought-tolerant cultivar AM and non-AM plants showed no significant differences in *ZmPIP1;3* expression levels under well-watered and under drought stress conditions. Drought stress only reduced the expression of this gene in AM plants as compared to non-AM plants under well-watered conditions (Figure 5B). Under drought stress conditions, significant differences in the expression of *ZmPIP1;3* gene between non-AM drought-sensitive and drought-tolerant plants were observed, being higher in the latter than in the former (Figure 5B).

ZmPIP1;6 was down-regulated by the AM symbiosis under well-watered conditions in the drought-sensitive cultivar, showing 60% of inhibition as compared to non-AM plants

(Figure 5C). Drought stress inhibited the expression of this gene in non-AM plants, while in AM plants no further inhibition was observed, as compared to well-watered conditions. In the drought-tolerant cultivar, the AM symbiosis up-regulated by 150% the expression of *ZmPIP1;6* under well-watered conditions (Figure 5C). However, when plants were subjected to drought stress such up-regulation was avoided, reaching similar values than non-AM plants. No changes in gene expression due to water regime were observed in non-AM plants for this gene. Non-AM plants exhibited higher expression levels of *ZmPIP1;6* gene in the drought-sensitive cultivar than in the drought-tolerant one, regardless of water regime. In contrast, under well-watered conditions, AM plants had significantly higher expression levels in the drought-tolerant cultivar than in the drought-sensitive one (Figure 5C).

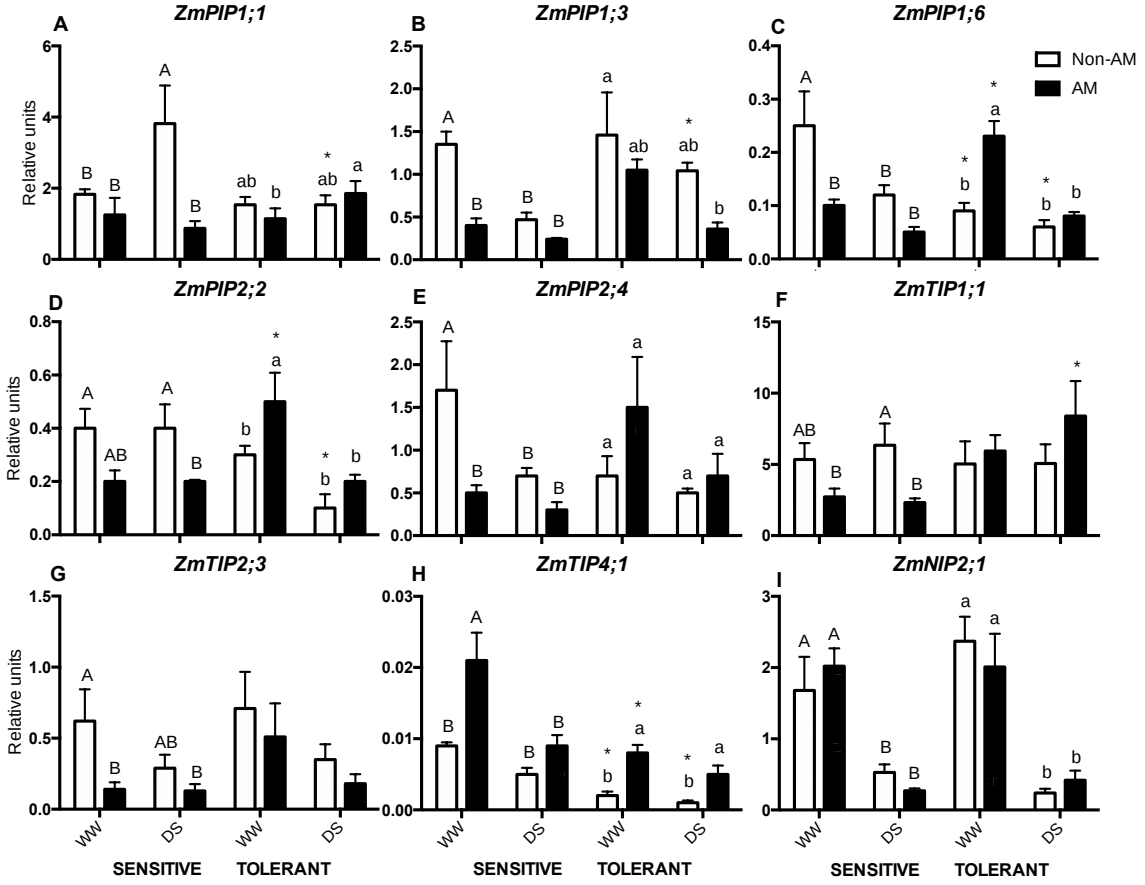

Figure 5. Expression of *ZmPIP1;1* (A), *ZmPIP1;3* (B), *ZmPIP1;6* (C), *ZmPIP2;2* (D), *ZmPIP2;4* (E), *ZmTIP1;1* (F), *ZmTIP2;3* (G), *ZmTIP4;1* (H) and *ZmNIP2;1* (I), in two maize genotypes differing in drought tolerance and inoculated or not with an arbuscular mycorrhizal fungus (AM). Values in the Y axis represent the expression levels in relative units. Data represents the means of 3 values ± S.E. Different letter indicates significant differences between treatments ($p<0.05$) based on Duncan's test for sensitive (uppercase) and tolerant (lowercase) genotypes. Asterisks indicate significant differences between drought-sensitive and drought-tolerant genotypes within each watering regime, according to Student's T test.

The expression of *ZmPIP2;2* in the drought-sensitive cultivar was significantly reduced by mycorrhization only under drought stress conditions (reduction by 62%) (Figure 5D). Under well-watered conditions this decrease was not significant. The expression of this gene in AM plants subjected to drought was also significantly lower (by 64%) than in non-AM plants under well-watered conditions. In the case of the drought-tolerant cultivar the behaviour was different since AM plants up-regulated this gene by 71% under well-watered conditions. In contrast, drought stress inhibited the expression of this gene in AM plants by 58% as compared to well-watered counterparts (Figure 5D). In AM plants cultivated under well-watered conditions the expression of *ZmPIP2;2* was higher in the drought-tolerant cultivar than in the drought-sensitive one. The opposite was observed in non-AM plants when cultivated under drought stress conditions (Figure 5D).

The mRNA level of *ZmPIP2;4* was reduced by 72% by mycorrhization in the drought sensitive cultivar when cultivated under well-watered conditions (Figure 5E). The expression of this gene was not further inhibited by drought stress in AM plants, while in non-AM plants it was reduced by 59%. In the drought-tolerant cultivar the expression of *ZmPIP2;4* did not show significant differences due to mycorrhization or water regime (Figure 5E).

In the drought-sensitive cultivar the expression of *ZmTIP1;1* gene was significantly affected by the AM symbiosis only under drought stress conditions, reducing its expression levels by 63% in AM plants as compared to non-AM ones (Figure 5F). Drought stress itself did not significantly affect the expression of this gene in both AM and non-AM plants. In the drought-tolerant cultivar the expression of *ZmTIP1;1* was unaltered by mycorrhization or water regime (Figure 5F). Under drought stress conditions, the expression of *ZmTIP1;1* was significantly higher in AM drought-tolerant plants than in AM drought-sensitive plants (Figure 5F).

The expression of *ZmTIP2;3* in the drought sensitive cultivar was inhibited by mycorrhization when cultivated under well-watered conditions, with a reduction of 77% (Figure 5G). The expression of this gene was not further inhibited by drought stress. In the drought-tolerant cultivar the expression of *ZmTIP2;3* was unaltered by mycorrhization or water regime (Figure 5G).

The *ZmTIP4;1* expression was up-regulated under well-watered conditions in the drought-sensitive cultivar as consequence of AM root colonization, with an increase in expression levels by 122% (Figure 5H). However, the drought stress reduced the expression of this gene by 58%, reaching similar expression levels than non-AM plants. In the case of the drought-tolerant cultivar, the expression of *ZmTIP4;1* gene in non-AM

plants was low and it was induced by the AM symbiosis both under well-watered (by 210%) and under drought stress conditions (by 310%) (Figure 5H). Non-AM plants had higher *ZmTIP4;1* expression levels in the drought-sensitive cultivar than in the drought-tolerant one, regardless of water regime. Moreover, under well-watered conditions, AM plants also exhibited significantly higher *ZmTIP4;1* expression in the drought-sensitive cultivar than in the drought-tolerant one (Figure 5H).

In the drought-sensitive cultivar the expression of *ZmNIP2;1* was only affected by drought stress, which reduced its expression in both non-AM (by 68%) and AM plants (by 87%) (Figure 5I). In the drought-tolerant cultivar a similar data was observed, with a reduction of gene expression by drought in non-AM plants (by 90%) and in AM plants (by 79%) (Figure 5I).

The expression of *GintAQP1* was slightly induced by drought stress in the drought-sensitive cultivar (Figure 6A). The expression of this gene was significantly higher in the drought-tolerant cultivar under well-watered conditions, but it resulted considerably inhibited (by 80%) by drought stress in this cultivar.

The gene *GintAQPF1* resulted similarly inhibited by drought stress in both cultivars (Figure 6B). However, the expression of this gene was lower than that of the other two fungal genes. Finally, the expression of *GintAQPF2* resulted unaltered by drought stress in both maize cultivars (Figure 6C).

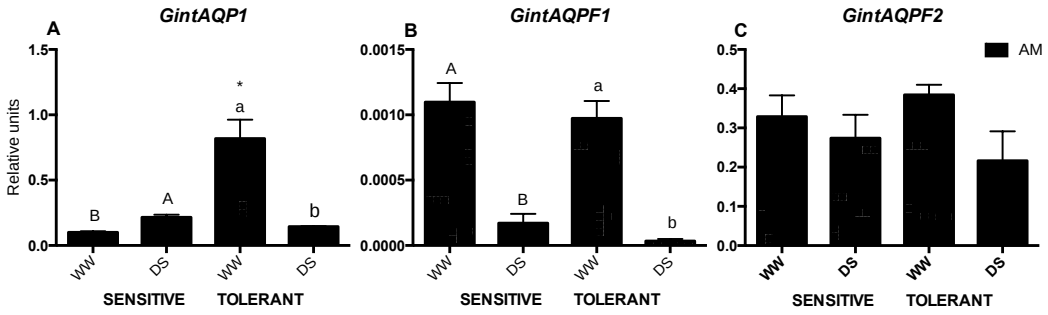

Figure 6. Expression of *GintAQP1* (A), *GintAQPF1* (B) and *GintAQPF2* (C) in two maize genotypes differing in drought tolerance and inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. Values in the Y axis represent the expression levels in relative units. Data are the means of 3 values ± S.E. Different letter indicates significant differences between treatments ($p<0.05$) based on Student's T test for sensitive (uppercase) and tolerant (lowercase) genotypes. Asterisks indicate significant differences between drought-sensitive and drought-tolerant genotypes within each watering regime, according to Student's T test.

Discussion

This study highlights the divergent responses to AM symbiosis of two maize genotypes differing in drought tolerance: PR34G13, a drought-tolerant cultivar, and PR34B39, a drought-sensitive cultivar (DuPont Pioneer Corporation). It particularly focused on the differential regulation of root aquaporins by the AM symbiosis under well-watered and drought stress conditions and its impact on plant performance. We also featured the influence of such factors on plant growth as well as on traits showing the effects of drought and AM symbiosis on plant physiology.

AM effects on plant physiological status

The AM fungal root colonization in both genotypes exceeded 50%, being not significantly affected by the drought stress treatment, probably due to its limited duration of only 12 days. The AM symbiosis has been previously reported to enhance drought tolerance of host plants (Augé, 2001).

In the present study, the beneficial effect of the AM fungus was firstly observed in plant biomass production. Indeed, plant biomass production is an integrative index of plant performance under stressful conditions and the efficiency of the AM symbiosis has often been measured in terms of host plant biomass improvement (Ruiz-Lozano *et al.*, 2012a). Droughted AM plants from the sensitive genotype presented higher SDW and RDW compared to non-inoculated plants. Contrariwise, no enhancement of SDW and RDW was observed in the case of the drought-tolerant genotype, highlighting genotype-depending responses to AM inoculation (Gianinazzi *et al.*, 2010; Subramanian and Charest, 1997; Subramanian *et al.*, 1995; Yooyongwech *et al.*, 2016). Anyway, water deficit negatively affected growth in both maize cultivars, but to a lesser extent in the drought-tolerant genotype.

Many of the physiological responses of plants to drought stress are directed toward the control of transpiration, of root hydraulic conductivity and of osmotic adjustment (Aroca *et al.*, 2012). Stomatal closure is a conserved mechanism in both maize genotypes studied, regardless of AM inoculation. A recent meta-analysis of 460 studies revealed that even if AM-inoculated C3 plants usually show higher *gs* values, C4 plants featured increases in *gs* of around 12% (Augé *et al.*, 2015). In agreement with this, AM symbiosis increased *gs* in both genotypes, especially in the case of the tolerant genotype under drought conditions. However, no differences were found in *gs* values in the drought-sensitive genotype subjected to the water stress. This could be probably related to the larger SDW of these plants with the consequent increased transpiring area, or to the fact

that drought-sensitive plants had generally lower *gs* values than the drought-tolerant ones. Nonetheless, it is noteworthy that the maize *g*s response to fungal inoculation could be described as inconsistent, ranging from unaltered to increased by AM (Boomsma & Vyn, 2008).

The alteration of plant physiology by AM is also confirmed with the better efficiency of Photosystem II, a highly sensitive-to-drought component from the plant photosynthetic apparatus (Ma *et al.*, 1995). The highest effect of the AM symbiosis was shown under drought in the drought-sensitive genotype, with enhanced performance of PSII by 72% as compared to 36% enhancement in the drought-tolerant cultivar. This indicates that photochemical apparatus of droughted AM plants did not lose functionality in light conversion, that is the proportion of the light absorbed by chlorophyll associated with PSII, to reaction centres (Johnson *et al.*, 2000), as it was reported in other species under several stresses (Hajiboland *et al.*, 2010; Porcel *et al.*, 2015; Yooyongwech *et al.*, 2016).

The percentage of membrane electrolyte leakage (EL), an estimation of cell membrane stability, has been postulated as a good indicator of the tolerance to water stress (Ortiz *et al.*, 2015). Accordingly, non-AM drought-tolerant plants had lowered EL values than the corresponding drought-sensitive ones. In addition, whereas in the case of the tolerant genotype droughted AM inoculated plants showed steady state levels, in sensitive plants AM symbiosis helped to stabilize the membranes both under well-watered and under drought stress conditions. In this sense, the higher membrane stability is often related to lower MDA levels (Abid *et al.*, 2016) accumulated as a result of lipid peroxidation. These results are in agreement with previous studies where MDA production was reduced by AM fungi (Liu *et al.*, 2016b). Furthermore, as expected, it is remarkable the similarity of results between EL and MDA concentration.

Plants need to maintain root osmotic potential below soil osmotic potential to take-up water. Previous studies have demonstrated that the AM fungi improve the plant osmotic adjustment by accumulation of different compounds (sugars, proline, free amino acids, etc) (Bheemareddy & Lakshman, 2011; Sheng *et al.*, 2011). This regulation by the AM symbiosis has been proposed as a mechanism allowing plants to grow under water stress (Ruiz-Lozano, 2003). In leaves of droughted plants, AM plants increased total soluble sugars in both genotypes, although to a lesser extent in the tolerant cultivar, suggesting an increased osmotic adjustment in AM plants during drought. The key effect of AM on sugar accumulation has been often reported under drought conditions (Wu *et al.*, 2007; Yooyongwech *et al.*, 2016; Zhang *et al.*, 2014) as it is also shown here in maize plants from both sensitive and tolerant genotypes.

In this study, when plants were subjected to drought stress the AM symbiosis induced a higher improvement of physiological parameters in drought-sensitive plants than in drought-tolerant plants. These include efficiency of photosystem II, membrane stability, accumulation of soluble sugars and shoot and root dry weights. Thus, drought-sensitive plants obtained greater physiological benefit from the AM symbiosis.

AM regulation of root hydraulic properties

Osmotic root hydraulic conductivity (Lo) can be 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 (Tyerman *et al.*, 1999). A reduction in Lo is usually reported in plants exposed to water deprivation (Javot and Maurel, 2002; Aroca *et al.*, 2012) probably as a mechanism for preventing water loss. This fact is consistent with our results, as a sharp drop in root hydraulic conductivity was observed in both genotypes when submitted to water stress. However, under drought stress the drought-tolerant genotype maintained a higher Lo values by 360% as compared to drought-sensitive genotype. Interestingly, AM increased Lo under drought compared to control plants in both genotypes, and this enhancement is in accordance with previous studies on AM plants under drought (Porcel *et al.*, 2005; Bárzana *et al.*, 2014; Sánchez-Romera *et al.*, 2016). The increase of Lo in AM plants could be related to an increased expression of plant or fungal aquaporins (Sánchez-Romera *et al.*, 2016). However, fungal aquaporins seems not to be involved in such increase since one gene was unaltered by drought, another gene was inhibited considerably in both maize cultivars, and the third one was only slightly induced in the drought-sensitive cultivar, but inhibited in the drought-tolerant one. Thus, the increase of Lo in AM plants may be due to additional mechanisms such as increased abundance and/or activity of the plants aquaporins due to post-translational modifications of these proteins (Chaumont & Tyerman, 2014) or to changes in density or size of plasmodesmata in AM roots (Blee & Anderson, 1998). Indeed, symplastic movement of water via plasmodesmata may also contribute significantly to Lo values (Galmés *et al.*, 2007).

Aquaporin abundance in root cortex cells may alter Lo, especially during water shortage (Maurel *et al.*, 2015), where aquaporins are thought to be regulated for the maintenance of the adequate water balance (Jang *et al.*, 2007a; 2007b). Among them, PIPs were proved to contribute to the adaptation of plants to drought episodes, also contributing to rehydration of the whole plant after water shortage (Maurel *et al.*, 2002). In addition to that, transcriptome analysis of drought tolerant and sensitive RILs in maize suggested that down-regulation of aquaporins is as a mechanism contributing to the

drought tolerance by upholding tissue turgor over longer time than drought-sensitive line (Min *et al.*, 2016).

In the present study, 16 maize aquaporins previously shown to be regulated by the AM symbiosis under different drought scenarios (Bárzana *et al.*, 2014) were analysed to check a possible differential regulation by the AM symbiosis in two maize cultivars with contrasting drought sensitivity. We first observed that there were differences in the expression of several of the studied aquaporins between the drought-sensitive and the drought-tolerant genotypes. But these differences depended on the water regime and also on the presence or absence of the AM fungus. In the sensitive genotype, a general down-regulation of aquaporins by the AM symbiosis, under drought and/or well-watered conditions (*ZmPIP1;1, ZmPIP1;3, ZmPIP1;4, ZmPIP1;6, ZmPIP2;2, ZmPIP2;4, ZmTIP1;1* and *ZmTIP2;3*) was featured (Figure 5, Figure S1). Similar result was also found in maize by Bárzana *et al.* (2014) and in other plant species (Liang *et al.*, 2013; Chitarra *et al.*, 2016). However, AM regulation of aquaporins in the drought-tolerant genotype was weaker, and only three aquaporins (*ZmPIP1;6, ZmPIP2;2* and *ZmTIP4;1*) were found to be altered. It is noteworthy that these three aquaporins were even up-regulated under well-watered conditions, which is an opposite behaviour than in the sensitive genotype, similar to results reported by Vinnakota *et al.* (2016) or by Liu *et al.* (2013) in two rice varieties and two *Malus* species with contrasting drought sensitivity. Also, upland rice and lowland rice with different responses to drought were compared to study the role of aquaporins in drought resistance and authors found important differences in PIP aquaporin transcriptional regulation in both types of rice (Lian *et al.*, 2006a).

During drought stress episodes, water conservation is critical for plant survival and productivity, and is achieved by an efficient uptake and stringently regulated water loss, in which aquaporins participate (Vinnakota *et al.*, 2016). Our results in the drought-sensitive cultivar are in line with the hypothesis that downregulation of aquaporins under water deprivation could be a way to minimize water loss, and the AM symbiosis could be helping the plant in this regulation. Through downregulation of aquaporin expression, roots from the drought-sensitive plants may be preventing drought damages by reducing water flow through cell membranes and upholding tissue turgor as a response to the soil water deficit (Liang *et al.*, 2013; Min *et al.*, 2016). Indeed, dehydration avoidance during drought stress is a consequence of a tight balance between stomatal movements, root water uptake capacity and water distribution along plant tissues (Aroca *et al.*, 2012; Ionenko *et al.*, 2012). Nevertheless, the drought-tolerant genotype may not need this adjustment as other naturally-occurring mechanisms such as deeper root development, improved turgor

adjustment and photosynthetic efficiency or altered hormonal levels (Min *et al.*, 2016) protected this genotype from the damage produced by drought.

It is also remarkable that under drought stress conditions *ZmPIP1;1*, *ZmTIP1;1* and *ZmPIP2;2* were downregulated by AM only in the drought-sensitive genotype. Among these three aquaporin genes, *ZmTIP1;1* is the most expressed TIP in maize (Chaumont *et al.*, 2001) and, besides water, it has the capacity to transport different compounds (urea, ammonia, boron, H_2O_2) (Bárzana *et al.*, 2014). *ZmPIP2;2* showed a high water permeability (Pf) when expressed in *Xenopus laevis* oocytes (Bárzana *et al.*, 2014). Thus, such tight regulation makes sense with a fine control of water balance in roots. Moreover, the specific regulation of these aquaporins by the AM symbiosis in the drought-sensitive cultivar point out a putative role of these three aquaporins in the AM-induced tolerance to drought stress, being possible targets for future studies.

In this sense, it must be considered that plant aquaporins can transport water, but also many other physiological substrates such as urea, glycerol, boric acid, silicic acid, hydrogen peroxide or gaseous molecules such as carbon dioxide, ammonia or oxygen (Heinen *et al.*, 2014; Li *et al.*, 2014; Zwiazek *et al.*, 2017). Among the different plant aquaporin subfamilies, NIPs is a versatile group with high diversity of substrates and a broad range of subcellular localization patterns (Maurel *et al.*, 2008). Regulation of NIP genes by the arbuscular mycorrhizal symbiosis has been shown in different plant species such as *Medicago truncatula* (Uehlein *et al.*, 2007), *Lotus japonicus* (Giovannetti *et al.*, 2012), *Zea mays* (Bárzana *et al.*, 2014) or *Solanum lycopersicum* (Chitarra *et al.*, 2016). MtNIP1 had putative plasma membrane localization and was induced by mycorrhization. LjNIP1 was expressed in the inner membrane system of arbuscule-containing cells and could transport water. ZmNIP1;1 was shown to transport glycerol as well as silicon, while ZmNIP2;2 could transport silicon. LeNIP3;1 was overexpressed in AM tomato plants subjected to drought stress. Altogether, their transport capacities and localizations suggest that the regulation of NIP genes by the AM symbiosis could be involved in cell turgor regulation and in the exchange of water and solutes between both symbionts (Uehlein *et al.*, 2007; Giovannetti *et al.*, 2012; Bárzana *et al.*, 2014; Chitarra *et al.*, 2016), which may be of physiological importance to cope with drought stress.

Given the diversity of substrates that can be transported by plant aquaporins, those isoforms regulated by the AM symbiosis may have a role in regulation of leaf and root hydraulics, stomatal movement, nutrient uptake and translocation along plant tissues, carbon fixation or signalling processes. In this context, regulation of aquaporins having urea or ammonium transport capacity suggests that these aquaporins could be involved in the fungus-based nitrogen nutrition of the host plants or in plant nitrogen mobilization

and metabolism (Bárzana *et al.*, 2014), as was also proposed for ectomycorrhizal fungi (Dietz *et al.*, 2011). Indeed, in the AM symbiosis, ammonium is suggested to be the major nitrogen compound transferred to the host plant, with urea playing a role as an intermediate solute (Tian *et al.*, 2010). Studies by Gustavsson *et al.* (2005) suggested that export of plant-derived glycerol may be important for symbiotic fungi. Thus, the regulation of plant aquaporins which can transport glycerol (i.e. ZmNIP1;1 and ZmTIP4;1) may be important for the AM symbiosis or for the plant-fungus interaction under drought stress conditions. Similarly, the regulation by the AM symbiosis of aquaporins with boron and/or silicon transport capacity could have structural functions in maize plants. Hydrogen peroxide is one of the most abundant reactive oxygen species continuously produced in the metabolism of aerobic organisms. As oxidant molecule, it reacts with various cellular targets causing cell damage, while at low concentration it acts as a signal molecule, controlling different essential processes in plants (Bienert *et al.*, 2006). Thus, aquaporins with H_2O_2 transport capacity such as ZmTIP1;1 could play a key role in the detoxification of excess H_2O_2 generated under stress conditions, or in signalling events mediated by H_2O_2 (Bárzana *et al.*, 2014). That means that elucidating the *in vivo* transport capacities of the aquaporins regulated by the AM symbiosis is required to understand the role of these proteins in the AM-induced drought tolerance.

Conclusion

In summary, under water limiting conditions AM plants enhanced maize growth, especially in the case of the drought sensitive cultivar as reflected by the larger biomass (shoots and roots) accumulation. This beneficial effect of the AM symbiosis was linked to a better efficiency of PSII, to the higher membrane stability and to lower lipid peroxidation.

It is noteworthy that *ZmPIP1;1, ZmPIP1;3, ZmPIP1;4, ZmPIP1;6, ZmPIP2;2, ZmPIP2;4, ZmTIP1;1, ZmTIP2;3* and *ZmTIP4;1* gene expression was regulated by the AM symbiosis in the drought-sensitive cultivar, while in the drought-tolerant cultivar only *ZmPIP1;6, ZmPIP2;2* and *ZmTIP4;1* genes were regulated by the AM symbiosis. In the drought-sensitive cultivar, the genes *ZmPIP1;1, ZmPIP2;2, and ZmTIP1;1* were down-regulated by the AM symbiosis when the plants were subjected to drought stress. Moreover, in this cultivar the genes *ZmPIP1;3, ZmPIP1;4, ZmPIP1;6, ZmPIP2;4, ZmTIP2;3* were also down-regulated when the plants grew under well-watered conditions and only *ZmTIP4;1* was up-regulated. In the drought tolerant cultivar, the three genes regulated by the AM symbiosis were indeed up-regulated under well-watered conditions and *ZmTIP4;1* was in addition up-regulated under drought stress.

Thus, the broader and contrasting regulation of these aquaporins by the AM symbiosis in the drought-sensitive than the drought-tolerant cultivar suggests a role of these aquaporins in water homeostasis or in the transport of solutes of physiological importance in both cultivars under drought stress conditions, which may be important for the AM-induced tolerance to drought stress. Grondin *et al.* (2016) found recently that a differential regulation of PIP aquaporins in six rice varieties was related to the drought stress tolerance of these varieties. Further research on the *in vivo* transport capacities by these aquaporins is needed to understand the specific role of these proteins in the AM-induced drought tolerance.

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Supporting Information

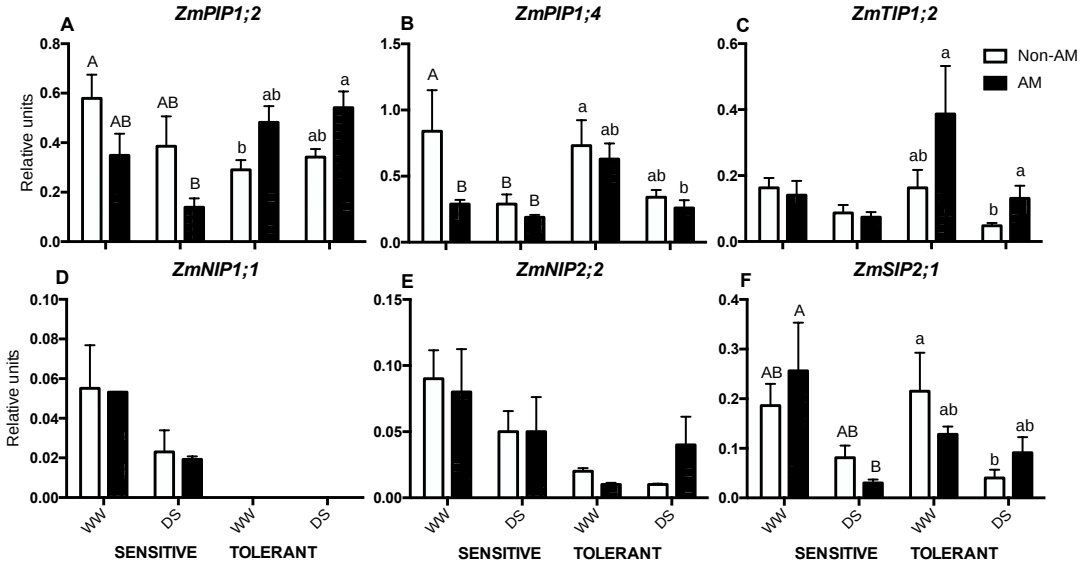

Figure S1. Expression of *ZmPIP1;2* (A), *ZmPIP1;4* (B), *ZmTIP1;2* (C), *ZmNIP1;1* (D), *ZmNIP2;2* (E) and *ZmSIP2;1* (F) in two maize genotypes differing in drought tolerance and inoculated or not with an arbuscular mycorrhizal fungus (AM). Data represents the means of 3 values ± S.E. Different letter indicates significant differences between treatments ($p<0.05$) based on Duncan's test for sensitive (uppercase) and tolerant (lowercase) genotypes. Asterisks indicate significant differences between drought-sensitive and drought-tolerant genotypes within each watering regime, according to Student's T test.

Table S1. Pearson correlation coefficients between Lo and expression of the different maize aquaporin genes in a drought-sensitive and a drought-tolerant genotype.

Sensitive genotype			**Tolerant genotype**		
	Lo			**Lo**	
Genes	**Pearson Corr**	**Sig**	**Genes**	**Pearson Corr**	**Sig**
ZmPIP1;1	-0.539	0.461	*ZmPIP1;1*	-0.347	0.653
ZmPIP1;2	0.474	0.526	*ZmPIP1;2*	-0.381	0.619
ZmPIP1;3	0.481	0.519	*ZmPIP1;3*	0.677	0.323
ZmPIP1;4	0.53	0.47	*ZmPIP1;4*	0.923	0.077
ZmPIP1;6	0.46	0.54	*ZmPIP1;6*	0.371	0.629
ZmPIP2;2	-0.03	0.97	*ZmPIP2;2*	0.571	0.429
ZmPIP2;4	0.409	0.591	*ZmPIP2;4*	0.309	0.691
ZmTIP1;1	-0.3	0.7	*ZmTIP1;1*	-0.355	0.645
ZmTIP1;2	0.421	0.579	*ZmTIP1;2*	0.472	0.528
ZmTIP2;3	0.285	0.715	*ZmTIP2;3*	0.886	0.114
ZmTIP4;1	0.539	0.461	*ZmTIP4;1*	0.158	0.842
ZmNIP1;1	**0,954***	0.046	*ZmNIP2;1*	**0,962***	0.038
ZmNIP2;1	0.874	0.126	*ZmNIP2;2*	-0.045	0.955
ZmNIP2;2	-0.151	0.849	*ZmSIP2;1*	**0,985***	0.015
ZmSIP2;1	0.512	0.488			

CHAPTER II

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

Adapted from:

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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 (Pf) and cell hydraulic conductivity (Lpc) 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 Pf and Lpc values of non-stressed plants, while in non-AM roots these values declined drastically as a consequence of water deficit. Interestingly, the phosphorylation status of PIP2 aquaporins increased in AM plants subjected to water deficit, and Pf 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 demonstrates 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.

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 water conductivity (Lpr) 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 Lpr (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 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 analysed (Aroca *et al.* 2007). In addition, the regulation of the plant aquaporins also depends on the severity and duration of the stress applied, as 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 (Pf) 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 (Lpc) 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 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), 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}.

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

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 $Lpc = \ln(2)V/(AT_{1/2}(\varepsilon+\pi_i)$, 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, 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% 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 was 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).

- RT-qPCR

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 ™), according to the manufacter's' instructions.

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 RT-qPCR 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, 2001b). 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 (A_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and instantaneous water use efficiency (iWUE = A_N/g_s) of fully expanded young leaves in five different plants were measured using portable photosystem system LI-6400 (LICOR Biosciences, Lincoln, NE, USA) after 8 weeks of growth. The response of A_N 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 A_N /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 A_N/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 described in Quiroga *et al.* (2017, 2018) in the second youngest leaf of 10 different plants of each treatment after 8 weeks of growth.

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).

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 analysed 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.

- 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.

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).

Half time of water exchange ($T_{1/2}$) was similar in AM and non-AM plants under well-watered conditions, but under water deficit conditions it was 45% lower in AM plants than in non-AM ones (Table 2).

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 (Lpc) values than non-AM plants (42% of decrease).

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
WD non-AM	0.33 ± 0.04 a	8.16 ± 1.8 a	5.44 ± 0.7 a
WD 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 analysed 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.

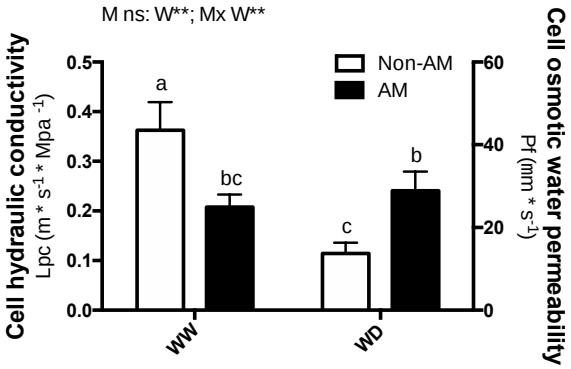

Figure 1. Experiment 1. Cell hydraulic conductivity (Lpc) and inferred Pf values of intact root cell of plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD), determined with the cell pressure probe. Data were analysed 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.

However, under water deficit, Lpc 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 Lpc values as compared to non-AM plants (Figure 1). The obtained Lpc values were converted into osmotic water permeability (Pf) values (Figure 1) for comparison with Pf data from the protoplast swelling assay (Volkov *et al.*, 2007).

Pf 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, Pf values decreased in non-AM plants, although the decrease was not significant. In contrast, in AM plants Pf values remained similar as under well-watered conditions, almost doubling the values of non-AM plants subjected to water deficit.

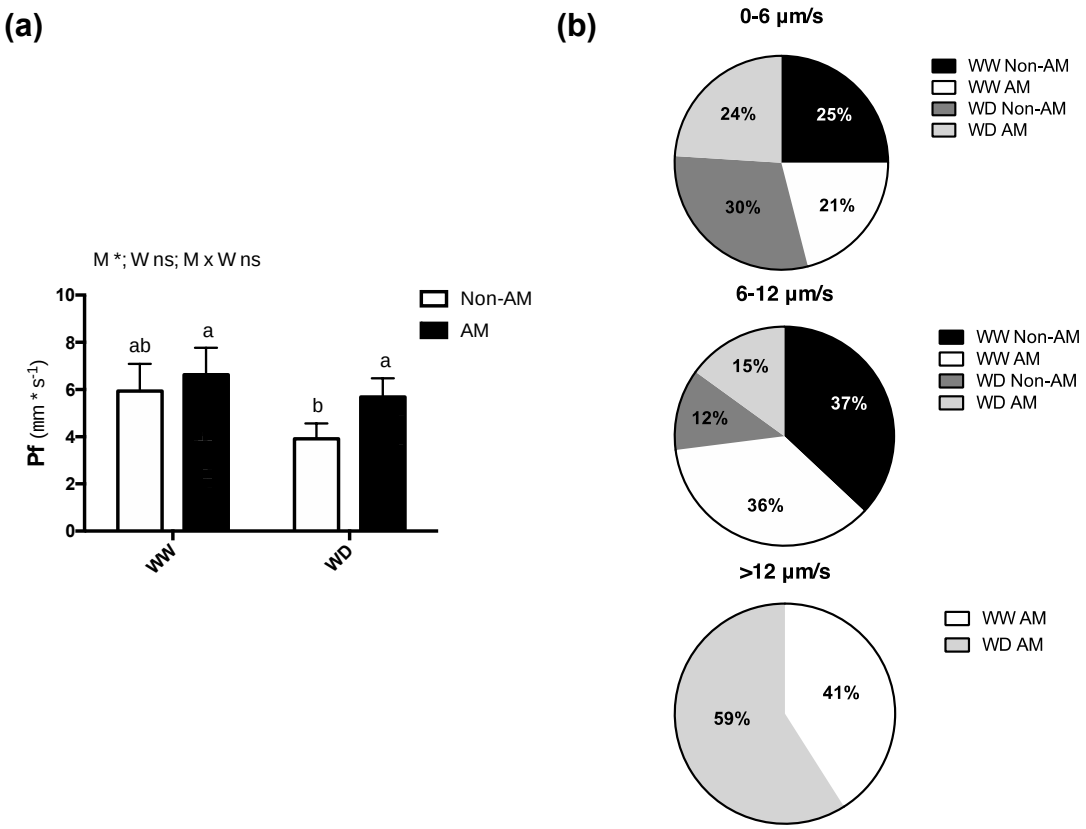

Figure 2. Experiment 1. **(a)** Osmotic water permeability coefficient (Pf) of isolated protoplasts from roots of plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data show the mean ± SE for 30 cells (at least three different plants). Data were analysed 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.

We analysed the distribution of Pf 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 Pf values over 12 µm/s. These higher Pf values were observed only in protoplasts coming from AM treatments, either under well-watered or water deficit conditions. Most Pf values were under 6 µm/s in

all treatments. However, AM plants exhibited a lower proportion of protoplast with Pf values under 6 µm/s than non-AM plants. Moreover, the highest proportion of Pf values within this category (92%) was found in droughted non-AM plants and the lowest proportion of Pf values within this category (65%) was found in well-watered AM plants. Altogether, the Pf values distribution could explain the differences in water permeability between the treatments.

- Aquaporin accumulation and phosphorylation status of PIP2s

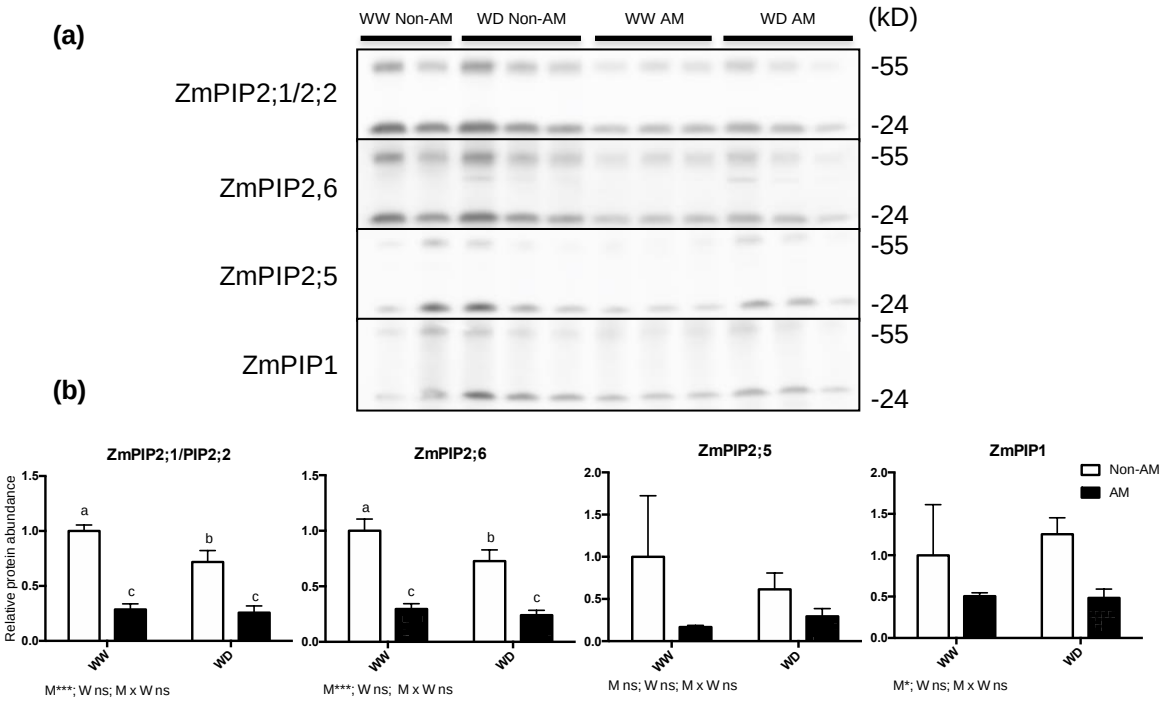

Figure 3. Experiment 1. **(a)** Accumulation of ZmPIP proteins in the microsomal fraction of roots from plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). The position of the molecular mass markers is indicated. **(b)** Quantification of signals on membranes (relative units, normalized to Commassie blue gel signal). Data were analysed 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.

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.

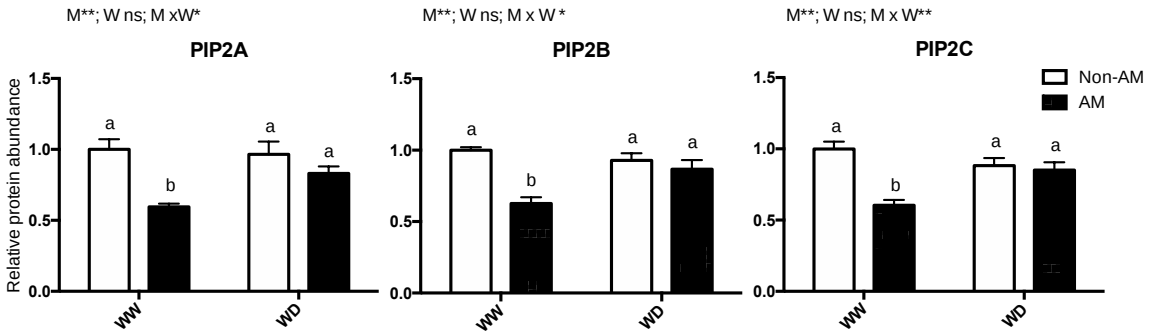

Figure 4. Experiment 1. PIP2A (Ph-Ser280), PIP2B (Ph-Ser283) and PIP2C (Ph-Ser280/Ser283) relative protein abundance in the microsomal fraction of roots from plants inoculated (black bars) or not (white bars) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analysed 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.

- 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.

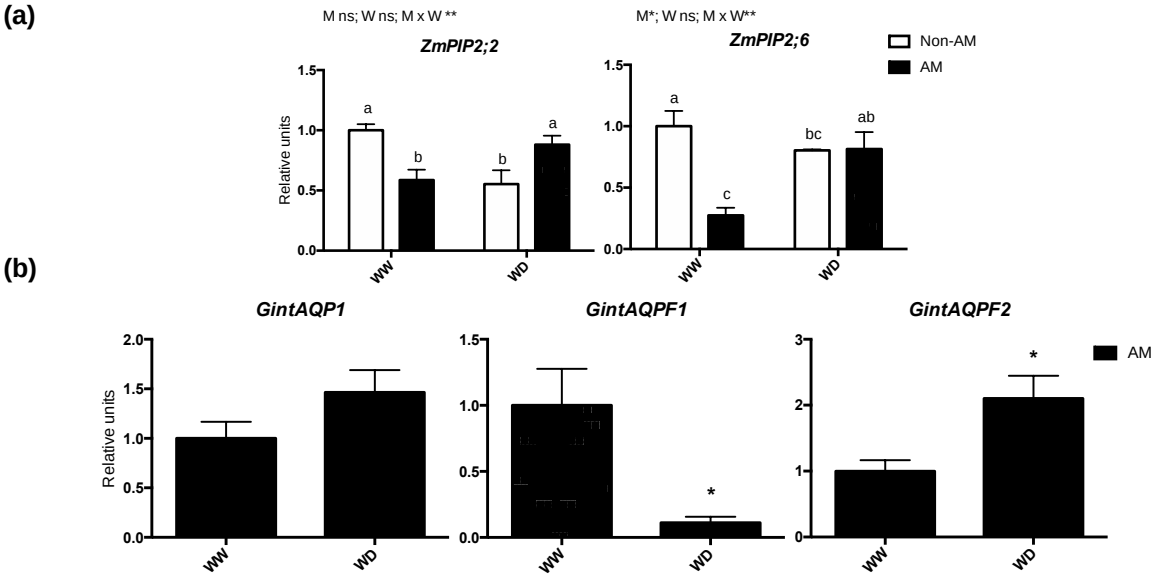

Figure 5. Experiment 1. **(a)** Relative mRNA levels of *ZmPIP2;2* and *ZmPIP2;6* normalized to *Zmtubulin* and *ZmGAPDH* genes. Plants were inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analysed by 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.

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).

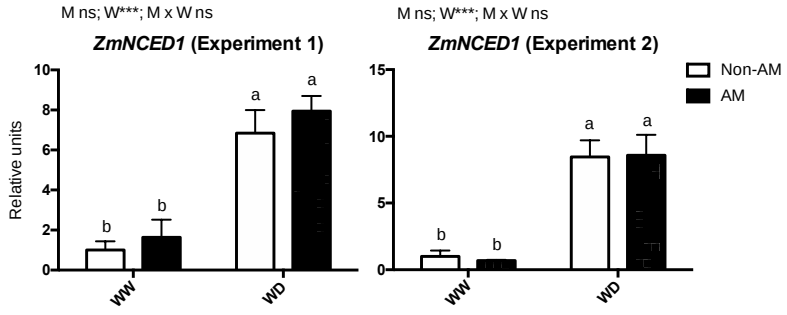

Figure 6. Relative mRNA levels of *ZmNCED1* gene normalized to *Zmtubulin* and *ZmGAPDH* genes. Plants were inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analysed by two-way ANOVA with mycorrhiza (M), 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.

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).

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 analysed 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.

- 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, A_N; stomatal conductance, *gs*, or intercellular CO_2 concentration, Ci) due to AM fungal inoculation (Table 4). Nevertheless, plants under water deficit exhibited lower A_N, *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 A_N (by 75%) and *gs* (90%) values. Similar results were obtained when analysing photosynthetic capacity at this growth period (Figure 7). Well-watered plants presented no differences in the maximum apparent rate of phosphoenolpyruvate carboxylase 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.

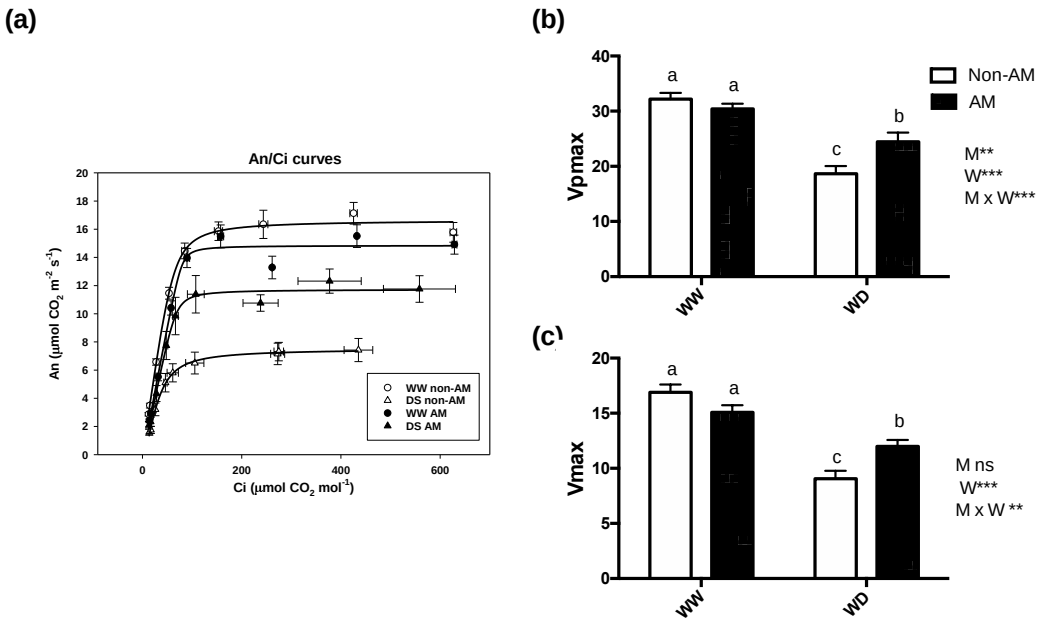

Figure 7. Experiment 2. **(a)** A_N /Ci curves, **(b)** Maximum carboxylation capacity of PEPc (Vpmax) and **(c)** CO_2-saturated photosynthetic rate (Vmax) of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or water deficit, WD). Data were analysed 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.

Table 4. Net photosynthesis (A_N), 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			
	A_N **(µmol CO_2 m^{-2} s^{-1})**	***gs*** **(mmol H_2O m^{-2} s^{-1})**	**Ci µmol 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 analysed 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.

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 (Bárzana *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 & 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 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.

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

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 Pf levels observed in non-stressed plants, while these levels declined drastically in the absence of the AM fungus (Figures 1 and 2a). Interestingly, Pf 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 protoplast isolation process (4 h) could affect gene and protein expression or the regulation of aquaporin activity in the membranes, leading to low Pf values (1-17 $\mu m\ s^{-1}$) compared to the pressure probe Pf values (10-100 $\mu m\ s^{-1}$) inferred from Lpc 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 Lpc and Pf 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 Pf 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-inoculated plants under these conditions, as it has been already reported for maize roots colonized by the same mycorrhizal fungus (Bárzana *et al.*, 2012).

$T_{1/2}$ parameter is considered to be a direct measure of hydraulic conductivity ($T_{1/2}$~ 1/Lpc) 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 Lpc 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 cell elastic properties during the water stress (Tomos & Leigh, 1999) that is modified by root AM fungal colonization.

Are aquaporins responsible for Pf and Lpc changes in AM roots?

Several aquaporin isoforms were analysed 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 with 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 Pf. Although ZmPIP2;2 and ZmPIP2;6 showed similar trends compared to Pf and Lpc results, differences due to fungal 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 Pf 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 Lpc 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 find 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.* 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 knockout 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 considered that Lpc and Pf 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 Lpc and Pf values with aquaporin gene expression or protein accumulation patterns may be due to the fact that Lpc and Pf were measured on isolated cells, while gene and protein levels were measured on the whole root 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 A_N/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 (A_N) 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 A_N under water deficit conditions. This effect could be 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

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 Lpc and Pf 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.

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CHAPTER III

Elucidating the possible involvement of maize aquaporins in the plant boron transport and homeostasis mediated by *Rhizophagus irregularis* under drought stress conditions

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Abstract

Boron (B) is an essential micronutrient for higher plants, having structural roles in primary cell walls, but also other functions in cell division, membrane integrity, pollen germination or metabolism. Plants need to maintain B concentration in their tissues within a narrow range by regulating transport processes, since either high or low B levels negatively impact crop performance. It was long considered that the B uptake and transport in plant roots was a passive transport. However, the presence of active transport and protein-facilitated diffusion through aquaporins, have also been demonstrated. Little information is available about arbuscular mycorrhizal (AM) symbiosis and B homeostasis in the host plant under drought stress conditions. This study aimed at elucidating the possible involvement in this process of some plant aquaporins, which potentially can transport B and are regulated by the AM symbiosis. Thus, AM and non-AM plants were cultivated under 0, 25 or 100 μM boron in the growing medium and subjected or not to drought stress. The accumulation of B in plant tissues and the regulation of plant aquaporins and other B transporters were analysed. This is the first report investigating a possible role of AM-regulated plant aquaporins in the *in planta* B transport and homeostasis. However, the general down-regulation of aquaporins and B transporters in AM plants suggests that, when the mycorrhizal fungus is present, other mechanisms contribute to B homeostasis, probably more related to the enhancement of water transport, which would concomitantly increase the passive transport of this micronutrient.

Keywords: arbuscular mycorrhizal symbiosis; aquaporins; boron, drought stress

Introduction

In higher plants the metalloid boron (B) is an essential micronutrient, mainly because of its functional and structural roles in primary cell walls, where it crosslinks the pectin polysaccharide rhamnogalacturonan-II (RG-II) to form a network (Kobayashi *et al.*, 1996; O'Neill *et al.*, 2004). Nevertheless, B may have other different functions in cell division and elongation, membrane integrity, pollen tube growth and germination or phenolic and nitrogen metabolism (Voxeur and Fry, 2014; Shireen *et al.*, 2018). Besides its main role in plant physiology, both, high or low B levels can negatively impact crop yield and quality. Thus, plants need to maintain B concentration in their tissues within a narrow range by regulating transport processes (Yoshinari & Takano, 2017).

Deficiency in B usually occurs in areas with high rainfall, since boric acid is quite soluble and easily leached by rainfall (Yoshinari & Takano, 2017). On the contrary, B toxicity naturally occurs in arid and semiarid soils, but it can also be the consequence of fertilization, irrigation or mining (Nable *et al.*, 1997). In Spain, B excess in soils has been attributed to the use of water from desalinating plants and waste treatment (Simón-Grao *et al.*, 2019). In any case, under sustained drought stress, the decrease in plant transpiration can lead to B deficiency, impacting negatively plant performance.

Boron is taken up by roots as boric acid at physiological pH values, thus, unlike other essential nutrients that are absorbed as ions, it is consumed as an uncharged molecule (Miwa *et al.*, 2009). Its cell membrane permeability is relatively high, and it was considered for a long time that the B uptake and transport in plant roots was only a passive process. Nonetheless, it was later demonstrated the presence of active transport and protein-facilitated diffusion for this nutrient (Dannel *et al.*, 2000; Stangoulis *et al.*, 2001; Takano *et al.*, 2006). Thus, depending on B availability, the transport of B can follow three different molecular pathways: (1) passive diffusion through biological membranes; (2) facilitated transport; (3) active transport.

Members of the Nodulin 26-like intrinsic protein (NIP) aquaporin subfamily have been identified as key channel molecules in the uptake and transport of B in roots. In *Arabidopsis thaliana*, *AtNIP5;1* was found to be crucial under B limitation (Takano *et al.*, 2006). BOR-1 is a plasma membrane protein identified in *A. thaliana* as a B efflux transporter. It is crucial under B limitation for xylem loading of the nutrient (Takano *et al.*, 2002). BOR-like transporters and NIPs are frequently present in the same cells but localized at opposite cell sides, allowing the transcellular flux of B within the plant organs (Shimotohno *et al.*, 2015). Functional orthologues of these proteins were found in different crops such as rice, wheat or barley (Sutton *et al.*, 2007; Schnurbusch *et al.*, 2010;

Leaungthitikanchana *et al.*, 2013; Hanaoka *et al.*, 2014). Moreover, an aquaporin isoform from sugar beet has been recently described with a role in plant B homeostasis and abiotic stress response (Porcel *et al.*, 2018).

Generally, monocots need less B for their normal growth than dicotyledonous species (Chatterjee *et al.*, 2017) and, in particular, maize was considered to be a low B-demanding cereal. However, B deficiency also affects this crop worldwide (Lordkaew *et al.*, 2011), especially at the reproductive stage, since this micronutrient is especially important for the adequate development of inflorescences and tassels (Blevins & Lukaszewski, 1998). Indeed, orthologues of the *Arabidopsis* B channels and transporters were found in maize. *ZmNIP3;1* (*TSL1*) was shown to be crucial for B transport within the plant, as well as, for its reproductive and vegetative development (Durbak *et al.*, 2014; Leonard *et al.*, 2014). Moreover, other maize aquaporins, ZmTIP1;1, ZmTIP2;1, ZmNIP1;1 and ZmNIP2;2, were found to transport B in yeast (Bárzana *et al.*, 2014). As an efflux transporter, the *ZmRTE* gene was found to be a functional orthologue of *AtBOR1* (Chatterjee *et al.*, 2014), and three additional B transporters genes (*ZmRTE2*, *ZmRTE3* and *ZmRTE6*) have been identified in maize (Chatterjee *et al.*, 2017). The diversity of B transporters highlights the tight regulation of B homeostasis in maize.

Most crop plants form mutualistic symbiosis between their roots and arbuscular mycorrhizal fungi. These fungi increase the surface of plant root systems, enhancing water and nutrient uptake and also providing tolerance to biotic and abiotic stresses (Kumar & Verma, 2018). In particular, the beneficial effects of AM fungi under drought stress have been widely studied (Augé, 2001; Ruiz-Lozano *et al.*, 2012c). Aquaporins were recognized as important elements in both water and nutrient exchanges during the AM symbiosis (Maurel & Plassard, 2011) and, in line with this, 16 out of 36 aquaporins from maize were found to be differentially regulated by the AM symbiosis during drought stress (Bárzana *et al.*, 2014). Given the diversity of substrates that can be transported by these AM-regulated aquaporin isoforms, they may have a role in the regulation of important physiological processes (Uehlein *et al.*, 2007), and thus, the elucidation of their *in planta* transport capacities is necessary to understand better the process of AM-induced drought tolerance.

Among the effects of the AM symbiosis in plant performance during drought stress, increased levels of ions are often observed (Filho *et al.*, 2017). Although no much information is available about AM symbiosis and B homeostasis in the host plant, a recent work has shown the beneficial effect of this symbiosis decreasing B toxicity in leaves and roots when applied to a citrus rootstock, which consequently increased plant tolerance to this stress (Simón-Grao *et al.*, 2019).

The present study aimed to assess whether B has a role *in planta* as an aquaporin substrate in the AM-enhancement of plant performance during drought stress. With this purpose, different concentrations of B were applied in the nutrient solution to non-inoculated and mycorrhizal plants that were submitted or not to a water deficit treatment. The results obtained improve our knowledge about the mechanisms of the AM symbiosis to enhance the tolerance to water deficit.

Materials and methods

Experimental design

The experiment consisted of a factorial design with three factors: (1) inoculation treatment, with plants inoculated with the AM fungus *Rhizophagus irregularis*, strain EEZ 58 (AM) and non-inoculated control plants (Non-AM); (2) watering treatment, so that half of the plants were subjected to drought stress (DS) for 15 days before harvest while the other half was grown under well-watered (WW) conditions throughout the entire experiment; (3) boron treatment, so that plants were irrigated with nutrient solution with three different B concentrations, plants without B in the nutrient solution (B0, only obtaining the B from the very low soil-containing substrate), plants irrigated with 25 μM of B in the nutrient solution (B25) and plants irrigated with 100 μM of B (B100), resulting in twelve different treatments with six replicates per treatment (n=6), giving a total of 72 plants.

Soil and biological materials

The growing substrate consisted of a mixture of soil and sand (1:9 v/v). The 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 undiluted soil had a pH of 8.1 (water); 0.85% organic matter, nutrient concentrations (mg kg^{-1}): P, 10 ($NaHCO_3$-extractable P); N, 1; K, 110. The soil texture was made of 47.1% silt, 38.3% sand and 14.6% clay.

Seeds of *Zea mays* L. were provided by Pioneer Hi-Bred (Spain), cultivar PR34B39 that was also used in previous studies (Quiroga *et al.*, 2017; 2018). Seeds were pre-germinated in sand and then transferred to 1.5 L pots containing 1250 g of the above described substrate. At planting time, half of the plants were inoculated with ten grams of AM inoculum with *Rhizophagus irregularis* (Schenck and Smith), strain EEZ 58. The inoculum consisted of spores, mycelia, infected root fragments and soil. Non-inoculated plants received a 10 mL aliquot of an inoculum filtrate (<20 μm), in order to provide the natural microbial population present in the inoculum, but free of AM propagules.

Growing conditions

Plants were grown under greenhouse conditions (average photosynthetic photon flux density 800 µmol m^{-2} s^{-1}, 25/20ºC, 16/8 light dark period and 50-60% RH) for a total of eight weeks. Plants were irrigated three times per week with 50 mL of Hoagland nutrient solution (Hoagland and Arnon, 1950) modified to contain only 25% of P, in order to avoid the inhibition of AM symbiosis establishment. Hoagland solution was also modified to provide the different Boron concentrations (0, 25 and 100µM). Plants received the same amount of water on alternate days.

Drought stress treatment was applied for the last 2 weeks. For that, plants were irrigated with half the water/Hoagland volume of well-watered ones (25 mL vs. 50 mL). In order to avoid a combination of drought stress plus nutrient deficiency, droughted treatments received 2X Hoagland nutrient solution, so that 25 mL provided the same nutrient levels as 50 mL of the 1X Hoagland nutrient solution used with well-watered plants. This water stress is considered as a severe stress and was similar to that imposed in previous studies (Quiroga *et al.*, 2017; 2018).

Parameters measured

- Biomass production

The shoot and root system of six replicates per treatment were fresh weighted at harvest (8 weeks after sowing). Two replicates per treatment were dried in a forced hot-air oven at 70 ºC for 2 days and the dry weight (DW) was measured. The determined dry matter content was used to calculate dry weight from the other plant replicates.

- Symbiotic development

Maize roots were stained following the procedure described by Phillips and Hayman (1970), in order to visualize and differentiate AM fungal structures. The extent of mycorrhizal colonization was calculated in three replicates per treatment according to the gridline intersect method (Giovannetti and Mosse, 1980).

- Stomatal conductance

Stomatal conductance (*gs*) was measured with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) two hours after the onset of photoperiod and following the manufacter's recommendations. The second fully expanded youngest leaf from five plants per treatment was used for this measurement. Measurements were taken one day before harvest.

- Leaf chlorophyll content

Leaf chlorophyll content was estimated four hours after sunrise on the second fully expanded youngest leaf for each plant by using a Chlorophyll Content Measurement System CL-01 (SPAD, Hansatech Instruments ltd., Norfolk, UK). This device determines relative chlorophyll content in leaf samples by measuring dual optical absorbances (620 and 940 nm wavelengths). Relative chlorophyll content was measured in five different plants per treatment one day before harvest.

- Photosynthetic efficiency

The efficiency of photosystem II was measured one day before harvest in light adapted maize leaves. We used a Fluor-Pen FP100 (Photon Systems Instruments, Brno, Czech Republic), as described previously in Quiroga *et al.* (2017, 2018, 2019), using the second fully expanded youngest leaf of five different plants per treatment.

- Mineral analysis

Analysis of Ca, K, Mg, S and P concentration (g/100g) as well as B, Cu, Fe, Mn, Zn and Si concentration (mg/kg) was determined in four samples (n=4) of shoots and roots of the different treatments by means of inductively coupled plasma-optical emission spectrometry (ICP-OES; THERMO ICAP 6000 DUO). The determination was performed by the Ionomic service of the CEBAS-CSIC institute of Murcia, Spain.

- RT-qPCR

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

The expression of eight previously selected maize aquaporins (Quiroga *et al.*, 2017), plus the aquaporin genes *ZmNIP1;1*, *ZmNIP2;2*, *ZmNIP3;1* and the B transporters-encoding genes *RTE*, *RTE2* and *RTE3* was measured by RT-qPCR using 1 µL of diluted cDNA (1:9) and PowerUp™ SYBR™ Green Master Mix in a QuantStudio™ 3 system (Thermo Fisher Scientific). The reaction was carried out at annealing temperature of 58ºC for all primers and repeated for 40 cycles. For normalization of gene expression values, four reference genes were measured in all the treatments. These genes were tubulin (gi:450292), poliubiquitin (gi:248338), elongation factor 1 (gi:2282583) and GAPDH (gi:22237) (Bárzana *et al.*, 2014). "NormFinder" algorithm (Andersen *et al.*, 2004) (https://moma.dk/normfinder-software) was used to choose the best-performing of these reference gene under our specific conditions. Thus, expression levels were normalized

according to the elongation factor 1 (gi:2282583). The relative abundance of transcripts was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001b). Three biological replicates were used per treatment and the threshold cycle (Ct) of each biological sample was determined in duplicate. Negative controls without cDNA were used in all PCR reactions.

- Aquaporins abundance and PIP2s phosphorylation status

For sub-cellular fractionation pieces of intact roots were grinded with 6 mL of a protein extraction buffer containing 250 mM Sorbitol, 2 mM EDTA, 50 mM Tris-HCl (pH 8), and protease inhibitors, according to Hachez *et al.* (2006a) with slight modifications. All steps were performed at 4ºC. The homogenate was centrifuged during 10 min at 770 *g* and the supernatant obtained was centrifuged again 10 min at 10000*g*. Finally, the subsequent supernatant was centrifuged during 2 hours at 144000*g* and the obtained pellet (containing the microsomal fraction) was resuspended in 20 µL of suspension buffer (5 mM KH_2PO_4, 3 mM KCl, 330 mM sucrose, pH 7.8) and sonicated twice for 5 s. A Bradford analysis was used to quantify total protein amounts. The abundance of specific proteins was measured by ELISA. A 2 µg aliquot of the microsomal fraction was incubated at 4ºC overnight in carbonate/bicarbonate coating buffer, pH 9.6. Afterwards proteins were cleaned by 3x 10 min washes with Tween Tris-buffered saline solution (TTBS) and blocked at room temperature with 1% bovine serum albumin (BSA) on TTBS for 1 hour. After three more washes with TTBS, proteins were incubated at room temperature for 1 hour with 100 µL of the primary antibody (1:1000 in TTBS v/v).

A total of eight different primary antibodies were used. Two antibodies recognize several PIP1s and PIP2s aquaporins, three antibodies recognize the phosphorylation of PIP2 aquaporins in the C-terminal region: PIP2A (Ser-280), PIP2B (Ser-283) and PIP2C (Ser-280/Ser-283) (Calvo-Polanco *et al.* 2014). Finally, we also used antibodies recognizing ZmPIP2;1/2;2, ZmPIP2;4 and ZmTIP1;1 (Hachez *et al.*, 2006a). As secondary antibody, a goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma-Aldrich Co.) was used at dilution 1:10000.

- Statistical analysis

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

Results

Plant biomass and symbiotic development

The total plant dry weight was not significantly affected by B levels under well-watered conditions, with the exception of non-AM plants under high B concentration (B100), which showed a smooth depression of growth. Mycorrhization enhanced significantly plant dry weight under medium and high B concentrations (B25 and B100) compared to non-AM plants at the same B concentrations (Table 1).

Under drought stress, AM plants presented higher plant dry weight compared to non-AM plants at the three B concentrations assayed (Table 1).

The percentage of root colonization was not affected by the B concentration under well-watered conditions (near 50%). Under water deficit conditions, mycorrhization was slightly increased at B100 as compared to B0 plants (Table 1).

Table 1. Percentage of mycorrhizal root length, plant dry weight (Plant DW), stomatal conductance (*gs*), SPAD values and photosystem II efficiency in the light-adapted state (ΔFv/Fm') in maize plants inoculated or not with the AM fungus Rhizophagus irregularis, submitted to two water regimes (well-watered-WW- or drought stress DS) and irrigate with three levels of B concentration in the nutrient solution (B0-B 0 μM, B25- B 25 μM and B100- B 100 μM. Data represents the means of three values ± SE for mycorrhization, six values ± SE for plant DW and five values ± SE for *gs*, SPAD and ΔFv/Fm'. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test.

			Mycorrhization (%)	Plant DW (g plant^{-1})	*gs* (mmol H_2O m^{-2} s^{-1})	SPAD	ΔFv/Fm'
WW	B0	Non-AM	n.d.	3.98 ± 0.18 AB	40.3 ± 8.05 C	10.6 ± 0.66 B	0.65 ± 0.01 A
		AM	54.3 ± 4.48 abc	4.06 ± 0.20 AB	148.4 ± 33.0 A	10.5 ± 0.54 B	0.66 ± 0.01 A
	B25	Non-AM	n.d.	3.71 ± 0.11 B	53.8 ± 10.3 C	10.6 ± 0.43 B	0.63 ± 0.01 AB
		AM	47.7 ± 2.60 bc	4.21 ± 0.13 A	105.4 ± 16.6 AB	10.6 ± 0.64 B	0.64 ± 0.01 AB
	B100	Non-AM	n.d.	3.13 ± 0.16 C	63.9 ± 6.36 BC	10.7 ± 0.55 B	0.66 ± 0.01 A
		AM	44.3 ± 1.20 c	4.33 ± 0.18 A	117.6 ± 23.4 A	12.6 ± 0.65 A	0.62 ±0.01 B
DS	B0	Non-AM	n.d.	2.86 ± 0.08 b	44.3 ± 4.01 ab	5.66 ± 0.53 b	0.52 ± 0.04 b
		AM	46.3 ± 2.33 bc	3.49 ± 0.13 a	16.9 ± 3.42 c	8.98 ± 0.29 a	0.61 ± 0.04 ab
	B25	Non-AM	n.d.	2.72 ± 0.09 b	47.6 ± 10.6 a	5.38 ± 0.41 b	0.58 ± 0.04 ab
		AM	58.7 ± 6.77 ab	3.63 ± 0.22 a	25.5 ± 5.11 c	8.49 ± 0.27 a	0.61 ± 0.03 ab
	B100	Non-AM	n.d.	2.72 ± 0.11 b	30.3 ± 3.96 bc	4.92 ± 0.18 b	0.62 ± 0.04 ab
		AM	64.7 ± 2.90 a	3.71 ± 0.0.9 a	27.2 ± 2.35 c	8.97 ± 1.02 a	0.63 ± 0.02 a

Stomatal conductance (*gs*)

Mycorrhization positively affected stomatal conductance when the plants were well watered despite the different B concentrations. However, the opposite effect occurred under drought stress, where mycorrhization decreased *gs* at B0 and B25. At B100, nonetheless, the differences were not significant (Table 1).

Chlorophyll content and efficiency of photosystem II

Chlorophyll content measured with SPAD did not show differences due to mycorrhization or B levels under well-watered conditions, with the exception of AM plants at B100, that slightly increased chlorophyll levels. In the case of drought stress, mycorrhization increased chlorophyll levels under all B concentrations compared to non-AM plants (Table 1).

The efficiency of photosystem II was not affected by B concentration in non-AM plants, but decreased in AM plants at B100, as compared to non-AM ones. During drought stress, there was no effect of any of the factors on the efficiency of photosystem II (Table 1).

Mineral content of roots and shoots

Boron concentration in roots increased in plants irrigated with B100 in both water treatments and regardless of AM fungal inoculation. However, B25 did not increase root B concentration compared to plants that did not receive B (Figure 1A). In leaves, the same trend was observed under well-watered conditions. However, under water deficit, AM plants slightly increased B levels at B100 as compared to non-AM plants, although both plant groups presented higher concentration when compared with the other B treatments (Figure 1B).

Ca concentration was significantly decreased due to mycorrhization in well-watered plants, only at B25. Under drought, however, this decrease was significant in AM plants at B100 (Figure 1C). In leaves, the same drop in Ca concentration occurred in well-watered AM plants at B0 and B25, but no significant differences were detected at B100 or during drought stress (Figure 1D).

P concentration in roots was increased by AM presence under all B concentration and water regimes (Figure 1E). In leaves, P concentration increased significantly with mycorrhization only under water deficit conditions, being not affected by B levels (Figure 1F). Interestingly, K concentration in roots presented a similar trend than Ca accumulation, and was decreased in AM plants at B25 under well-watered treatment.

K concentration didn't vary under drought stress or in leaves with any of the treatments (Figures G and H). Mg concentration in roots did not show significant differences due to AM inoculation, B concentration or water regime (Figure 2A). However, in leaves it was increased by mycorrhization under all the different conditions (although not significant under well-watered conditions) (Figure 2B).

S concentration presented a similar trend than Mg. In roots, the changes in S concentration were not significant (Figure 2C), but the concentration of this compound in leaves generally increased with mycorrhization, while it was only significant at B0 or B25 during drought stress treatment (Figure 2D).

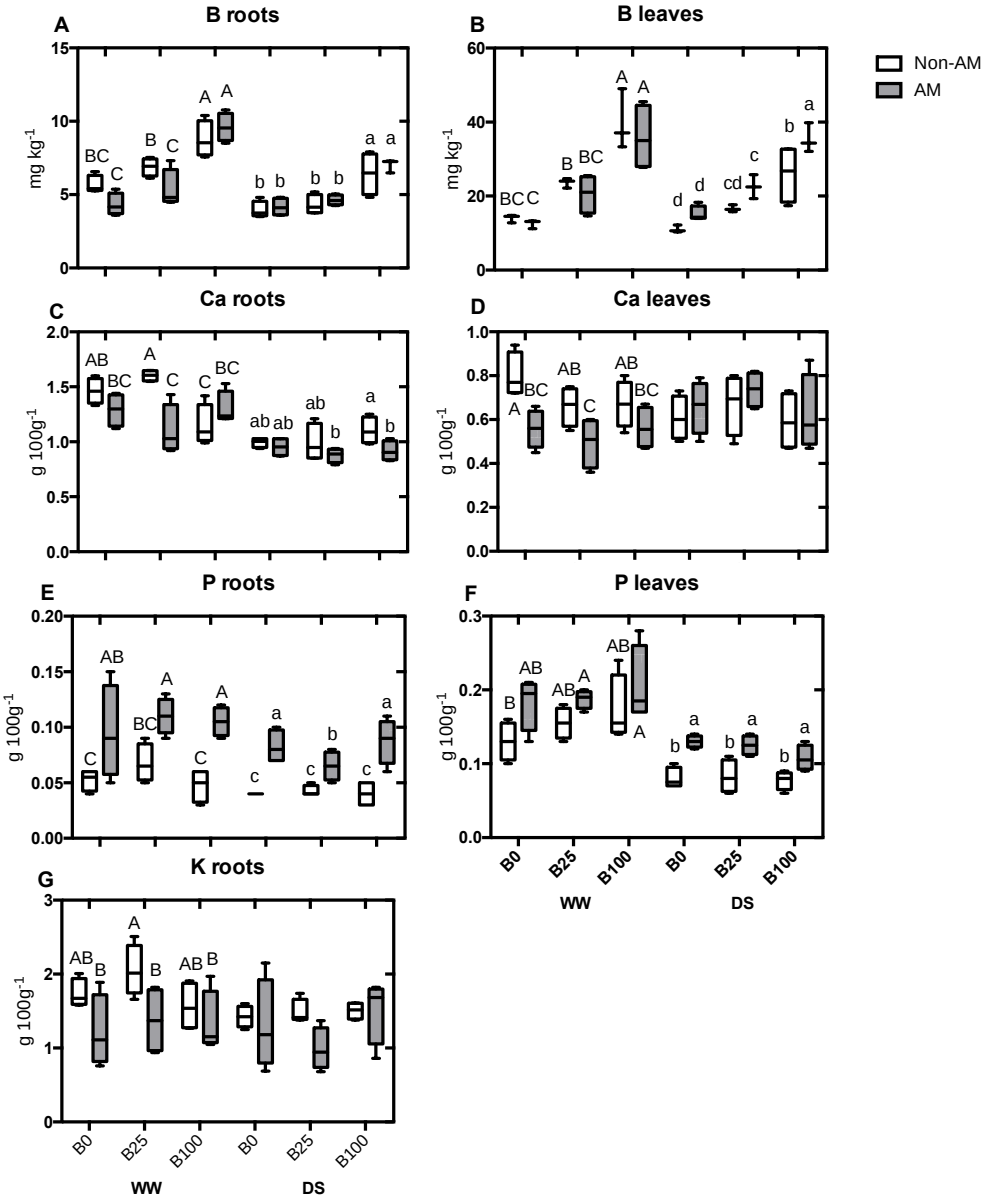

Figure 1. Boxplots representing root and leaf concentrations of B, Ca, P and K. Boxes represent the interquartile range, with the line representing the median, whiskers represent maxima and minima within 1.5 times the interquartile range. Different letters indicate significant differences among treatments (P<0.05) based on Duncan's test.

Cu content in roots was regulated by AM during drought stress, increasing levels of this nutrient to achieve almost concentrations of well-watered plants (Figure 2E). Nonetheless, no changes were observed in leaves of the different treatments (Figure 2F).

Concentration of Fe in roots was generally high, but significantly enhanced in AM roots of droughted plants at B25 and B100 (Figure 2G). Once again, the concentration of this element in leaves was not affected by the different treatments (Figure 2H).

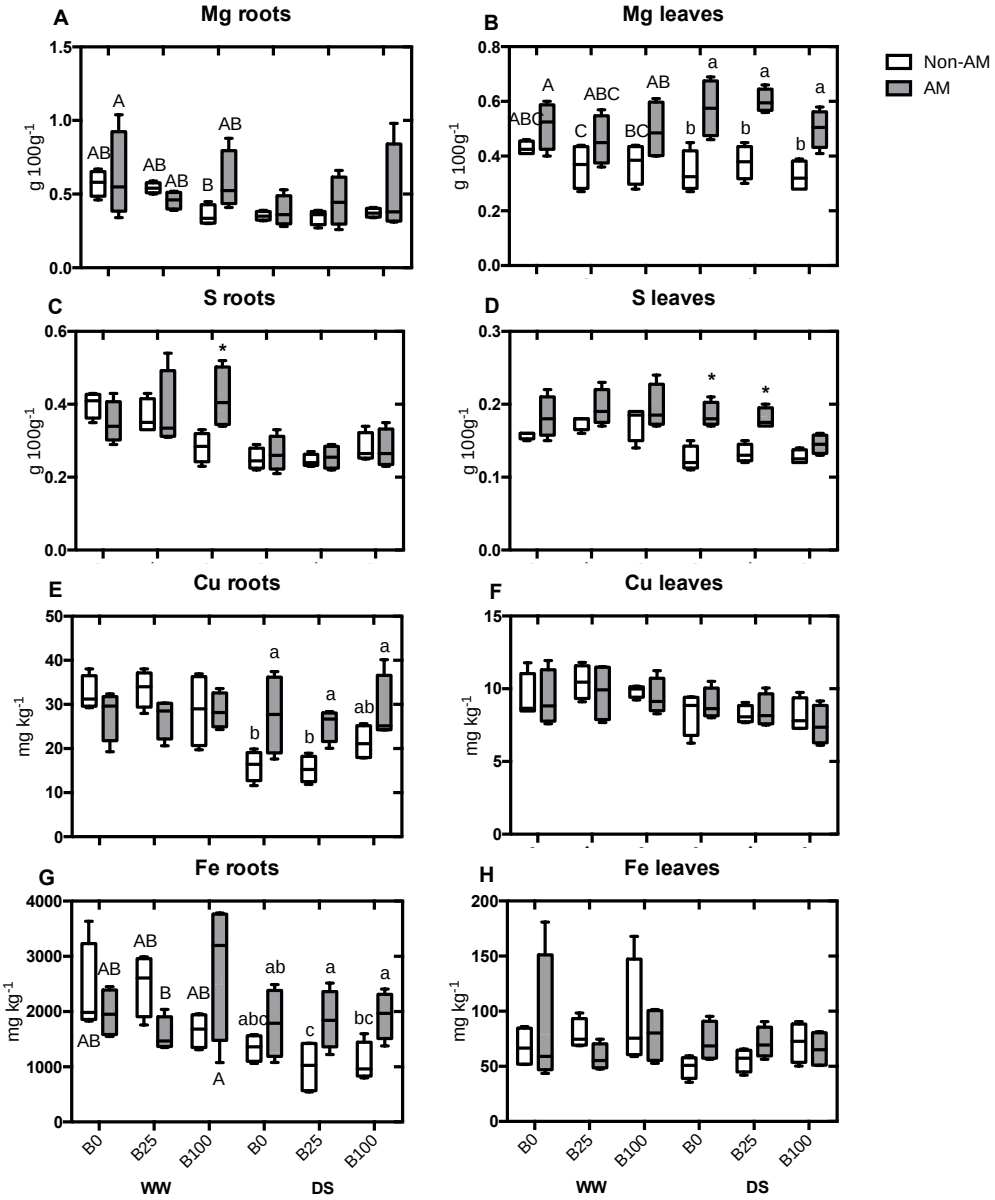

Figure 2. Boxplots representing root and leaf concentrations of Mg, S, Cu and Fe. Boxes represent the interquartile range, with the line representing the median, whiskers represent maxima and minima within 1.5 times the interquartile range. Different letters indicate significant differences among treatments (P<0.05) based on Duncan's test. Asterisks indicate significant differences between non-AM and AM plants within each treatment, according to t-test.

In the case of Mn, root concentration decreased with mycorrhization under well-watered conditions at B0 and B25, not being affected by AM at B100. Under drought stress, a significant decrease of Mn concentration with AM inoculation was only observed at B0 (Figure 3A). In leaves, the opposite effect was observed during drought at B0, with AM increasing the concentration of this nutrient (Figure 3B).

Si concentration in roots was increased by AM at B0 and B25 under well-watered conditions, while at B100, levels of non-AM plants were higher and similar to those of AM-plants. Under water deficit, no differences were observed (Figure 3C), as well as in leaves (Figure 3D).

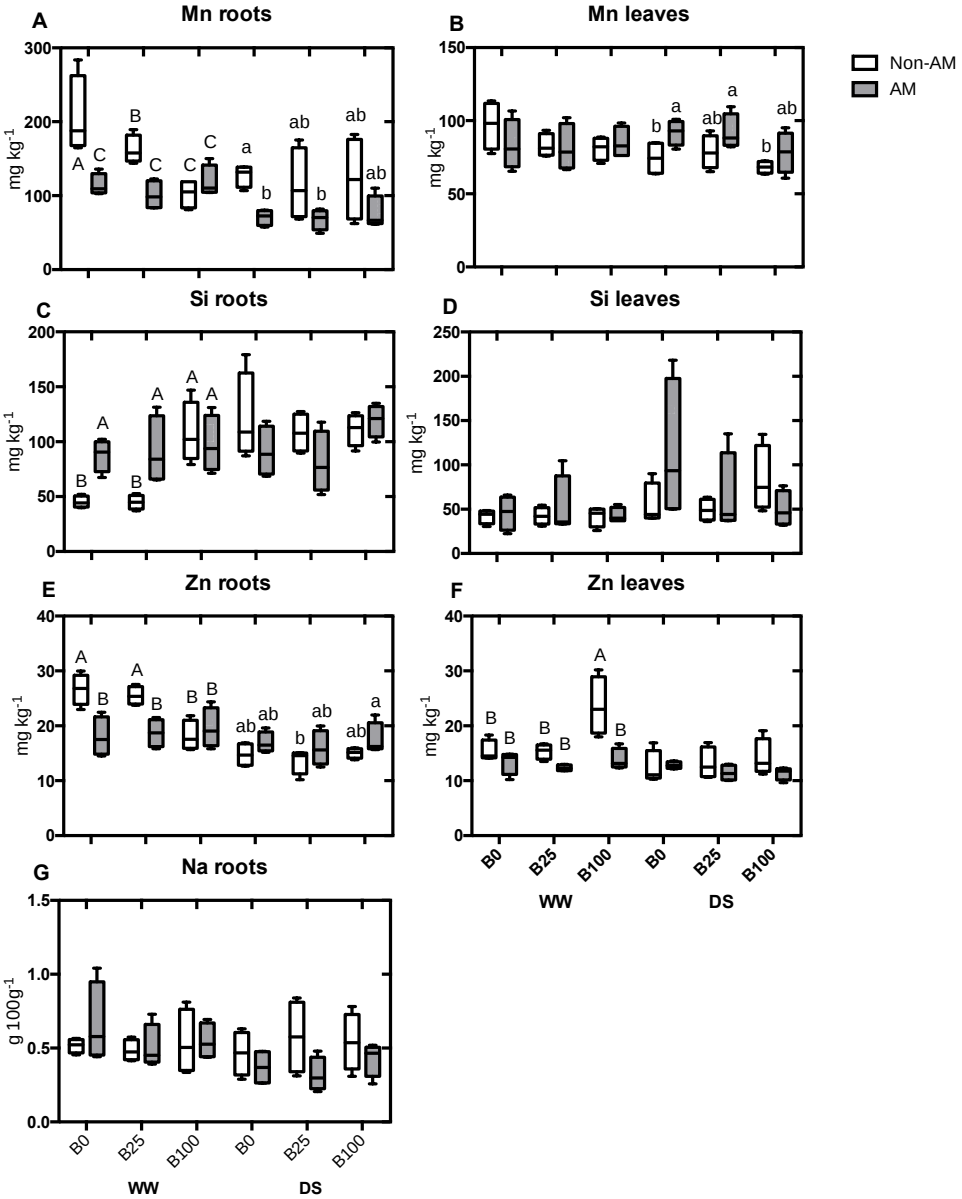

Figure 3. Boxplots representing root and leaf concentrations of Mn, Si, Zn and Na. Boxes represent the interquartile range, with the line representing the median, whiskers represent maxima and minima within 1.5 times the interquartile range. Different letters indicate significant differences among treatments (P<0.05) based on Duncan's test.

Zn content in roots decreased in well-watered AM-plants at B0 and B25. At B100 levels of Zn decreased in both non-AM and AM plants. Under drought, no significant differences were detected (Figure 3E). In leaves Zn concentration increased in non-AM plants at B100 during WW conditions (Figure 3F). Na concentration was not affected either in roots or leaves by any of the applied treatments (Figure 3G and H).

mRNA relative transcript abundance of aquaporins and *RTE* genes

Eight plant aquaporins selected in previous studies due to their AM regulation (Quiroga *et al.* 2017) were analysed. Three additional aquaporins that potentially transport B, *ZmNIP1;1*, *ZmNIP2;2* and *ZmNIP3;1* were also studied. Moreover, three B efflux transporters, *RTE*, *RTE2* and *RTE3* (Chatterjee *et al.*, 2017), were also included in this study. *ZmPIP1;1* and *ZmPIP1;3* mRNA levels were not regulated by B under full irrigation conditions, although AM plants decreased transcript levels compared to non-AM ones at B100. Under water deficit conditions, no different were observed among treatments (Figure 4A, 4B).

In the case of *ZmPIP2;2*, a down-regulation due to mycorrhization was evident at B0 in well-watered plants. On the contrary, an important up-regulation of this gene occurred in non-AM plants at B100, increasing mRNA levels four times compared to B0 plants (Figure 4C). *ZmPIP2;4* transcript levels decreased with mycorrhization under well-watered conditions, although the effect was only significant at B25 and B100. However, this effect was not observed under drought stress (Figure 4D).

No significant changes were observed in *ZmTIP1;1* transcript abundance in well-watered plants with any of the applied treatments. However, during drought stress, mycorrhizal plants up-regulated this gene at B25 compared to non-AM plants and to the other B concentrations (Figure 4E).

ZmTIP2;3 mRNA levels increased at B100 in non-AM plants under well-watered conditions. However, under drought stress conditions no significant differences in gene expression were found (Figure 4F).

Generally, AM plants decreased *ZmTIP4;1* transcript abundance, although the effect was only significant for well-watered plants at B25 or B100. These plants also have higher levels of *ZmTIP4;1* transcripts than plants at B0 (Figure 4G).

In the case of *ZmNIP1;1*, a strong up-regulation occurred in well-watered non-AM plants at B100, but no significant differences were observed under drought stress (Figure 2H). No significant differences in transcript accumulation were observed for *ZmNIP2;1* gene (Figure 4I).

ZmNIP2;2 transcript abundance slightly increased with high B (B100) in both AM and non-AM plants under well-watered conditions. Drought did not affect significantly the expression of this gene (Figure 4J).

Mycorrhization decreased *ZmNIP3;1* expression of well-watered plants at B0 and B100, while any significant effect was observed under drought stress (Figure 4K).

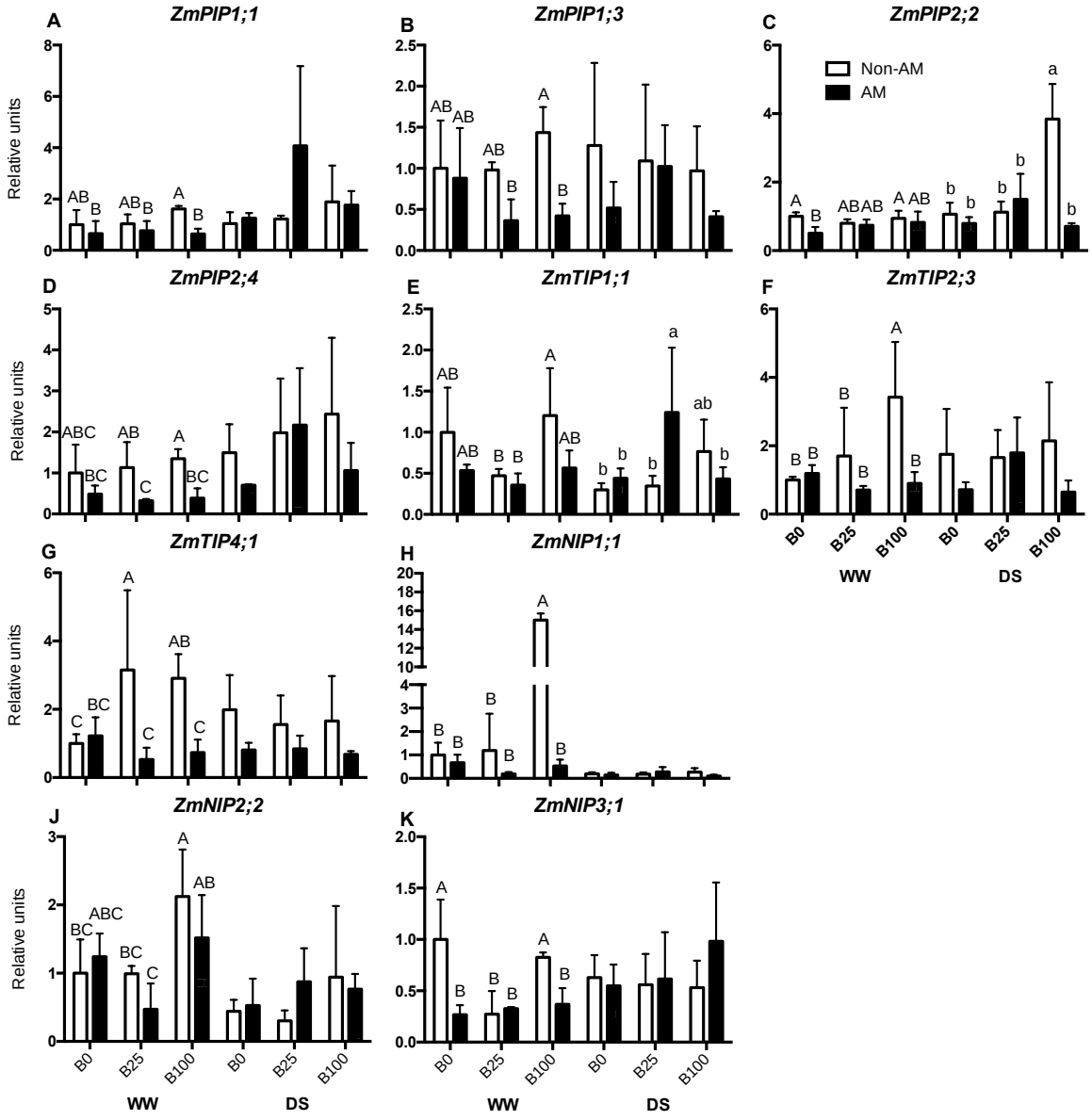

Figure 4. Relative mRNA levels of *ZmPIP1;1*, *ZmPIP1;3*, *ZmPIP2;2*, *ZmPIP2;4*, *ZmTIP1;1*, *ZmTIP2;3*, *ZmTIP4;1*, *ZmNIP1;1*, *ZmNIP2;1*, *ZmNIP2;2* and *ZmNIP3;1,* normalized to *ZmEF1* gene. Plants were inoculated or not with the AM fungus *R. irregularis*, grown under different B concentrations (0, 25 or 100 μM B) and submitted to two water regimes (well-watered [WW] or drought stress [DS]). Data indicate the mean ± SE for three biological replicates. Different letters indicate significant differences between treatments ($P < 0.05$) based on Duncan's test.

Interestingly, *RTE* was generally down-regulated in all treatments compared to non-AM plants at B0, and this result was similar for *RTE2*, although only significant in well-watered AM plants at B25 and B100 (Figure 5A and B). In the case of *RTE3*, however, a significant up-regulation occurred with mycorrhization at B0 under well-watered conditions (Figure 5C).

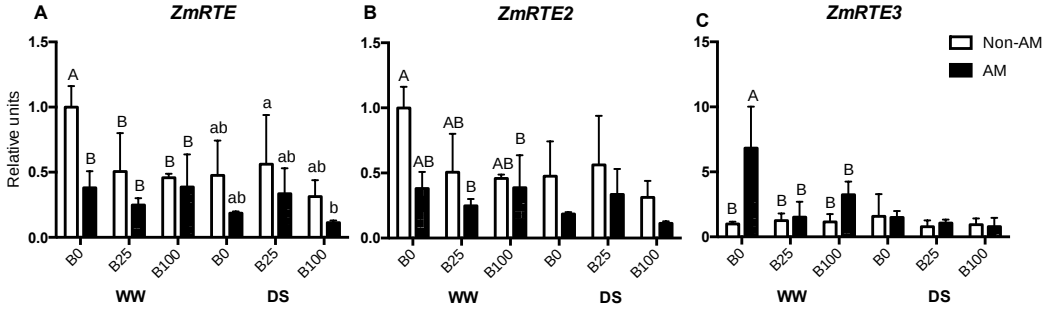

Figure 5. Relative mRNA levels of *ZmRTE, ZmRTE2* and *ZmRTE3* normalized to *ZmEF1* gene. Plants were inoculated or not with the AM fungus *R. irregularis*, grown under different B concentrations (0, 25 or 100 μM B) and submitted to two water regimes (well-watered [WW] or drought stress [DS]). Data indicate the mean ± SE for three biological replicates. Different letters indicate significant differences between treatments ($P < 0.05$) based on Duncan's test.

Aquaporin protein accumulation and PIP2s phosphorylation status

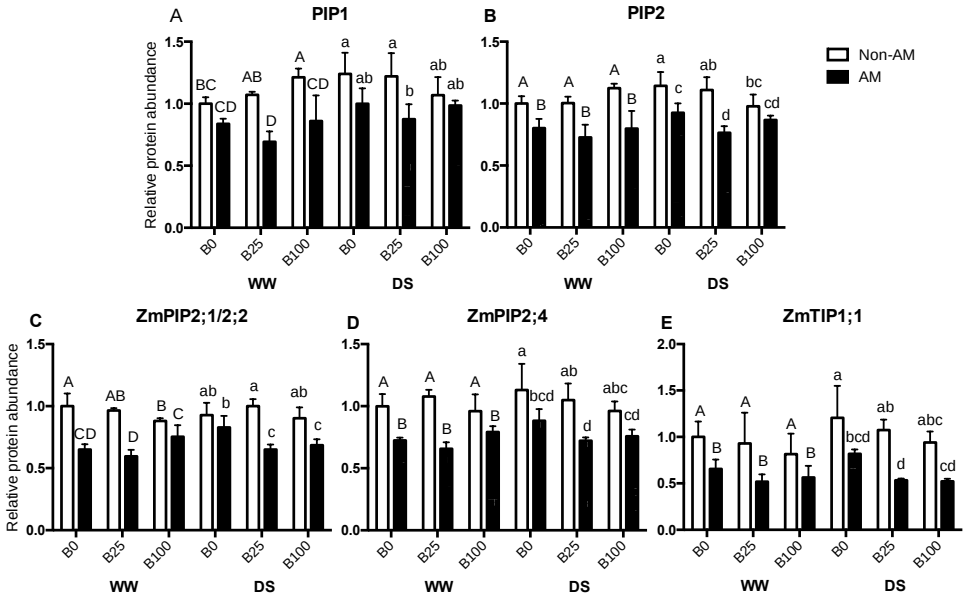

Figure 6. Relative protein abundance in the microsomal fraction of roots from plants inoculated or not with the AM fungus *R. irregularis*, grown under different B concentrations (0, 25 or 100 μM B) and submitted to two water regimes (well-watered [WW] or drought stress [DS]). Data indicate the mean ± SE for three biological replicates. Different letters indicate significant differences between treatments ($P < 0.05$) based on Duncan's test.

A general decrease of PIP accumulation was observed in AM plants with all the analysed antibodies: the general anti-PIP1 and PIP2 and the isoform specific anti-ZmPIP2;1/2;2, antiPIP2;4 and anti-TIP1;1. This effect was significant regardless of the water or the B treatment. The different B concentration did not impact protein accumulation (Figure 6A, B. C. D and E).

The phosphorylation of PIP2 proteins in different serine residues (PIP2A-Ser 280, PIP2B-Ser 283 and PIP2C-Ser 280/283) was also generally decreased by mycorrhization, but not affected by B concentration or drought stress treatment (Figure 7A, B and C).

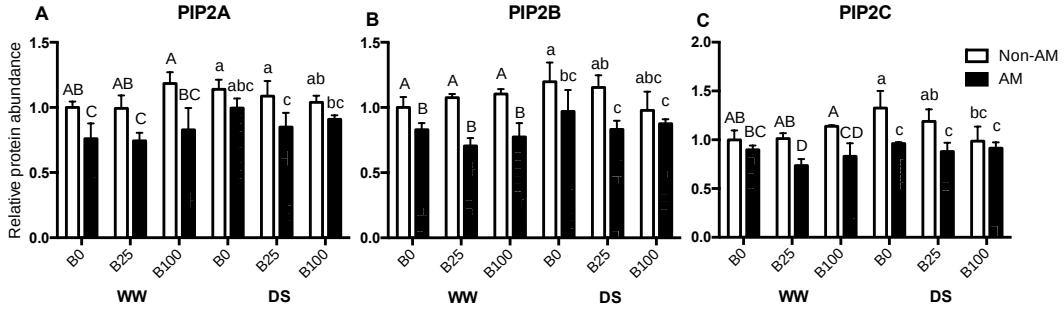

Figure 7. PIP2A (Ph-Ser280), PIP2B (Ph-Ser283) and PIP2C (PhSer280/Ser283) relative protein abundance in the microsomal fraction of roots from plants inoculated or not with the AM fungus *R. irregularis*, grown under different B concentrations (0, 25 or 100 μM B) and submitted to two water regimes (well-watered [WW] or drought stress [DS]). Data indicate the mean ± SE for three biological replicates. Different letters indicate significant differences between treatments ($P < 0.05$) based on Duncan's test.

Discussion

The present study aims to understand whether maize aquaporins regulated by the AM symbiosis are involved in the B transport and homeostasis *in planta* under water deficit conditions. There is not much information available about the role of AM symbiosis in plant B homeostasis, and, as far as we know, this is the first study dealing with this topic in AM maize plants under drought stress.

At physiological level, no detrimental effects of either low or high B concentrations were observed in this experiment, under well-watered or drought stress conditions (Table 1). In fact, the benefits of AM inoculation (especially under drought stress) were similar under the range of B concentrations assayed (as evidenced by plant dry weight or chlorophyll contents). As explained above, the apparent lack of physiological response of the plant may be due to the low B requirement of maize, as a monocot species (Chatterjee *et al.*, 2017). In fact, during vegetative stages, monocots rarely develop deficiency symptoms (Wimmer & Eichert, 2013).

Different B requirements between dicots and monocots may be related to the different composition of their cell walls (Calderan-Rodrigues *et al.*, 2019). In addition, the tolerance of each species to B deficiency or toxicity is highly variety-dependent (Nable *et al.*, 1997; Pallotta *et al.*, 2014; Pommerrenig *et al.*, 2018), and some low-demanding cultivars may increase B use efficiency, which allows them to develop with a limited amount of this nutrient (Pommerrenig *et al.*, 2018). Recently, it has been also suggested that B is neither a beneficial nor an essential element for plant growth. Instead, it was hypothesized to be a toxic element which is maintained in a homeostatic balance within the plant, thanks to the natural selection of constitutive biochemical mechanisms (Lewis, 2019).

When B concentrations are not deficient, it moves in the plant during the active transpiration, accumulating where water is lost through stomata in the leaf (Dannel *et al.*, 2002; Hrmova & Gilliham, 2018). Moreover, even under non-optimal soil B conditions, transpiration stream was found to be a significant source of B for maize plants (Matthes *et al.*, 2018). Therefore, in this study it is not surprising the higher concentration of B observed in leaves (ranging from 20 mg kg^{-1} to 50 mg kg^{-1}) compared to roots (ranging from 4 mg kg^{-1} to 10 mg kg^{-1}) (Figure 1A and B) regardless of the B concentration applied. In fact, even under B toxicity, roots generally do not reflect any visible symptoms, and B concentrations remain relatively low compared to leaves (Nable *et al.*, 1997). In general, mycorrhization did not have an effect on tissue B concentrations, although during drought, B concentrations in leaves slightly increased with mycorrhizal presence, being only significant at B100 (Figure 1B). As explained earlier, the observed effect may be due to the enhancement of water transport in those plants. In relation to this, ectomycorrhizal fungi were found to enhance B uptake in *Betula pendula*, but the effect was mild and dependent on fungal species (Ruuhola & Lehto, 2014).

In contrast, mycorrhization decreased uptake of B and concentrations in wheat plants under both, with and without B supply (Sonmez *et al.*, 2009). In a recent study with citrus rootstocks and *R. irregularis*, the symbiosis decreased the toxicity of high B application, accumulating less B in leaves (Simón-Grao *et al.*, 2019). The disparity of results obtained in different studies suggests that the effect of mycorrhization on plant B homeostasis is very dependent on the plant-fungal combination, as well as, on the specific conditions of the experiment.

Generally, mycorrhization enhanced the uptake of nutrients, especially under water deficit, as revealed by the higher levels in roots and/or leaves of P, Mg, S, Cu, Fe or Mn (Figure 1,2 and 3). This effect is one of the most obvious benefits from AM symbiosis, and it is due to the efficiency of extra-radical mycelial network to penetrate deeper in soils,

extracting water and nutrients even under drought stress. This AM-improvement of nutrient uptake has been extensively reported in numerous plant-fungal combinations (Chen *et al.*, 2017; Essahibi *et al.*, 2017; Liu *et al.*, 2018). Nonetheless, concentrations of those nutrients were not affected in this study by the different B conditions.

High B levels or B toxicity produce changes in plant water balance, probably as a mechanism to prevent the excessive B accumulation. Thus, PIP aquaporins are probably involved in this process, as recently observed in *Arabidopsis* plants (Macho-Rivero *et al.*, 2018). This statement agrees with our results, as *ZmPIP2;2* mRNA levels were upregulated in non-AM plants under high B supply during water stress (Figure 4C), which can be a measure to increase water flow in roots and diminish B excess. In fact, ZmPIP2;2 isoform showed high water transport capacity in *Xenopus laevis* oocytes (Moshelion *et al.*, 2009; Bárzana *et al.*, 2014) and was found to contribute to root cell water permeability changes in maize protoplasts (Quiroga *et al.*, 2019b). However, AM plants under the same conditions decreased *ZmPIP2;2* transcript abundance, which suggest that mycorrhizal plants have other mechanisms for regulation of B excess or that these plants have a different B toxicity threshold. The up-regulation of *ZmTIP2;3* in WW non-AM plants at B100 suggest that this aquaporin may be also involved in B homeostasis under high B concentrations (Figure 4F).

NIP aquaporins were found to be crucial for the uptake and transport of B within roots (Takano *et al.*, 2006). In maize, *ZmNIP3;1* (*TSL1*), an ortholog of *AtNIP5;1*, has been implicated in the transport of boron under B deficient conditions. It was mainly expressed in inflorescences, although it was also found in other plant tissues such as roots (Leonard *et al.*, 2014). In agreement with this, *ZmNIP3;1* mRNA levels were higher at B0 in non-AM plants compared to other treatments under well-watered conditions, although its levels increased again at B100 also in non-AM plants (Figure 4K). However, differential transcriptional regulation of this gene was not observed under drought. In the present study, a strong up-regulation of *ZmNIP1;1* transcript abundance occurred at B100 in non-AM plants under well-watered conditions (Figure 4H). Interestingly, this aquaporin was found to transport B when expressed in yeast (Bárzana *et al.*, 2014). Thus, *ZmNIP1;1* is a good candidate as a B transporter under high B concentrations. NIPs generally present lower transcript levels compared to other aquaporin subfamilies (Chaumont & Tyerman, 2014) and *ZmNIP1;1* levels were very low during drought in this study (Figure 4H). This may be the reason why no differences in mRNA levels were detected under the different B concentrations.

ROTTEN EAR (*RTE*) is a functional homolog of *AtBOR1* and represents the main B efflux transporter in maize, required for vegetative and reproductive development under

B deficiency (Chatterjee *et al.*, 2014). In this study, we analysed other two transporters that also contribute to B transport in different tissues, *RTE2* and *RTE3*. Chatterjee *et al.* (2017) showed that the three genes were expressed in all tissues, but *RTE* and *RTE2* were found in roots with identical expression patterns. Our results are in agreement with this, as *RTE* and *RTE2* showed similar expression patterns in roots, and enhanced levels were found under B0, although only in non-AM plants under WW conditions (Figure 5A and B). Under DS the regulation of the genes was not strong enough to display differences among treatments. Interestingly, *RTE3* was upregulated only in AM plants also at B0 under WW conditions (Figure 5C), suggesting that this gene is differently regulated by the AM symbiosis under B deficiency.

The lack of correlation among B concentrations and B transporters analysed may be due to the existence of additional uncharacterized B transporters in maize, as previously hypothesized in other studies (Matthes *et al.*, 2018). Moreover, AM symbiosis generally down-regulated aquaporins and *RTE* genes under all B concentrations and drought conditions. However, during drought, leaf levels of B increased in AM plants. This could mean than the enhancement of water uptake and transport generally found in mycorrhizal plants (Quiroga *et al.*, 2017; 2019a) leads also to an enhanced passive B transport.

A general drop in AQP protein levels was observed with the presence of the AM fungus (Figure 6). This is in line with previous results under similar conditions (Quiroga *et al.*, 2019b; Ruiz-Lozano *et al.*, 2012). Phosphorylation of specific serine residues in plant aquaporins induces conformational changes that control gating or changes in the subcellular localization (Luu & Maurel, 2013; Santoni, 2017). B concentrations did not affect aquaporin phosphorylation status, and it seems that drought did not influence phosphorylation neither (Figure 7A, B and C). However mycorrhization decreased phosphorylation levels in the three cases, coinciding again with previous results (Quiroga *et al.*, 2019a).

In summary, although a range of B concentrations was applied to AM and non-AM plants during well-watered and water deficit conditions, no apparent physiological effect was found in any of the treatments. This result may be due to the low B requirement of maize, or to tolerance related to the specific cultivar. Some aquaporins (*ZmPIP2;2*, *ZmTIP2;3* and *ZmNIP1;1*) and boron efflux transporters (*RTE*, *RTE2* and *RTE3*) were regulated under low or high B concentration, mainly in non-AM plants. In the case of *RTE* genes, the result confirms their previously proposed role in B transport under deficient conditions. In the case of the stated aquaporins, this is the first report investigating a possible role of AM-regulated plant aquaporins in the *in planta* B transport and

homeostasis. However, the general down-regulation of aquaporins and B transporters in AM plants suggests that, when the mycorrhizal fungus is present, other mechanisms contribute to B homeostasis, probably more related to the enhancement of water transport, which would concomitantly increase the passive transport of this micronutrient.

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CHAPTER IV

Arbuscular mycorrhizal symbiosis and salicylic acid regulate aquaporins and root hydraulic properties in maize plants subjected to drought

Adapted from:

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Abstract

Climate change is leading to the intensification of drought effects worldwide, which considerably reduce crop production. A better understanding of the drought-tolerance mechanisms would lead into a more productive agriculture. The arbuscular mycorrhizal (AM) symbiosis has been shown to improve plant tolerance to drought. Salicylic acid (SA) is a phenolic compound involved in many aspects of plant growth and development. Apart from its role in biotic interactions, it is also involved in the regulation of important plant physiological processes, including plant water relations under stressful conditions. However, despite the importance of SA in plant physiology and in AM colonization, little is known about its effect on regulation of root water transport. Thus, the aim of this work was to study the combined effect of AM symbiosis and SA on root hydraulic properties under drought stress, with special focus on how these factors can alter radial root water transport pathways through aquaporin regulation. Also, the crosstalk between SA and other phytohormones was considered. Results showed that the AM symbiosis modifies root hydraulic responses to drought episodes. Under these conditions, AM plants showed increased Lpr and Lo. Exogenous SA application decreased Lpr and Lo under drought. SA modulation of water conductivity could be due to a fine-regulation of root aquaporins (as *ZmPIP2;4* or *ZmTIP1;1*). Furthermore, SA application differently modulated the percentage of water flowing by the apoplastic pathway, decreasing its contribution to total root water flow in AM plants and increasing it in non-AM plants. An intricate relationship between Lpr, aquaporins and endogenous levels of SA, ABA and jasmonic acid was observed. Future studies should explore more in detail the crosstalk mechanism between these hormones in the regulation of water transport in AM roots, in order to better understand the mechanism through which the AM symbiosis copes with drought stress.

Keywords: aquaporin; arbuscular mycorrhizal symbiosis; drought; root hydraulic conductivity

Introduction

Climate change is leading to the intensification of drought effects and cultivable soils are progressively drying worldwide (Trenberth *et al.*, 2014), with more often drought events that considerably reduce crop production (Lesk *et al.*, 2016). Agricultural drought reduces plant growth and affects essential plant physiological and biochemical processes as stomatal conductance, transpiration, root water uptake, photosynthesis or membrane functions. It also increases the production of reactive oxygen species (ROS), producing oxidative stress that damages cells and even leads to plant death (Hasanuzzaman *et al.*, 2014). Thus, a better understanding of the mechanisms that help plants to improve their water status during water stress would lead into a more productive agriculture.

Phytohormones play essential roles and coordinate different signalling pathways during abiotic stress responses (Wani *et al.*, 2016). Among these, salicylic acid (SA) is a phenolic compound involved in many aspects of growth and plant development as well as in the regulation of the response to different abiotic and biotic stresses (Miura & Tada, 2014; Khan *et al.*, 2015). Salicylic acid has been studied mainly in relation to plant-pathogen interactions since it has the ability to induce systemic acquired resistance to different pathogens in plants (Gunes *et al.*, 2007). Indeed, it coordinates the plant's defence against biotrophic pathogens (Lu, 2009) and Foo *et al.* (2013) suggested that SA might also have a role during arbuscular mycorrhizal (AM) colonization. Previous studies point in this direction, with a short-lived rise in SA levels during the early stages of AM colonization (Blilou *et al.*, 1999). Herrera-Medina *et al.* (2003) showed that the rate of AM colonization was affected by the SA content. They found that transgenic plants with reduced SA levels exhibited a more rapid AM colonization while wild-type plants with constitutive SA biosynthesis retarded AM colonization of roots, although the final level of colonization was unaltered.

Apart from this role in biotic interactions, SA is also involved in the regulation of important plant physiological processes such as nitrogen metabolism, photosynthesis, antioxidant defence system and plant water relations under stress conditions and thereby provides protection in plants against abiotic stresses (Faried *et al.*, 2017; Khan *et al.*, 2015). SA has been found to improve plant tolerance to salt stress (Miura & Tada, 2014; Jini & Joseph, 2017) and to affect plant physiology in maize plants subjected to salinity (Gunes *et al.*, 2007). Indeed, exogenous SA may induce stomatal closure (Miura & Tada, 2014), regulates biosynthesis of osmolytes (Misra & Saxena, 2009; Li *et al.*, 2017) and increases antioxidative defences in stressed tissues (Nazar *et al.*, 2011). However, SA is thought to interact in a complex way with other hormonal compounds such as ethylene

(Gharbi *et al.*, 2016). Thus, its effects on plant physiology can be direct or indirect, through alteration of other plant hormones. Finally, SA influences plant functions in a dose dependent manner, where induced or inhibited plant functions can be possible with low and high SA concentrations, respectively (Khan *et al.*, 2015).

There are increasing evidences of enhanced drought tolerance when exogenous SA is applied (Alam *et al.*, 2013; Miura and Tada, 2014; Li *et al.*, 2016). However, this regulation is orchestrated in a complex cross-talk between different phytohormones (auxins, cytokinins, ABA, gibberellins) under optimal and stressful conditions (Munné-Bosch and Müller, 2013). On the other hand, AM fungi (which establish a mutualistic relationship with most crop plants) have been described to improve water and nutrient uptake, enhancing tolerance to abiotic stresses such as drought (Ruiz-Lozano *et al.*, 2012a) being a possible alternative to the use of inorganic fertilizers (Zopellari *et al.*, 2014). This amelioration is achieved by allowing plants the access to distant water from the soil, and by altering root hydraulic properties (Bárzana *et al.*, 2012).

Water transport in roots, according to the composite model (Steudle & Peterson, 1998) occurs as the sum of three pathways: apoplastic (via the cell wall continuum), symplastic (via plasmodesmata) and transcellular (across the cell membranes). The last two pathways cannot be differentiated empirically, being reduced to the so-called cell-to-cell pathway. Aquaporins play an important regulatory role in this last pathway, and within this protein family, water channel activity is mainly found in the PIP2 subfamily (Maurel *et al.*, 2008). By measuring root hydraulic conductivity (Lpr), root water transport capacity can be estimated, providing information on plant water status and water mobilization capacity of roots.

It is known that under non-stressful conditions the radial water flow is mainly apoplastic, following the hydrostatic gradient created by transpiration. However, when transpiration is restricted (as under drought), water goes mainly by the cell-to-cell pathway following an osmotic gradient between soil solution and xylem sap. Thus, relative contribution of these two pathway to overall water uptake or hydraulic conductivity may change substantially (Martínez-Ballesta *et al.*, 2003; Hachez *et al.*, 2006a; Vadez *et al.*, 2013b) and, under drought conditions, root hydraulics is adjusted by switching between both pathways (Ranathunge *et al.*, 2004). It is expected that aquaporins play a key role in the regulation of water flow in plants under conditions of water limitation, affecting important parameters such as the root hydraulic conductivity (Hachez *et al.*, 2006a; Zarrouk *et al.*, 2016). Moreover, there is growing evidence that the contribution of aquaporin-mediated water transport to root water uptake is much larger than previously thought, even under conditions of high transpiration (Knipfer and Fricke, 2010, 2011).

Previous studies have investigated the effects of the AM symbiosis on water pathways in the roots of host plants, combined with the use of an inhibitor of aquaporins activity (Bárzana *et al.*, 2012). Results showed that roots of AM plants enhanced significantly the water circulating by apoplastic pathway as compared to non-AM plants, both under well-watered and under drought stress conditions. Data also showed that the presence of the AM fungus in the roots of the host plants could modulate the switching between cell-to-cell and apoplastic water transport pathways. This was interpreted as a way to provide higher flexibility in the response of AM plants to water shortage according to the demands from the shoot (Bárzana *et al.*, 2012). Other recent evidences suggest that the modulation of ABA, auxins and/or SA levels in the host plant may contribute to this switching between water pathways mediated by the AM fungus (Calvo-Polanco *et al.*, 2014; Sánchez-Romera *et al.*, 2016). Indeed, ABA was found to increased Lpr at root cortical cell and organ levels in maize, facilitating water uptake under water limiting conditions (Hose *et al.*, 2000) and ABA was identified as a possible aquaporin regulator (Wan *et al.*, 2004; Boursiac *et al.*, 2008). Studies in Arabidopsis indicated that indole acetic acid (IAA) acts through an Auxin Response Factor 7 (ARF7)-dependent path to inhibit the expression of most PIPs at both transcriptional and translational levels (Péret *et al.*, 2012). Similarly, SA down regulates PIP aquaporins and root hydraulic conductivity by a ROS-mediated mechanism which provoked membrane internalization of PIP aquaporins (Boursiac *et al.*, 2008).

Despite the importance of SA in plant physiology and in AM colonization, as well as its putative role under drought conditions, little is known about its effect on root hydraulic conductivity and regulation of water transport in roots, and to the best of our knowledge, studies about the combined effect of exogenous SA application and AM symbiosis are lacking. Thus, the aim of this research was to study the combined effect of AM symbiosis and SA on root hydraulic properties under drought stress, being specially focused on how these factors can alter radial root water transport pathways through aquaporin regulation. For that, we applied exogenous SA or an inhibitor of its biosynthesis (2-aminoindan-2-phosphonic acid, AIP; Pan *et al.*, 2006). Also, the crosstalk between SA and other plant hormones under the former conditions will be discussed. The results of this study could lead to a better understanding of water uptake mechanisms and plant tolerance to drought when the AM fungus is present, increasing our knowledge of its effect on plant water balance.

Material and methods

Experimental design

The experiment consisted of a factorial design with three factors: (1) inoculation treatment, with non-inoculated control plants (C) and plants inoculated with the AM fungus *Rhizophagus irregularis*, strain EEZ 58 (Ri); (2) chemical treatment, so that one group of each inoculation treatment was maintained without hormone (untreated), another group of plants was treated with salicylic acid (SA), and the last group was treated with 2-aminoindan-2-phosphonic acid (AIP), as inhibitor of SA biosynthesis; (3) 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 for 15 days before harvest (DS). The different combination of these factors gave a total of 12 treatments. Each treatment had 10 replicates, giving a total of 120 plants.

Biological material and growth conditions

A loamy soil was collected at the grounds of IFAPA (Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm) (1:9, soil:sand, v/v) and sterilized by steaming (100°C for 1 h on 3 consecutive days). The soil had a pH of 8.1 (water); 0.85% organic matter, nutrient concentrations (mg kg^{-1}): N, 1; P, 10 ($NaHCO_3$-extractable P); K, 110. The soil texture comprised 38.3% sand, 47.1% silt and 14.6% clay.

Seeds *of Zea mays* L. cultivar PR34B39 were provided by Pioneer Hi-Bred, Spain (DuPont Pioneer Corporation). Maize plants were grown in 1L pots filled with 1250 g of a mixture of soil/sand (1:9) for 8 weeks. At the time of planting, half of the plants were inoculated with ten grams of mycorrhizal inoculum from *Rhizophagus irregularis* (Schenck and Smith), strain EEZ 58. Mycorrhizal inoculum consisted of soil, spores, mycelia and infected root fragments. Non-inoculated plants received a 5 mL aliquot of a filtrate (<20 μm) of the AM inoculum in order to provide the natural microbial population free of AM propagules.

Plants were grown for 8 weeks in a greenhouse at 19/25ºC, 16/8 light/dark period, 50-60% relative humidity and an average photosynthetic photon flux density of 800 μmol m^{-2} s^{-1}, as measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Plants were irrigated three times per week with 50 mL of Hoagland nutrient solution (Hoagland & 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). This water stress was similar as in a previous work

with similar experimental design (Quiroga *et al.*, 2017). It could be considered as a severe stress as evidenced by a drop of stomatal conductance by around 75% (Table 1).

Salicylic acid 20 µM and AIP 75 µM were applied with the nutrient solution 6 hours before harvesting. Dose of the phytohormone and its inhibitor, as well as, the exposure time needed to affect root hydraulic conductivity were established in previous experiments ranging from 20 to 150 µM SA, 25 to 100 µM AIP, and exposure times of 1h, 6h, 12h and 24h.

Measurements

- Biomass production and symbiotic development

At harvest the shoot and root system of ten replicates per treatment were collected and used for fresh weight recording. Then, 5 replicates per treatment were dried in a hot-air oven at 70ºC for 2 days and dry weights were recorded. The other 5 replicates were immersed in liquid nitrogen and stored at -80ºC until they were used.

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.

- Stomatal conductance

Stomatal conductance (*gs*) was measured two hours after the onset of photoperiod in the second youngest leaf from 10 plants per treatment with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the manufacter's instructions. Measurements were taken one day before harvest.

- Photosynthetic efficiency

The efficiency of photosystem II was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll a fluorescence. FluorPen quantifies the quantum yield of photosystem II as the ratio between the actual fluorescence yield in the light-adapted state (FV') and the maximum fluorescence yield in the light-adapted state (FM'), according to Oxborough and Baker, 1997. Measurements were taken in the second youngest leaf of 10 different plants of each treatment one day before harvest.

- Membrane electrolyte leakage

Leaf electrolyte leakage was determined in 10 plants per treatment. Leaf samples were washed with deionized water to remove surface-adhered electrolytes. The samples were placed in 15 mL falcon tubes containing 10 mL of deionized water and incubated at 25 $_{o}$C on a rotary shaker (at 100 rpm) during 3 hours, and the electrical conductivity of the solution (E_0) was determined using a conductivity meter (Mettler Toledo AG 8603, Switzerland). Samples were then placed at -80ºC for 2 hours. Subsequently, tubes were incubated again at room temperature under smoothly agitation and the final electrical conductivity (E_f) was obtained after 3 hours under these conditions. The electrolyte leakage was defined as follows: $[(E_0 - E_{water})/(E_f - E_{water})] \times 100$, where E_{water} is the electrical conductivity of the deionized water used to incubate the samples.

- Osmotic root hydraulic conductivity (Lo)

Lo was measured at noon on detached roots exuding under atmospheric pressure by the free exudation method (Benabdellah *et al.*, 2009). Under these conditions, water is only moving following an osmotic gradient. Therefore, the water would be moving through the cell-to-cell path (Steudle & 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 and used for Lo calculation, according to Aroca *et al.* (2007). Lo was calculated as $Lo = Jv/\Delta\Psi$, where *Jv* 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 6 hours after starting the chemical treatment.

- Hydrostatic root hydraulic conductivity (Lpr)

Lpr was determined at noon in five plants (n=5) per treatment with a Scholander pressure chamber, 6 hours after starting the chemical treatment 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 after 2 minutes at the three pressure points. Sap flow was plotted against pressure, with the slope being the root hydraulic conductance (*L*) value. Lpr was determined by dividing *L* by root dry weight (RDW) and expressed as mg H_2O g RDW^{-1} MPa^{-1} h^{-1}. Aliquots of the collected sap were used for subsequent hormonal determination.

- Relative apoplastic water flow

Relative changes in apoplastic water flux were estimated using light green dye (light green SF yellowish; Sigma-Aldrich Chemical, Gillingham, Dorset; colour index

42095, molecular weight 792.85 g mol^{-1}), which has the ability to move apoplastically but not symplastically (López-Pérez *et al.*, 2007). Detopped root systems were immersed in 250 μmol L^{-1} light green solution inside the pressure chamber 5 min before pressure application 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 concentration of the dye in the whole collected sap was determined immediately at 630 nm (Bárzana *et al.*, 2012). The average baseline fluorescence 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 dye concentration 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%.

- Sap and root hormonal content

In sap, IAA, ABA, SA and JA were analysed according to Albacete *et al.* (2008) with some modifications. Briefly, xylem sap samples were filtered through 13 mm diameter Millex filters with 0.22 μm pore size nylon membrane (Millipore, Bedford, MA, USA). Ten μl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 μg L^{-1}).

In plant roots, IAA, ABA, SA, JA and JA-Ile were analysed using high-performance liquid chromatography-electrospray ionization-high-resolution accurate mass spectrometry (HPLC-ESI_HRMS) as described in Ibort *et al.* (2017).

- PIP aquaporins abundance and phosphorylation status

Microsomal fraction isolation and ELISA were performed as described previously by Calvo-Polanco *et al.* (2014). We used five different primary antibodies (at a dilution of 1:1000), two antibodies that recognize several PIP1s and PIP2s, and 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).

- Gene expression analysis through RT-qPCR

Total RNA was extracted from five biological replicates of maize roots harvested 8 weeks after sowing and conserved at -80ºC prior to use. Isolation was carried out by a

phenol/chloroform extraction method followed by precipitation with LiCl (Kay *et al.*, 1987). The integrity of RNA was checked electrophoretically and quality assessment of total RNA was measured with NanoDrop (Thermo Scientific™; NanoDrop 1000). First-strand cDNA was synthesized using 1 μg of purified RNA with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific™), according to the manufacter's protocol. To rule out the possibility of a genomic DNA contamination, all the cDNA sets were checked by running control PCR reactions with aliquots of the same RNA that have been subjected to the DNase treatment but not to the reverse transcription step.

The expression of a group of maize aquaporins previously selected as regulated by the AM symbiosis (Bárzana *et al.*, 2014; Quiroga *et al.* 2017) was studied by RT-qPCR by using iCycler system (Bio-Rad, Hercules, CA, USA) adjusting protocols to optimize the PCR reaction to each gene. The primer sets used to amplify each aquaporin gene were designed in the 3' and 5' untranslated regions of each gene in order to avoid unspecific amplification of the different aquaporin genes (Hachez *et al.*, 2006a; Bárzana *et al.*, 2014). Polymerase chain reactions were performed in a 96-well plate with an iCycler 5 system (Bio-Rad, Hercules, California, USA), using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KAPABIOSYSTEMS, Boston, Massachusetts, United States). The following standard thermal profile was used for all PCR reactions: Enzyme activation (95 ºC for 3 min), denaturation, annealing and extension cycles repeated 40 times (95 ºC for 25 seconds, 60 ºC for 25 seconds, 72 ºC for 30 seconds) and dissociation curve (70 ºC for 2 min, 55 º C for 10 seconds).

The Elongation Factor 1 (*gi:2282583*) was used as reference gene for normalization, as it was the best-performing reference gene under the specific growing conditions. The relative abundance of transcripts was calculated from three biological and two technical replicates by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001a). Negative controls without cDNA were used in all PCR reactions.

- Statistical analysis

Within each watering regime, data were analysed using SPSSStatistics (version 23, IBM Analytics) and subjected to analysis of variance (ANOVA) with inoculation treatment and chemical treatment as sources of variation. Post-hoc comparisons with Duncan test were used to find out differences between means at α=0.05. Correlations between the different parameters were performed by calculating the Pearson correlation coefficients.

Results

Root mycorrhization, plant growth and ecophysiological parameters

Plants inoculated with *Rhizophagus irregularis* (AM) presented around 65% of mycorrhizal root length, showing no significant differences between water treatments, whereas non-inoculated plants did not show AM colonization (Table 1).

Shoot and root dry weight decreased significantly by 40% in average due to drought stress treatment, but AM plants maintained higher plant dry weight than non-AM ones, regardless of water regime (Table 1). Thus, under well-watered conditions the shoot dry weight was 13% higher in AM plants and under drought stress conditions the increase was by 11%. Membrane electrolyte leakage (EL) was significantly enhanced by 79% in non-AM plants after drought stress. In contrast, AM plants maintained steady state levels as compared to well-watered treatments (Table 1). Stomatal conductance (*gs*) was significantly reduced after two weeks of water limited conditions both in AM and in non-AM plants (Table 1). No differences were found in the efficiency of photosystem II due to water availability or AM fungal inoculation (Table 1).

Table 1. Percentage of mycorrhizal root length, shoot dry weight (SDW), root dry weight (RDW), electrolyte leakage (EL), stomatal conductance (*gs*) and photosystem II efficiency in the light-adapted state (ΔFv/Fm') in maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered-WW- or drought stress DS). Data represents the means of six values ± SE for mycorrhization, twelve values ± SE for *gs*, ΔFv/Fm' and EL; and thirty values ± SE for SDW and RDW. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test.

	Mycorrhization (%)	SDW (g plant^{-1})	RDW (g plant^{-1})	EL (%)	*gs* (mmol H_2O m^{-2} s^{-1})	ΔFv/Fm'
WW non-AM	n.d.	6.85 ± 0.14 b	7.42 ± 0.46 b	10.07 ± 1.07 b	88.90 ± 15.77 a	0.700 ± 0.004 a
WW AM	64.8 ± 3.2 a	7.77 ± 0.20 a	10.78 ± 0.85 a	8.24 ± 0.87 b	88.88 ± 7.35 a	0.683 ± 0.011 a
DS non-AM	n.d.	4.23 ± 0.09 d	4.91 ± 0.16 c	18.06 ± 3.04 a	27. 09 ± 2.42 b	0.702 ± 0.005 a
DS AM	65.9 ± 5.7 a	4.71 ± 0.08 c	5.99 ± 0.26 c	8.09 ± 1.51 b	31.31 ± 3.47 b	0.704 ± 0.003 a

Hydrostatic and osmotic root hydraulic conductivities and percentage of apoplastic water flow

Under well-watered conditions Lpr was enhanced significantly by SA application in non-AM plants (Figure 1A). AM inoculation also enhanced Lpr as compared to non-AM plants, but no further enhancement was observed in these plants due to SA application. The application of AIP in non-AM plants maintained Lpr values similar to control untreated plants.

Under drought stress conditions Lpr values were also higher in AM plants than in non-AM plants (Figure 1A). The application of SA inhibited Lpr by 47% in AM plants, while the application of AIP maintained steady-state Lpr values. In non-AM plants Lpr exhibited the lowest values and no effects of either SA or AIP were observed.

Under well-watered conditions Lo resulted unaffected by AM inoculation, SA or AIP application (Figure 1B). Under drought stress conditions Lo values where always considerably and consistently higher in AM plants than in non-AM plants. The application of SA inhibited Lo both in AM plants (by 23%) and in non-AM plants (by 51%). The application of AIP maintained steady-state Lo values in both kinds of plants.

The percentage of apoplastic water flow under well-watered conditions was similar in AM and non-AM plants (Figure 1C). The SA application reduced significantly this value only in AM plants, while the application of AIP reduced this value both in AM and non-AM plants. Under drought stress conditions the application of SA had contrasting effects in AM and non-AM plants. Thus, SA enhanced by 71% the percentage of apoplastic water flow in non-AM plants, but decreased it by 50% in AM plants. Again, the application of AIP did not affect the apoplastic water flow as compared to untreated plants.

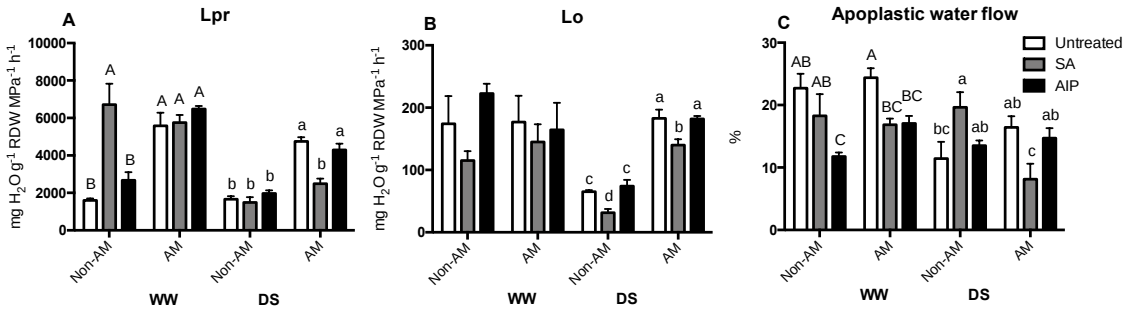

Figure 1. Hydrostatic root hydraulic conductivity (Lpr) (A), osmotic root hydraulic conductivity (Lo) (B) and relative apoplastic water flow (C) in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered or drought stress). Plants remained untreated or received exogenous salicylic acid (SA) or an inhibitor of SA biosynthesis (AIP). Data represents the means of five values ± SE. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Expression of maize aquaporin genes

In this study we analysed the expression of 8 maize aquaporin genes shown in a previous report to be consistently regulated by the AM symbiosis under drought stress (Quiroga *et al.*, 2017). In well-watered non-AM plants *ZmPIP1;1* aquaporin relative expression level was unaltered by SA application but it showed a significant increase due to AIP treatment (Figure 2A). In such conditions, AM inoculation resulted in *ZmPIP1;1*

expression drop in the case of untreated plants and plants treated with AIP. On the other hand, droughted non-AM plants featured a significant decrease in *ZmPIP1;1* expression in presence of external SA but no effect of AM inoculation was detected.

Similar results were obtained for *ZmPIP2;2* relative expression of well-watered plants (Figure 2B), no effect after SA application but significant decreases due to AM inoculation in untreated plants and in plants treated with AIP. Under drought conditions SA and AIP led to a significant decline in *ZmPIP2;2* relative expression in non-AM plants, whereas no effect of AM inoculation was featured.

Under well-watered conditions *ZmPIP1;3* relative expression was unaltered, but when plants grew under water limited conditions, its expression was reduced in non-AM plants after AIP application (Figure 2C). AM inoculation also significantly decreased its expression but only in untreated plants (Figure 2C).

When analysing the *ZmPIP2;4* aquaporin mRNA it was highlighted that when non-AM plants grew well irrigated SA application decreased its expression, but AIP maintained steady-state expression level as comparted to control untreated plants (Figure 2D). In such conditions, AM reduced *ZmPIP2;4* relative expression in the case of untreated well-watered plants or after AIP treatment. Under drought stress conditions no chemical effect was shown and AM inoculation only provoked *ZmPIP2;4* expression to drop in the case of SA treated plants. Interestingly, a similar pattern was found when comparing *ZmTIP1;1* relative expression (Figure 2E). Fully-watered non-AM plants also showed a significant decrease in gene expression after SA application and a significant increase when treated with AIP. AM symbiosis contributed to *ZmTIP1;1* relative expression drop in untreated or AIP-treated plants. Contrariwise, none of the studied factors altered *ZmTIP1;1* expression under drought stress conditions.

Well-watered plants did not show significant differences regarding *ZmTIP2;3* relative gene expression, but under water limited conditions a significant inhibition was observed due to AM symbiosis in untreated plants (Figure 2F).

Well-watered plants did not feature changes regarding *ZmTIP4;1* relative gene expression (Figure 2G). However, under drought stress, SA application led to gene expression enhancement in non-AM plants, which were maintained under steady-state levels after AIP treatment. This behaviour was not observed when plants were inoculated with *R. irregularis,* since no changes were detected in *ZmTIP4;1* relative gene expression after chemical treatment (Figure 2G).

Concerning the relative expression of *ZmNIP2;1* aquaporin no significant alterations were featured due to the studies treatments, regardless of the water regime (Figure 2H).

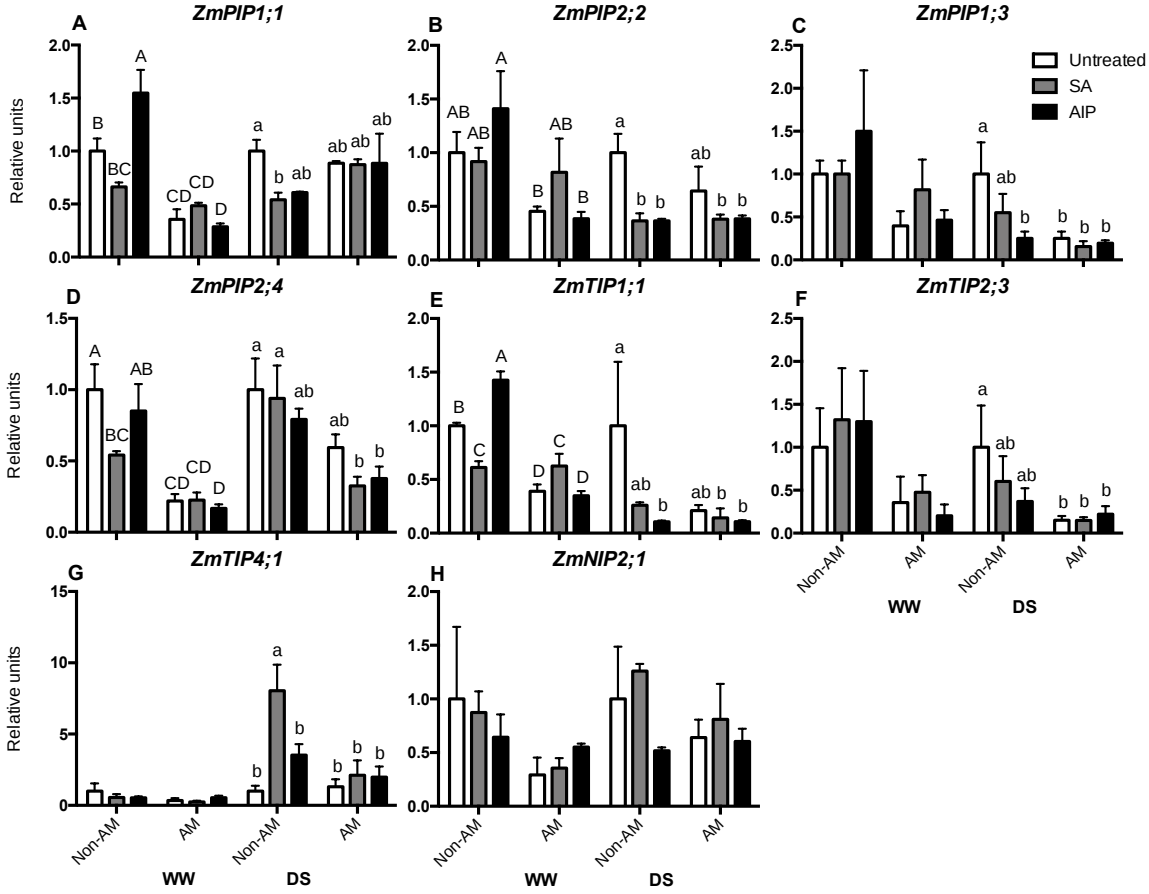

Figure 2. Relative expression of *ZmPIP1;1* (A), *ZmPIP2;2* (B), *ZmPIP1;3* (C), *ZmPIP2;4* (D), *ZmTIP1;1* (E), *ZmTIP2;3* (F), *ZmTIP4;1* (G), and *ZmNIP2;1* (H) genes in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered or drought stress). Plants remained untreated or received exogenous salicylic acid (SA) or an inhibitor of SA biosynthesis (AIP). Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Aquaporin protein abundance

PIP1 and PIP2 aquaporin proteins abundance were measured. Besides, it was quantified the PIP2 phosphorylation state in roots as aquaporin water channel activity depends on this post-transcriptional modification. In this line, the content of PIP2 protein phosphorylated at Ser-280 (PIP2A), at Ser-283 (PIP2B) and double phosphorylated at Ser-280 and Ser-283 (PIP2C) was also quantified. In addition, specific antibodies were used to quantify the abundance of ZmTIP1;1 and ZmPIP2;4 proteins.

Under well-watered conditions PIP1 protein abundance was unaffected by

chemical treatment but significantly decreased due to AM fungus inoculation (Figure 3A). However, when plants grew under drought stress conditions no significant alteration of PIP1 proteins in roots was registered as result of the studied factors alone or in combination.

Regarding the PIP2 proteins abundance, it is remarkable that fully-irrigated plants showed no differences due to chemical treatment or fungal inoculation (Figure 3B). Nevertheless, non-AM droughted plants increased PIP2 proteins content after SA application.

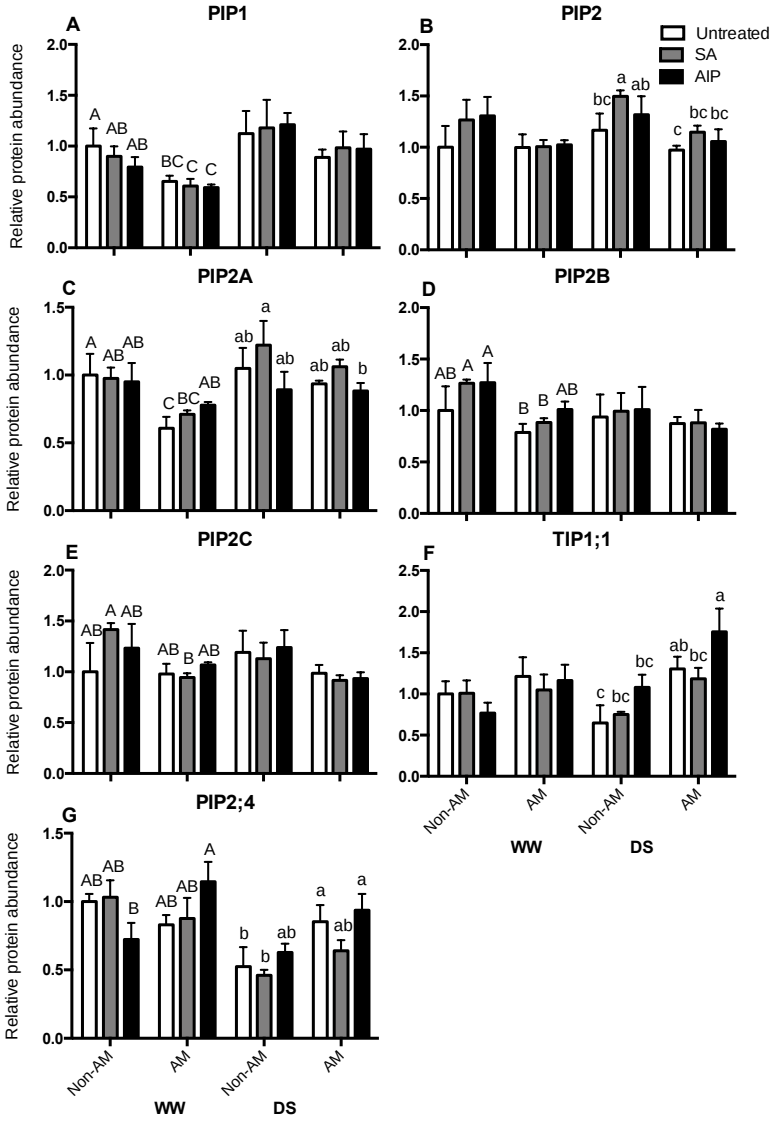

Figure 3. PIP1 (A), PIP2 (B), PIP2A (C), PIP2B (D), PIP2C (E), ZmTIP1;1 (F) and ZmPIP2;4 (G) relative protein abundance in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered or drought stress). Plants remained untreated or received exogenous salicylic acid (SA) or an inhibitor of SA biosynthesis (AIP). Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Phosphorylated PIP2 proteins at Ser-280 (PIP2A) featured no relevant changes due to chemical treatment under well-watered conditions (Figure 3C), but a significant decrease after AM inoculation in untreated plants was observed. In the case of drought stressed treatments, no changes were observed as result of chemical treatment or AM symbiosis. PIP2 proteins phosphorylated at Ser-283 (PIP2B) of fully-watered plants showed no significant differences because of the chemical treatment (Figure 3D), but a significant drop due to AM inoculation in the case of SA-treated plants was observed. PIP2B protein of plants subjected to drought stress did not feature any significant change related to chemical treatments or AM symbiosis. Interestingly, PIP2C (PIP2 proteins phosphorylated at Ser-280 and Ser-283) showed a similar pattern than PIP2B (Figure 3E). In both cases, when plants grew fully-watered no alteration was due to the chemical treatment but they showed a significant decrease in their protein abundance because of the AM inoculation in plants submitted to SA application.

The relative abundance of ZmTIP1;1 aquaporin was unaltered by the studied parameters under well-watered conditions (Figure 3F). Nevertheless, under water limitation, AM inoculation led to a significant increase in ZmTIP1;1 abundance when plants were either untreated or treated with AIP, and this effect was not found in SA-treated plants (Figure 3F).

Fully watered plants did not show significant changes in ZmPIP2;4 relative abundance due to SA or AIP application and AM inoculation. These plants only featured enhanced aquaporin content when plants were AM inoculated and treated with AIP (Figure 3G). Under drought conditions ZmPIP2;4 abundance was not significantly altered by chemical application, SA or AIP. In such circumstances AM inoculation led to significant increase of ZmPIP2;4 protein abundance in untreated plants.

Sap and root phytohormone contents

Sap IAA content under well-watered conditions was unaffected by any the studied chemical treatment or even AM inoculation (Figure 4A). However, under drought conditions a greater IAA content in non-AM plants was shown after SA application. In contrast, this chemical treatment showed a significant drop in sap IAA when plants were AM-inoculated. Sap ABA concentration featured no differences due to chemical treatment or fungal inoculation regardless of the water regime (Figure 4B). Similar trend was registered for sap SA content in well-watered plants which were unaffected by chemical treatment (Figure 4C).

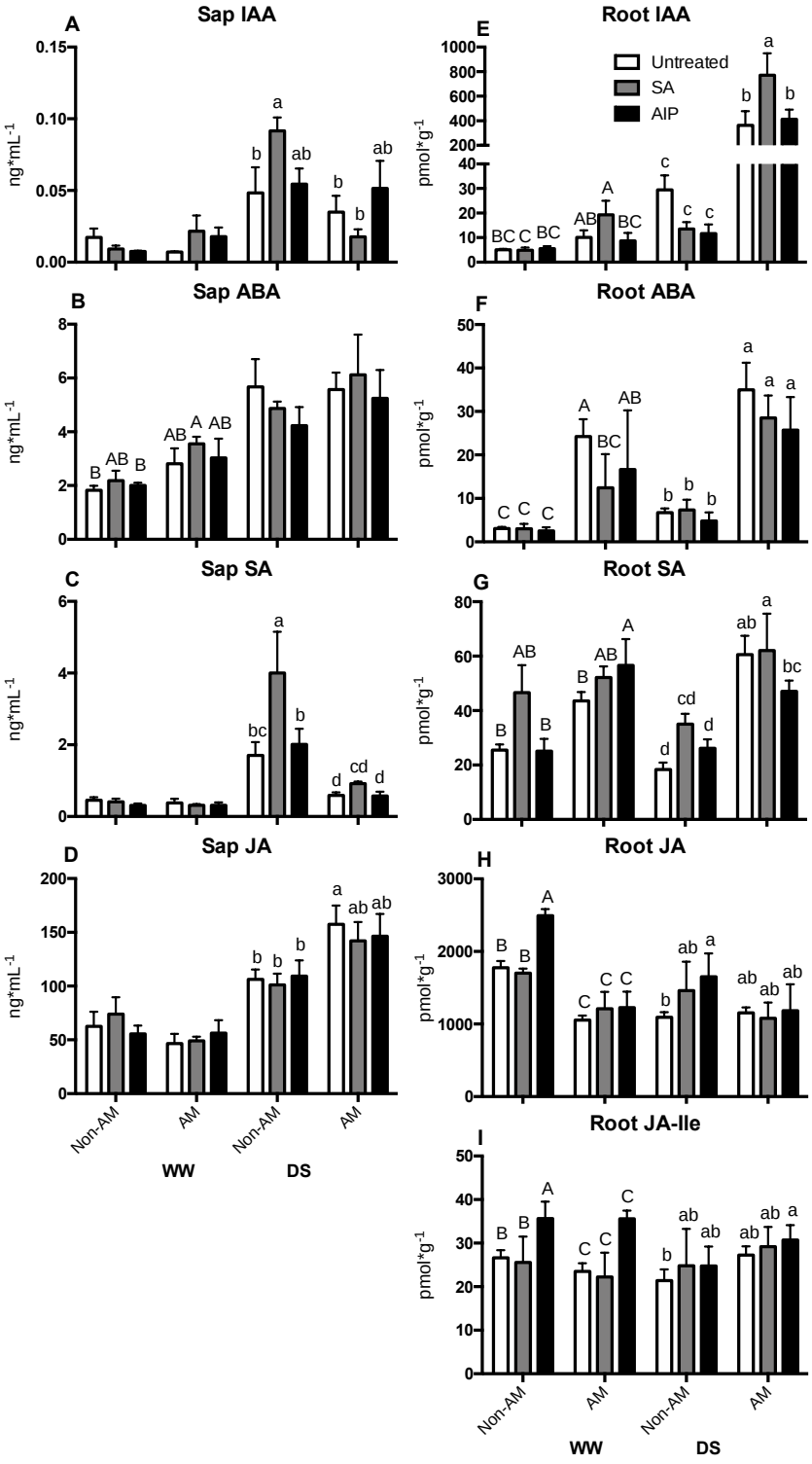

Figure 4. IAA, ABA, SA and JA concentration in sap (A to D), and IAA, ABA, SA, JA and JA-Ile concentration in roots (E to I) in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered or drought stress). Plants remained untreated or received exogenous salicylic acid (SA) or an inhibitor of SA biosynthesis (AIP). Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Nevertheless, under drought stress conditions, SA application provoked the sap SA content to increase in non-AM plants. Remarkably, in such stressed plants a significant decrease in sap SA content was found due to AM inoculation.

Sap JA concentration was unaffected by the studied hormone treatment when plants grew under full water regime (Figure 4D) but under drought stress conditions, AM inoculation significantly increased JA levels of untreated plant.

Root IAA content was unaffected by chemical treatment or AM inoculation under well-watered conditions (Figure 4E). Only in SA-treated plants AM inoculation significantly increased IAA concentration. However, when plants were submitted to water deficit, AM inoculation led to a significant root IAA increase in all chemical treatments, particularly after SA application.

Under well-watered conditions, plants featured no changes in root ABA concentration due to chemical application but a significant increase because of AM inoculation was observed in the case of untreated plants or plants treated with AIP (Figure 4F). In droughted plants root ABA was not susceptible to change after chemical treatment but in all cases AM inoculation led to significant increases in ABA content.

Regarding the root SA concentration, plants were not significantly altered by chemical treatment regardless of the water regime (Figure 4G), but AM-inoculated plants significantly increased their contents, except in the case of well-watered plants treated with SA. It was also highlighted that under well-watered conditions the combination of AM inoculation and AIP augmented root SA concentration.

Under fully-watered conditions AIP application significantly increased root JA content in non-AM plants (Figure 4H). In such water regime *R. irregularis* led to root JA drop. Besides, when plants were submitted to drought stress, AIP application also promoted root JA accumulation in non-AM plants. However, under drought stress, no significant changes as consequence of AM inoculation were featured regarding root JA.

Root JA-Ile of well-watered plants presented increased content after AIP treatment in both AM-inoculated and non-inoculated plants (Figure 4I), but when plants were submitted to drought treatment no significant changes were observed due to the chemical or fungal treatments.

Correlations among root hydraulic properties and the studied parameters

Under well-watered conditions hydrostatic root hydraulic conductivity (Lpr) was found to be negatively correlated with *ZmPIP2;4* and *ZmTIP1;1* gene expression as reflected by the Pearson correlation test (Table 2).

Table 2. Pearson correlation coefficients between hydrostatic root hydraulic conductivity (Lpr), osmotic root hydraulic conductivity (Lo), relative apoplastic water flow, root and sap SA concentration and measured sap and root hormones, root aquaporin abundance and root aquaporin gene expression in well-watered and drought plants (n = 6). * Significant at p < 0.05; ** Significant at p < 0.01; *** Significant at p < 0.00.

		Well-watered				Drought			
		Lpr	**Lo**	**% Apoplastic water flow**	**Sap SA**	**Lpr**	**Lo**	**% Apoplastic water flow**	**Sap SA**
Sap hormones	ABA	0.577	-0.124	0.338	0.771	0.705	**0.836***	-0.235	**0.898***
	JA	0.739	-0.338	0.318	0.753	**0.876***	**0.962****	-0.188	0.798
	IAA	0.429	-0.231	0.123	**0.989 ****	0.792	0.733	0.263	0.235
	SA	0.472	-0.28	0.006	1	0.421	0.675	-0.612	1
Root hormones	ABA	0.489	-0.209	0.371	**0.862***	0.788	**0.851***	-0.309	0.666
	JA	-0.588	0.534	-0.75	-0.551	0.076	-0.145	0.728	-0.553
	JA-Ile	-0.629	0.64	-0.754	-0.487	0.213	0.322	-0.339	0.129
	IAA	0.316	0.052	0.585	0.464	0.496	0.73	-0.525	**0.987****
	SA	0.438	-0.773	-0.285	0.016	0.136	0.249	-0.202	0.542
Root protein abundance	PIP1	-0.607	-0.029	0.162	-0.776	**-0.876***	**-0.926*****	0.154	-0.739
	PIP2	-0.062	0.128	-0.664	-0.593	**-0.826***	**-0.894***	0.359	-0.572
	PIP2A	-0.521	0.033	-0.319	-0.734	-0.633	-0.646	0.219	-0.103
	PIP2B	-0.155	0.052	-0.663	-0.661	-0.808	**-0.901***	0.249	-0.694
	PIP2C	0.177	-0.226	-0.432	-0.632	-0.694	**-0.818***	0.195	**-0.857***
	ZmPIP2;4	0.396	-0.606	0.249	0.019	**0.954****	**0.937****	0.005	0.404
	ZmTIP1.1	0.6	-0.451	0.708	0.464	**0.836***	**0.872***	-0.041	0.474
Root gene expression	*ZmPIP1.1*	-0.785	0.626	-0.51	-0.613	0.429	0.562	-0.564	0.499
	ZmPIP1.3	-0.461	0.177	-0.6	-0.555	-0.545	-0.622	0.058	-0.532
	ZmPIP2.2	-0.662	0.393	-0.559	-0.479	-0.076	-0.109	-0.195	-0.188
	ZmPIP2.4	**-0.867***	0.391	-0.123	-0.742	-0.654	**-0.840***	0.394	**-0.855***
	ZmTIP1.1	**-0.816***	0.633	-0.517	-0.513	-0.41	-0.417	-0.195	-0.315
	ZmTIP2.3	-0.45	0.071	-0.346	-0.73	-0.681	-0.747	0.054	-0.629
	ZmTIP4.1	-0.659	0.131	0.22	-0.685	-0.508	-0.648	0.643	-0.458
	ZmNIP2.1	-0.469	-0.128	-0.002	-0.731	-0.61	-0.666	0.293	-0.248
Root hydraulic parameters	Lpr	1	-0.674	0.022	0.472	1	**0.938****	0.128	0.421
	Lo	-0.674	1	-0.288	-0.28	**0.938****	1	-0.199	0.675
	% Apoplastic water flow	0.022	-0.288	1	0.006	0.128	-0.199	1	-0.612

However, no correlation was found between osmotic root hydraulic conductivity (Lo) and the measured parameters. Also, none of the measured parameters showed significant correlation with the percentage of water flowing thought the apoplastic route (Table 2). Our data also revealed the absence of correlation between root SA concentration and these variables. However, sap SA concentration was correlated positively with sap IAA and root ABA content (Table 2).

In contrast, under drought stress conditions Pearson correlation test revealed the positive correlation between Lpr and Lo (Table 2). Both measurements of root hydraulic conductivity, hydrostatic and osmotic, were significantly and positively correlated with sap JA concentration, ZmPIP2;4 and ZmTIP1;1 protein abundance and negatively correlated with PIP1 and PIP2 proteins content. Besides, Lo also showed positive correlation with sap ABA concentration and root ABA content and was negatively correlated with PIP2B and PIP2C proteins content, as well as, with *ZmPIP2;4* gene expression. Relative apoplastic water flow and root SA concentration did not correlate with any of the measured parameters. Contrariwise, sap SA concentration was positively correlated with sap ABA concentration and root IAA content, as well as, negatively correlated with PIP2C protein abundance and *ZmPIP2;4* gene expression (Table 2).

Discussion

In this study, we shed light on the differential root water transport regulation under water shortage when an AM fungus, in this case *R. irregularis*, is present in plant roots. Our group has already reported the modulation between different water transport pathways by the AM symbiosis in maize plants compared to non-inoculated plants under drought stress (Bárzana *et al.*, 2012). Now we go further on this mechanism by studying for the first time the implication of external SA application in water transport regulation of AM plants.

AM enhances plant performance under drought

The water stress imposed in this study produced a drop of *gs* by around 75%, regardless of fungal AM inoculation (Table 1), as in isohydric cultivars this is one of the earliest responses to water deprivation (Hasanuzzaman *et al.*, 2014). Indeed, the reduction in plant biomass production caused by drought stress has been related to direct effects on the plant photosynthetic capacity due to reduced stomatal conductance (Sheng *et al.*, 2008). Moreover, drought stress caused a significant reduction in plant biomass production in all treatments, although AM plants always maintained higher values of SDW and RDW than non-AM plants. Thus, AM-improved drought tolerance in maize plants was firstly demonstrated by the higher biomass production by these plants under water deprivation treatment (Table 1). The positive effect of mycorrhization was also observed under well-watered conditions both in shoot and root dry weights (Table 1). The enhancement of drought tolerance in maize and other plant species by AM colonization involves greater plant biomass (Boomsma & Vyn, 2008) thanks in part to a better plant mineral nutrition (Smith & Smith, 2011). However, a higher capacity for CO_2 fixation in AM

plants may also have accounted for this improved plant growth since enhanced Rubisco activity in AM grapevine and rice plants has been described under drought and salinity, respectively (Valentine *et al.*, 2006; Porcel *et al.*, 2015).

In this study, AM symbiosis did not lead to the enhancement of *gs* values probably due to the higher biomass of AM plants, which implies an also higher total transpiration rate in AM plants (Baslam *et al.*, 2012). However, better membrane stability of droughted AM plants compared to non-AM ones was observed by measuring the percentage of electrolyte leakage (EL). In these plants, EL maintained the steady-state values of well-watered plants (Table 1). This effect of AM association is consistent with our previous observation under drought stress conditions (Quiroga *et al.*, 2017).

Root water transport is regulated by AM and SA application

To cope with water scarcity, plants have developed a variety of strategies, including regulation of tissues permeability to water movement (Calvo-Polanco *et al.*, 2016). Root hydraulic conductivity (Lpr) was measured as an estimation of the root water transport potential and to determine its role under limited water availability. Drought stress produced a drop of Lpr and Lo (Figure 1A and B), often addressed in the literature under water deprivation or other abiotic stresses like salinity (Martre *et al.*, 2001; Martínez-Ballesta *et al.*, 2003; Boursiac *et al.*, 2005; Meng & Fricke, 2017). Some authors argued that this phenomenon and the consequent decrease of water uptake by roots could be a mechanism for the avoidance of water loss when soils start to dry (Aroca *et al.*, 2012). However, AM plants enhanced Lpr of droughted plants (Figure 1A), and this positive effect was already observed in other studies (Sánchez-Romera *et al.*, 2016), probably because these plants do not suffer dehydration as much as non-inoculated plants.

Moreover, in this study the effects of the applied chemical compound are mainly observed in water stressed plants (Figure 1A). This could be due to a different dynamic of droughted roots for water uptake as compared to well-watered roots, and therefore, to different efficiency for chemical uptake from the nutrient solution. SA decreased Lpr of droughted-AM plants, while the application of AIP maintained steady-state Lpr levels (Figure 1A).

In addition, osmotic component of root hydraulic conductivity (Lo), that gives information of water flowing through the cell-to-cell pathway, where aquaporins participate (Maurel *et al.*, 2008), presented the same trend under drought stress than Lpr (Figure 1B), diminishing its levels when applying SA in both non-AM or AM plants. This suggests that SA may be also altering aquaporin regulation, as it was previously pointed by Boursiac *et al.* (2008) and Du *et al.* (2013). However, in this study the effects of SA on root hydraulic

properties and aquaporin gene expression were not evident. The lack of a clear correlation between SA-mediated root hydraulic properties and SA-mediated aquaporin gene expression may be due to the fact that some aquaporins genes cannot be regulated by SA because of the lack of SA-responsive elements in their promoter region, as evidenced by Tungngoen *et al.* (2011) for a *Hevea brasilensis* PIP aquaporin. No information is available currently about the presence or absence of such elements in the promoter regions of the maize aquaporins. Moreover, a delay between hormonal treatment (IAA) and aquaporin gene expression has been described in Arabidopsis (Péret *et al.*, 2012), which may also occur here with SA. Thus, the way through which SA regulates these membrane proteins is uncertain, and the two studies mentioned above presented contradictory results about the regulation of aquaporin internalization by the hormone.

Du *et al.* (2013) found that increased SA levels hinder the constitutive recycling of membrane proteins like aquaporins, increasing the abundance of some of them in the plasmalemma, as a mechanism to control their activity. In contrast, Boursiac *et al.* (2008) described a stimulus-induced internalization of PIP proteins after SA application meditated by reactive oxygen species (ROS). In any case, aquaporin modulation was extensively reported to substantially contribute to total root water flow (Martínez-Ballesta *et al.*, 2000; Martre *et al.*, 2001; Boursiac *et al.*, 2008; Knipfer & Fricke, 2011; Vandeleur *et al.*, 2014). In our study, significant correlation between aquaporin accumulation and Lo was found exclusively under drought stress treatment (Table 2), but not under well-watered conditions. This supports the idea of ROS involvement, as they may accumulate under drought conditions, leading to relocalization of aquaporins as reported by independent studies (Boursiac *et al.*, 2008; Velikanov *et al.*, 2015).

Since plants undergo frequent environmental changes, the activity of aquaporins must be regulated by mechanisms that allow rapid responses to these changes. Aquaporins regulate cell water flow either through changes in their abundance or channel gating (Tyerman *et al.*, 2002). Post-translational modifications are also necessary to achieve such rapid regulation (Vandeleur *et al.*, 2014), including phosphorylation/de-phosphorylation of specific serine residues, the first post-translational regulation mechanism found in aquaporins (Prak *et al.*, 2008; Prado *et al.*, 2013). This modification generates conformational changes allowing aquaporin gating or modifying its subcellular localization in the membrane (Johansson *et al.*, 1998; Prak *et al.*, 2008; Prado *et al.*, 2013) and has been proposed as a mechanism to prevent water loss (Bárzana *et al.*, 2015). Phosphorylation of C-terminal residues Ser-280 and Ser-283 of PIP2 aquaporins was correlated to the regulation of Lpr in plants (Prado *et al.*, 2013). The present data show that PIP2B (Ser-283) and PIP2C (Ser-280 and Ser-283) both negatively correlated with

Lo under DS, as well as PIP2 and PIP1 protein levels (Table 2). However, when analysing ZmPIP2;4 root aquaporin abundance, one of the most abundant aquaporins in maize roots, with prominent role in water transport (Chaumont *et al.*, 2001), it correlated positively with Lo under water shortage (Table 2). This could be contradictory, but it must be taken into account that the PIP2 antibody recognizes several different isoforms within the PIP2 subfamily, that may have different roles in water transport regulation. ZmTIP1;1, protein abundance presented the same trend previously described for ZmPIP2;4 protein and consequently also correlated with Lpr (Table 2), suggesting that these two proteins could be of high interest in regulating water transport in our experimental conditions. Surprisingly, *ZmPIP2;4* transcript levels negatively correlated with Lo (Table 2). However, expression levels of aquaporins do not always correlate with their protein abundance, as both change along time and with the growing conditions (Chaumont & Tyerman, 2014). Expression patterns of most analysed aquaporins differed in AM and non-AM plants (Figure 2), which in general involved their downregulation in AM plants, as was shown in previous reports (Bárzana *et al.*, 2014; Quiroga *et al.*, 2017).

Interestingly, it has been highlighted the different behaviour of non-AM and AM plants under drought after SA application. Whereas non-AM plants increased the percentage of apoplastic water flow in presence of exogenous SA, plants inoculated with the AM fungus decreased water circulating through this pathway when the hormone was applied (Figure 1C). This effect can result from the differential effect of this hormone on Lpr in plants inoculated with the symbiotic fungus under drought. This is consistent with previous results, where AM plants were suggested to have a higher plasticity for switching between water transport pathways (Bárzana *et al.*, 2012).

In addition, this differential effect of SA on the apoplastic water flow in AM and non-AM plants may be mediated by altered nitric oxide (NO) content in these plants, since it has been recently shown that SA-induced NO regulates maize water content and hydraulic conductivity under drought (Shan & Wang, 2017). Moreover, Sánchez-Romera *et al.* (2017) have suggested that NO favours apoplastic water pathway inside roots and suggested that different outcomes in root hydraulic conductivity observed between AM and non-AM plants could be mediated by differences in NO content. Thus, a higher NO content in non-AM plants than in AM ones could explain the SA-induced enhancement of apoplastic water flow in non-AM plants and the opposite effect in AM plants.

Implication of phytohormones in root water transport regulation

Salicylic acid has been previously shown to alter plant water relations under drought or salt stress conditions (Farooq *et al.*, 2010; Khan *et al.*, 2015; Faried *et al.*,

2017). On the light of our results, no clear relationship between exogenous SA application and root or sap SA concentration increase was found, although in sap, an enhancement in such hormone occurred under drought (Figure 4C, G and L). However, it is noteworthy that, as mentioned above, SA effects on plant functions are dose-dependent (Miura & Tada, 2014; Khan *et al.*, 2015). Indeed, an important aspect regarding the effect of SA application is the dose of SA and the method of application (via foliar or via hydroponic solution). Generally, low concentrations (less than 0.5 mM) of SA increase drought tolerance, while high concentrations (2-3 mM) decrease drought tolerance (Miura & Tada, 2014). Thereby, the selected dose in this study (0.02 mM, applied via hydroponic solution) is enough to affect Lpr, but may not be sufficient to alter hormonal tissue content.

Nonetheless, SA could be modifying Lpr through the alteration of other phytohormones in roots, as a complex crosstalk among these compounds may take place, controlling plant performance under different environmental conditions (Munné-Bosch & Müller, 2013). In fact, a consistent response in AM plants was the increase of IAA, ABA and SA in roots under drought conditions compared to non-AM plants (Figure 4E, F and G). In this sense, SA has been described to play a role in the regulation of AM root colonization, although the precise mechanism is not clear yet (Herrera-Medina *et al.*, 2003). Moreover, SA was also reported to induce genes involved in ABA biosynthesis, as well as to modify ABA transport to the shoots (Horváth *et al.*, 2015). Regarding the functioning of the AM symbiosis, ABA was related to arbuscule formation, thus being necessary for efficient AM symbiosis establishment and functioning (Miransari *et al.*, 2012). An enhancement of the ABA content by the AM symbiosis was clearly reflected by our results (Figure 4F). Taking together these data and the previously described enhancement of Lpr by ABA (Aroca *et al.*, 2008; Parent *et al.*, 2009) we hypothesize that enhanced ABA in AM inoculated plants may favour the increase in root conductivity under drought conditions. This idea is also supported by the higher Lpr and Lo found in AM plants under drought stress (Figure1A and B).

In addition to this, IAA is considered to be essential for AM infection, especially during pre-symbiotic interactions (Hanlon & Coenen, 2011) and our result showed an important enhancement of root IAA content after AMF root colonization, especially in SA-treated plants. This enhanced IAA levels in AM SA-treated plants may have contributed to the reduction in the hydraulic parameters measured here, since Péret *et al.* (2012) showed that exogenous IAA application inhibited most aquaporins genes in *A. thaliana* and reduced root hydraulic conductivity both at the cell and whole-organ level. In relation with AM development, some authors also found alterations in root JA levels with root colonization (Liu *et al.*, 2013; Pedranzani *et al.*, 2016). However, no changes were found

in other studies (López-Ráez *et al.*, 2010; Sánchez-Romera *et al.*, 2016).

ABA, JA and SA were hypothesized to have some common regulatory elements in their signalling pathways, although their clear relationship was not established yet (Proietti *et al.*, 2013). SA and JA interaction has normally been reported to be antagonistic in defence response (Koornneef *et al.*, 2008). Although from hormonal content we cannot observe any clear relationship between them, sap JA levels positively correlated with Lo and Lpr under drought (Table 2) and this is in line with previous results of JA on Lpr (Sánchez-Romera *et al.*, 2014, 2016). An opposite effect was induced by SA on these parameters, which agree with results by Volobueva *et al.* (2004), who reported decreased water conductance in maize roots by SA addition and results by Boursiac *et al.* (2008) showing down-regulation of root water transport by SA in Arabidopsis plants. Thus, from our data we could deduce that there is a relationship between these hormones in response to drought, which is regulated by AM colonization, even if further research is needed to explain accurately the way these hormones interact.

Conclusions

In the present work we demonstrated that AM symbiosis can modify root hydraulic response to drought episodes. Under these conditions, AM plants showed both increased Lpr and Lo. This, together with the better exploration and exploitation of the soil water resources by the fungal hyphae that has been widely described in literature (Marulanda *et al.*, 2003; Allen, 2007, 2009; Ruth *et al.*, 2011), may results in greater amount of water available to the AM plants and better performance of AM plants under water deprivation.

Exogenous SA application altered root hydraulic parameters, decreasing Lpr and Lo under drought, while application of its inhibitor, AIP maintained steady state levels for these parameters. SA modulation of water conductivity could be due to a fine-regulation of root aquaporins (as ZmPIP2;4 or ZmTIP1;1). Furthermore, SA application differently modulated the percentage of water flowing by the apoplastic pathway under the imposed stress, decreasing its contribution to total root water flow in AM plants and increasing it in non-AM plants.

Intricate relationship between Lpr, aquaporins and endogenous levels of phytohormones, especially SA, ABA and JA was observed, revealing a complex network controlling water transport in roots. Future researches should analyse the promoter regions of the maize aquaporin genes to search for hormone responsive elements and to explore more in detail the crosstalk mechanism between these hormones in the regulation of water transport in AM roots, in order to better understand the mechanism through which

the AM symbiosis copes with root dehydration and contributes to improved root hydraulic properties under drought conditions.

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CHAPTER V

Radial water transport in arbuscular mycorrhizal maize plants under drought stress conditions as affected by indole-acetic acid (IAA) application

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Abstract

Drought stress is one of the most devastating abiotic stresses, compromising crop growth, reproductive success and ultimately yield. The arbuscular mycorrhizal (AM) symbiosis has been demonstrated to be beneficial in helping the plant to bear with water deficit. In plants, development and stress responses are largely regulated by a complex hormonal crosstalk and auxins play significant roles in plant growth and development, in responses to different abiotic stresses or in the establishment and functioning of the AM symbiosis. Despite these important functions, the role of indole-3acetic acid (IAA) as a regulator of root water transport and stress response is not well understood. In this study, the effect of exogenous application of IAA on the regulation of root radial water transport in AM plants was analysed during well-watered and drought stress conditions. Exogenous IAA application affected root hydraulic parameters, mainly osmotic root hydraulic conductivity (Lo), which was decreased in both AM and non-AM plants under water deficit conditions. Under drought, the apoplastic water flow was differentially regulated by the hormonal application in non-AM and AM plants. The effect of IAA on the internal cell component of root water conductivity suggests that aquaporins are involved in the IAA-dependent inhibition of this internal cell pathway.

Keywords: arbuscular mycorrhizal symbiosis, drought stress, IAA, radial water transport

Introduction

Drought stress is one of the most devastating abiotic stresses, compromising crop growth, reproductive success and ultimately yield (Hasanuzzaman *et al.*, 2014). In addition, climate change is intensifying the effects of drought worldwide (Trenberth *et al.*, 2014). Roots are the first organs to sense the stress, as they are in contact with soil moisture changes. Therefore, they must adapt to it morphologically and physiologically in order not to be damaged. In this sense, the arbuscular mycorrhizal symbiosis between *Glomeromycotina* fungi and the roots of most terrestrial plants has been demonstrated to be beneficial in helping the plant to bear with water deficit (Chitarra *et al.*, 2016; Essahibi *et al.*, 2017; Yooyongwech *et al.*, 2016). This association contributes to the uptake of water and nutrients thanks to a vast network of extraradical mycelium in exchange of carbon compounds and lipids. In addition to a better access of nutrients and water in soil, the relieve of drought stress is achieved by the alteration of root hydraulic properties (Aroca *et al.*, 2007; Bárzana *et al.*, 2012; Quiroga *et al.*, 2019a).

In roots, radial water movement occurs through three main parallel pathways according to the composite transport model: the apoplastic (around the cell walls), symplastic (crossing cells via plasmodesmata) and transcellular (involving water passage across cell membranes). The last two pathways are commonly referred to as cell-to-cell pathway (Steudle and Peterson, 1998). During non-stressful conditions the apoplastic pathway usually dominates, following the transpiration stream. However, during water deficit conditions in the soil such pathway is hampered due to stomatal closure and transpiration decline and the cell-to-cell pathway is enhanced. Aquaporins play a key regulatory role of root cell water transport in higher plants both under normal and under stressful conditions (Maurel *et al.*, 2008), participating in the cell-to-cell water transport. Arbuscular mycorrhizal symbiosis has been shown to modulate the switch between water transport pathways in roots, and a previous study showed an increase in the relative apoplastic water flow in AM plants under both well-watered and drought stress conditions (Bárzana *et al.*, 2012). In addition, these changes in root water conductivity (Lpr) by AM symbiosis were found to be largely mediated by changes in plant aquaporins (Ruiz-Lozano and Aroca, 2017, 2010). In fact, several maize aquaporins were differently regulated by the AM fungus depending on the drought stress imposed (Bárzana *et al.*, 2014).

In plants, development and stress responses are mainly regulated by a complex hormonal crosstalk (Munné-Bosch and Müller, 2013). Besides their other fundamental functions, the exogenous application of hormones may help to improve plant yield (Singh *et al.*, 2017). Among phytohormones, auxins play significant roles in plant growth and

development, as well as in response to different abiotic stresses (Ullah *et al.*, 2018). In concert with other hormones, the roles of auxin include meristem maintenance, leaf primordia and lateral root initiation, tropic responses, development of vascular tissues, root and shoot elongation and control of apical dominance. At cell level, auxins also affect cell division, elongation, differentiation and polarity (Naser and Shani, 2016).

During AM symbiosis, a complex molecular dialog is established between both symbiotic partners and phytohormones also play an important role on this process. In fact, some evidences showed that auxin signalling is required for normal AM infection, and the exchange of diffusible signals between plant and fungus is mediated by host auxin responses (Hanlon and Coenen, 2011). In fact, auxin was found to be required for arbuscule development (Etemadi *et al.*, 2014). In trifoliate orange, higher root indole-3acetic acid (IAA) levels were found in AM plants and this enhancement (together with other hormonal increases) was positively related with drought tolerance in different studies (Liu *et al.*, 2018, 2016).

Despite the important functions of IAA, its role as a regulator of root water transport and stress response is not well understood (Wani *et al.*, 2016). Auxin-mediated growth inhibition under abiotic stress is one of the strategies for the acclimation to the changing environment (Naser and Shani, 2016). Some evidences point to a role in drought stress tolerance. Aux/IAA proteins accumulate in response to auxin signalling. In the absence of auxin, these proteins dimerize with Auxin Response Factors (ARF) to prevent ARF-mediated transcriptional regulation of early auxin response genes. However, when auxin is present, Aux/IAA proteins are ubiquitinated, allowing ARF-mediated transcriptional regulation of response genes. Some Aux/IAA genes in rice were induced by drought stress, and in particular OsIAA6 was confirmed to be involved in drought stress responses (Jung *et al.*, 2015). In another study, plants overexpressing *YUCCA6*, a gene involved in the tryptophan-dependent IAA biosynthesis pathway, was associated with drought stress tolerance in poplar (Ke *et al.*, 2015). Additional studies revealed that under osmotic stress conditions, abscisic acid (ABA) regulated growth through the interaction with cytokinin, ethylene and auxin (Rowe *et al.*, 2016).

Rather than participating in stress tolerance, De Diego *et al.* (2012) found that IAA played a role as a drought signal also in poplar, even more than ABA. Additionally, auxin treatment was found to regulate tissue hydraulics and reduce root hydraulic conductivity, at cell and whole-organ level, and to repress aquaporin genes through the auxin response factor ARF7 (Péret *et al.*, 2012).

Based on all these previous results, we hypothesized that hormonal treatment can affect the AM modulation of water transport in roots, especially during drought stress conditions and probably through aquaporin regulation. To confirm this hypothesis, we externally applied IAA or 6-Fluoroindole (6-FI), an inhibitor of the tryptophan-dependent IAA biosynthesis (Ludwig-Müller *et al.*, 2010), to AM or non-AM plants subjected or not to drought stress. The aim was to analyse the effect of this hormone on root water transport pathways and aquaporins during AM colonization. The results obtained from this study shed further light on the AM regulation of root water transport during drought stress conditions.

Materials and methods

Experimental design

The experiment consisted of a factorial design with three factors: (1) inoculation treatment, with plants inoculated with the AM fungus *Rhizophagus irregularis*, strain EEZ 58 (AM) and non-inoculated control plants (non-AM); (2) watering treatment, so that half of the plants were subjected to drought stress (DS) for 15 days before harvest while the other half was grown under well-watered (WW) conditions throughout the entire experiment; (3) chemical treatment, so that one group of each inoculation treatment was maintained untreated, another group of plants was treated with 20 µM of the auxin indole-3acetic acid (IAA), and the last group was treated with 75 µM of 6-fluoroindol (6-FI), as an inhibitor of IAA biosynthesis. The different combination of these factors gave a total of 12 treatments. Each treatment had 10 replicates, giving a total of 120 plants.

Biological material and growth conditions

The growing substrate consisted of a mixture of soil and sand (1:9 v/v). The 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 undiluted soil had a pH of 8.1 (water); 0.85% organic matter, nutrient concentrations (mg kg^{-1}): P, 10 ($NaHCO_3$-extractable P); N, 1; K, 110. The soil texture was made of 47.1% silt, 38.3% sand and 14.6% clay.

Seeds of *Zea mays* L. were provided by Pioneer Hi-Bred (Spain), cultivar PR34B39 that was also used in previous studies (Quiroga *et al.*, 2017; 2018). Seeds were pre-germinated in sand and then transferred to 1.5 L pots containing 1250 g of the above-described substrate. At planting time, half of the plants were inoculated with ten grams of AM inoculum with *Rhizophagus irregularis* (Schenck and Smith), strain EEZ 58. The

inoculum consisted of spores, mycelia, infected root fragments and soil. Non-inoculated plants received a 10 mL aliquot of an inoculum filtrate (<20 μm), in order to provide the natural microbial population present in the inoculum, but free of AM propagules.

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

Indole-3-acetic acid 20 μM and 6-FI 75 μM were applied with the nutrient solution 6 hours before harvesting. The dose of phytohormone and its inhibitor, as well as, the exposure time needed to affect root hydraulic conductivity were previously established in experiments ranging from 0.5 to 20 μM IAA, 25 to 100 μM 6-FI, and exposure times of 1h, 6h, 12h and 24h.

Measurements

- Biomass production and symbiotic development

Five replicates per treatment were collected from roots and shoots and dried in a hot-air oven at 70ºC for 2 days to measure dry weight.

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

- Stomatal conductance

Stomatal conductance (*gs*) was measured in the second youngest leaf from 10 plants per treatment two hours after the onset of photoperiod and one day before harvest with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK), following the manufacter's recommendations.

- Photosynthetic efficiency

Photosystem II efficiency was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll a fluorescence. It quantifies the quantum yield of photosystem II as the ratio between the current fluorescence yield in the light-adapted state (FV‘) and the maximum fluorescence yield in the light-adapted state (FM‘), according to Oxborough and Baker (1997). Measurements were taken one day before harvest in the second youngest leaf of 10 different plants of each treatment.

- Membrane electrolyte leakage

Leaf samples from 10 plants per treatment were washed with deionized water to remove surface-adhered electrolytes. Samples were placed in 15 mL falcon tubes containing 10 mL of deionized water and incubated on a rotary shaker (at 100 rpm) at 25 $_{o}$C during 3 hours. Then the electrical conductivity of the solution (E_0) was determined using a conductivity meter (Mettler Toledo AG 8603, Switzerland). Samples were subsequently placed at -80ºC for 2 hours. Afterwards, tubes were incubated again at room temperature under smoothly agitation for 3 hours and the final electrical conductivity (E_f) was obtained. The electrolyte leakage was defined as follows: $[(E_0 - E_{water})/(E_f - E_{water})] \times 100$, where E_{water} is the electrical conductivity of the deionized water used to incubate the samples.

- Osmotic root hydraulic conductivity (Lo)

Lo was measured at noon by the free exudation method (Benabdellah *et al.*, 2009) on detached roots exuding under atmospheric pressure. Under such conditions, water moves through the cell-to-cell path following only an osmotic gradient (Steudle and Peterson, 1998). The exuded sap was collected and weighed after 2 hours of exudation. A cryoscopic osmometer was used to measure the osmolarity of the exuded sap and the nutrient solution, needed for Lo calculation, according to Aroca *et al.* (2007). Lo was calculated as $Lo = Jv/\Delta\Psi$, where *Jv* 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 6 hours after starting the chemical treatment.

- Hydrostatic root hydraulic conductivity (Lpr)

Lpr was determined at noon in five plants (n=5) per treatment with a Scholander pressure chamber, 6 hours after starting the chemical treatment as described by Bárzana *et al.* (2012). The detached roots received a gradual increase of pressure (0.2, 0.3 and 0.4 MPa) at 2-minutes intervals. Sap was collected after 2 minutes at the three pressure

points. Then the sap flow was plotted against pressure, with the slope being the root hydraulic conductance (*L*) value. Finally, Lpr was determined by dividing *L* by root dry weight (RDW) and expressed as mg H_2O g RDW^{-1} MPa^{-1} h^{-1}. The collected sap was also used for subsequent hormonal determination.

- Relative apoplastic water flow

Relative changes in apoplastic water flux were estimated using a high molecular weight dye (light green SF yellowish; Sigma-Aldrich Chemical, Gillingham, Dorset; colour index 42095, molecular weight 792.85 g mol^{-1}), which has the ability to move only through the apoplast (López-Pérez *et al.*, 2007). For that, detopped roots were immersed in 250 μmol L^{-1} dye solution inside the pressure chamber 5 min before pressure application 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 concentration of the dye was determined at 630 nm (Bárzana *et al.*, 2012) in the whole collected sap. The percentage of apoplastic pathway was calculated from the ratio between dye concentration in the sap flow and in the nutrient solution, being the concentration of dye in the nutrient solution of each treatment considered to be 100%.

- Sap and tissues hormonal content

In sap, IAA, ABA, salicylic acid (SA) and jasmonic acid (JA) contents were analysed according to Albacete *et al.* (2008) with some modifications. Thus, xylem sap samples were filtered through 13 mm diameter Millex filters with nylon membrane having 0.22μm pore size (Millipore, Bedford, MA, USA). Ten μl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 μg L^{-1}).

In plant roots and leaves, IAA, ABA, SA, JA and jasmonate isoleucine (JA-Ile) were analysed using high-performance liquid chromatography-electrospray ionization-high-resolution accurate mass spectrometry (HPLC-ESI_HRMS) as described in Ibort *et al.* (2017).

- PIP aquaporins abundance and phosphorylation status

Isolation of microsomal fraction and ELISA were performed as described previously by Calvo-Polanco *et al.* (2014). As primary antibodies we used two antibodies

recognizing several PIP1s and PIP2 isoforms, and three antibodies recognizing 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) at (dilution 1:1000).

- Statistical analysis

Within each watering regime, data were analysed using SPSSStatistics (version 23, IBM Analytics) and subjected to analysis of variance (ANOVA) with inoculation treatment and chemical treatment as sources of variation. Post-hoc comparisons were performed with Duncan's test (P<0.05). Correlations between the different parameters were performed by calculating the Pearson correlation coefficients.

Results

Root mycorrhization, plant growth and ecophysiological parameters

The chemical treatment for only 6h did not affect parameters presented in Table 1, thus only the inoculation treatment and the water regime are considered in these data.

Uninoculated plants did not show AM root colonization. Mycorrhizal root length of plants inoculated with *Rhizophagus irregularis* (AM) represented around 65% of the root system and no significant differences were observed between water treatments (data not shown).

Table 1. Plant dry weight (DW), electrolyte leakage (EL), stomatal conductance (*gs*) and photosystem II efficiency in the light-adapted state (ΔFv/Fm') in maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Data represents the means of thirty values ± SE for plant DW or twelve values ± SE for EL, *gs* and ΔFv/Fm'. Different letter indicates significant differences between treatments (p < 0.05) based on Duncan's test.

	Plant DW (g plant^{-1})	**EL (%)**	***gs* (mmol H_2O m^{-2} s^{-1})**	**ΔFv/Fm'**
WW non-AM	14.3 ± 0.51 b	10.5 ± 1.12 b	210.5 ± 21.4 a	0.61 ± 0.01 a
WW AM	18.6 ± 0.95 a	8.1 ± 0.68 b	112.3 ± 9.39 b	0.64 ± 0.01 a
DS non-AM	9.15 ± 0.22 c	17.7 ± 2.33 a	115.3 ± 10.8 b	0.55 ± 0.02 b
DS AM	10.7 ± 0.27 c	6.43 ± 0.23 b	199.0 ± 15.8 a	0.52 ± 0.01 c

Drought stress negatively affected plant dry weight (between 36 and 42% of decrease), but AM plants maintained higher plant dry weight than non-AM ones, regardless of water regime (Table 1). Membrane electrolyte leakage (EL) increased significantly in non-AM plants after drought stress. In contrast, AM plants maintained levels of well-watered plants (Table 1). Stomatal conductance (*gs*) was significantly reduced after

two weeks of drought stress treatment in non-AM plants (Table 1), while AM plants exhibited higher *gs* levels than under well-watered conditions. The efficiency of photosystem II was reduced by water deficit both in AM and in non-AM plants (Table 1).

Hydrostatic and osmotic root hydraulic conductivities and percentage of apoplastic water flow

Hydrostatic root hydraulic conductivity (Lpr) was not significantly affected by IAA treatment or its inhibitor under well-watered conditions, regardless of AM inoculation. In contrast, under drought stress AM plants had considerably higher Lpr values (118%) than non-AM ones. Application of IAA enhanced Lpr levels in non-AM plants under drought stress, almost doubling control values, while AM ones maintained their high Lpr values but with no further increment due to IAA. The application of 6-FI, the IAA biosynthesis inhibitor, decreased significantly Lpr levels only in AM plants (Figure 1A).

Lo levels remained also unchanged in the case of well-watered plants, both in non-AM and AM treatments. However, an evident drop of Lo levels occurred under drought conditions in both non-inoculated and inoculated plants. Moreover, under these conditions an additional inhibition of Lo by IAA occurred in both AM and non-AM plants (more than 70% inhibition in both cases), and the inhibitor 6-FI had a weak effect restoring Lo levels but without reaching the control levels (Figure 1B).

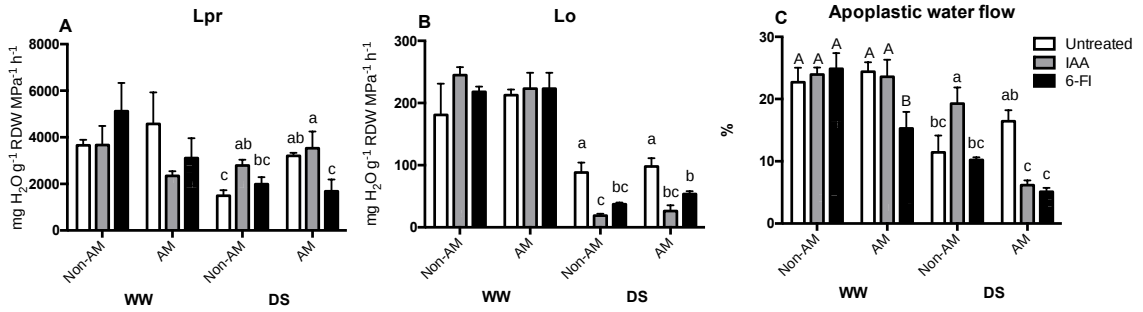

Figure 1. (A) Hydrostatic root hydraulic conductivity (Lpr), (B) osmotic root hydraulic conductivity (Lo) and (C) relative apoplastic water flow in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

As for the other parameters, well-watered plants did not featured changes in the relative percentage of water circulating by the apoplastic pathway, with the exception of the inhibition produced by 6-FI in AM plants. This effect seems to be compensated with Lo contribution in these plants, thus not being reflected in changes of Lpr levels. Under

drought stress, IAA application differently affected the percentage of water circulating by the apoplastic pathway in non-AM and AM plants. Hence, in non-AM plants IAA produced a significant increase of this percentage, while in AM plants the hormone significantly diminished the flux of water circulating through this pathway. The use of 6-FI restored this parameter in non-AM plants and had a similar effect than IAA in AM ones (Figure 1C).

Aquaporin protein abundance and post-translational regulation

The abundance PIP1 and PIP2 aquaporin proteins was measured. Moreover, the PIP2 phosphorylation state was quantified in roots, as aquaporin water channel activity is affected by this post-translational modification. In this context, the contents of PIP2 protein phosphorylated at Ser-280 (PIP2A), at Ser-283 (PIP2B) and double phosphorylated at Ser-280 and Ser-283 (PIP2C) were quantified.

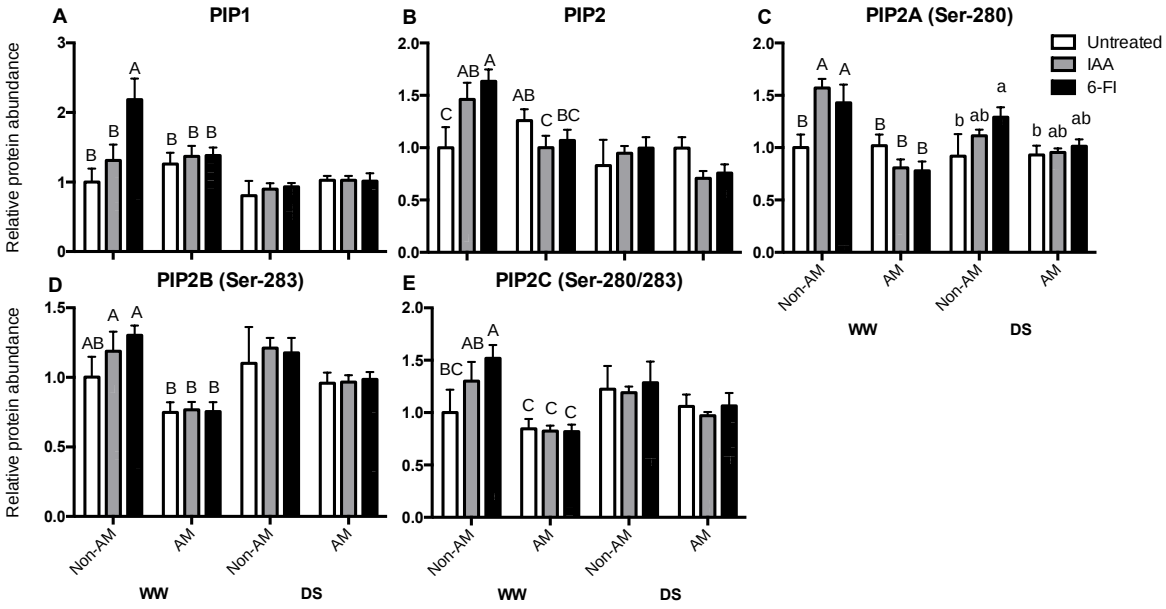

Figure 2. (A) PIP1, (B) PIP2, (C) PIP2A, (D) PIP2B, (E) PIP2C relative protein abundance in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

There was not a clear response of aquaporin accumulation to the chemical treatment, probably due to the short time of application. Under well-watered conditions, 6-FI increased PIP1 and PIP2 protein levels in non-AM plants. IAA also increased PIP2 accumulation in non-AM plants under these conditions. However, it had the opposite effect in AM plants. No significant effect on protein levels was observed under drought stress

conditions either by the hormones or by fungal inoculation (Figure 2A and B).

AM inoculation generally decreased the abundance of phosphorylated proteins under well-watered conditions in plants treated with IAA or 6-FI. No significant changes in phosphorylation levels were observed in droughted plants (Figure 2C, D and E).

Sap and tissues phytohormones contents

In sap IAA content resulted not significantly affected by the applied chemical treatments, AM inoculation or water regime (Figure 3A). Under well-watered conditions, sap ABA concentration was higher in AM plants compared to non-AM ones when IAA or 6-FI were applied. The application of the inhibitor did not affect sap ABA levels in non-AM plants but increased its levels in AM plants under the same conditions. No significant changes were observed under water deprivation conditions (Figure 3B).

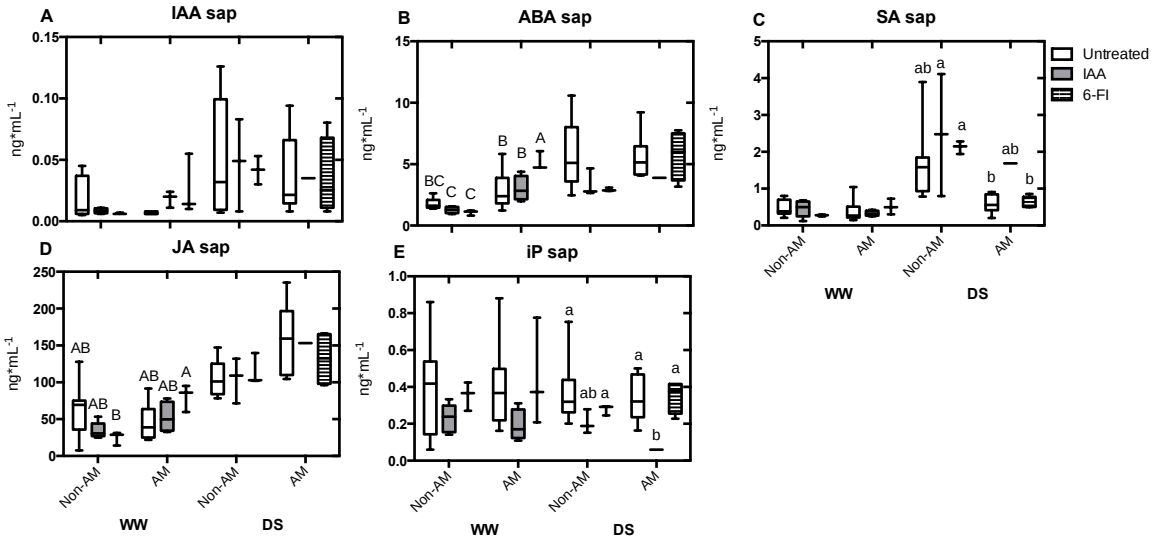

Figure 3. (A to E) Boxplots representing the sap concentrations of IAA, ABA, SA, JA and iP in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

No clear effect was observed in sap SA and JA levels under any of the studied conditions (Figure 3C and 3D). In the case of sap iP, a precursor of cytokinins, no significant effect was observed under well-watered conditions. However, under drought stress, IAA treatment significantly decreased sap iP levels of AM plants, being their levels restored by 6-FI (Figure 3E).

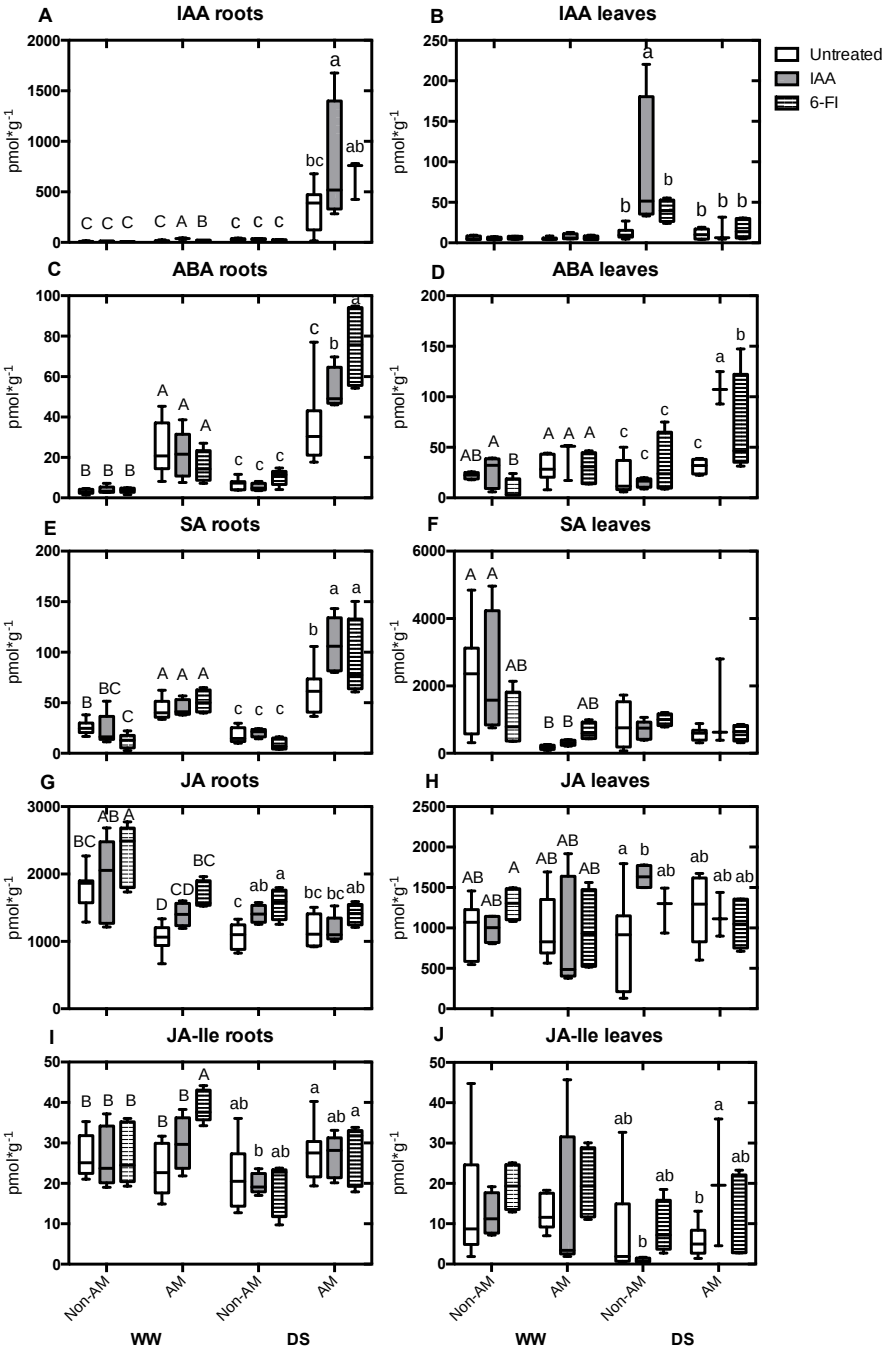

Figure 4. (A to J) Boxplots representing root and leaf concentrations of IAA, ABA, SA, JA and JA-Ile in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

In roots, IAA content increased significantly after application of this compound in AM plants under fully-irrigation conditions, being this effect reversed by the application of the inhibitor 6-FI. Under drought stress conditions, AM plants presented higher root IAA

concentration compared to non-AM plants, and the application of IAA further increased IAA levels (Figure 4A). However, in leaves, the increase in IAA content after the application of the hormone was only observed in non-AM plants during drought stress (Figure 4B).

Root ABA concentration increased with AM inoculation regardless of the water treatment. However, during drought stress, this increment was significant only with the application of the hormone or its inhibitor, which strongly elevated ABA levels (Figure 4C). In leaves, the trend was similar, but the increase was only significant in well-watered AM roots treated with 6-FI and in droughted AM roots with both chemical treatments (Figure 4D). Root SA content presented the same pattern than ABA, being increased by the fungal presence in both watering conditions, especially under drought stress (Figure 4E). In contrast, SA concentration in leaves decreased in AM plants under well-watered conditions when untreated or treated with IAA. Under drought stress, SA content in leaves was not significantly affected by the different treatments (Figure 4F).

Root JA content suffered a drop in well-watered AM plants regardless of hormonal treatment, and 6-FI slightly increased JA levels in both non-AM and AM plants under these conditions. The same increment was observed in non-AM plants during drought after application of IAA or 6-FI (Figure 4G). In leaves, a slight increase in JA concentration occurred during drought in non-AM plants when IAA was applied (Figure 4H).

Interestingly, JA-Ile content in roots was also increased by 6-FI in roots of AM plants under well-watered conditions. Under drought stress, levels of this hormone were unaffected by any of the treatments (Figure 4I). In the case of leaves, JA-Ile levels were not modified under well-watered conditions, but they were increased by IAA application in AM plants under drought stress (Figure 4J).

Correlations among root hydraulic properties and the different parameters analysed

Under well-watered conditions, Lpr was negatively correlated with root IAA content, as reflected by Pearson correlation coefficient (Table 2). However, this correlation was not found in the case of Lo (Table 2). The percentage of apoplastic water flow showed a strong negative correlation with sap IAA, ABA and JA, as well as, with root JA-Ile (Table 2). Sap IAA content was also positively correlated with sap JA content and root JA-Ile. Moreover, root IAA content showed a positive correlation with IAA content in leaves (Table 2).

Table 2. Pearson correlation coefficients between hydrostatic root hydraulic conductivity (Lpr), osmotic root hydraulic conductivity (Lo), relative apoplastic water flow, sap and root IAA concentration and measured sap and root hormones and root aquaporin abundance in well-watered and drought plants (n = 6). * Significant at $p < 0.05$; ** Significant at $p < 0.01$; *** Significant at $p < 0.001$.

		Well-watered					Drought				
		Lpr	**Lo**	**% Apoplastic water flow**	**Sap IAA**	**Root IAA**	**Lpr**	**Lo**	**% Apoplastic water flow**	**Sap IAA**	**Root IAA**
Sap hormones	IAA	-0.757	-0.171	**-0,854***		0.499	-0.451	-0.061	0.477		**-0.841***
	ABA	-0.525	0.047	**-0.860***	0.795	0.502	-0.293	**0.810***	-0.173	-0.232	0.290
	SA	-0.381	-0.010	-0.687	0.556	-0.106	0.014	-0.644	0.318	0.722	-0.577
	JA	-0.609	-0.332	**-0.846***	**0.915***	0.324	0.661	0.235	-0.278	**-0.906****	0.758
	iP	0.408	-0.509	-0.523	0.235	-0.529	-0.706	0.752	0.141	0.184	-0.387
Root hormones	IAA	**-0.825***	0.301	-0.194	0.499		0.377	-0.123	-0.675	**-0.841***	
	ABA	-0.313	0.117	-0.152	0.195	0.638	0.145	-0.011	-0.689	**-0.854***	**0.943***
	SA	-0.609	0.077	-0.602	0.634	0.651	0.440	-0.074	-0.598	**-0.835***	**0.993*****
	JA	0.321	0.100	0.032	-0.130	-0.458	-0.267	-0.601	-0.031	0.052	-0.229
	JA-Ile	-0.530	0.186	**-0.929****	**0.861***	0.396	0.384	0.319	-0.394	**-0.838***	**0.869***
Leaf hormones	IAA	-0.669	-0.023	-0.165	0.550	**0.802***	0.085	-0.649	0.604	0.526	-0.506
	ABA	0.274	0.743	0.231	-0.178	-0.383	0.108	**0.922****	0.365	-0.068	-0.148
	SA	0.078	-0.110	0.142	-0.125	-0.540	0.269	-0.466	-0.469	-0.021	0.216
	JA	0.791	0.041	0.283	-0.520	-0.550	-0.267	-0.605	0.601	0.040	-0.192
	JA-Ile	0.093	-0.078	-0.591	0.447	-0.065	0.278	-0.195	**-0.824***	-0.583	0.767
Root protein abundance	PIP1	0.531	0.309	0.206	-0.384	-0.169	0.588	-0.116	-0.321	**-0.965****	0.792
	PIP2	0.766	0.453	0.465	-0.784	-0.552	0.026	0.187	0.759	0.286	-0.743
	PIP2A	0.598	0.396	0.557	-0.768	-0.600	-0.209	-0.569	0.088	0.230	-0.450
	PIP2B	0.557	0.143	0.461	-0.572	-0.616	-0.345	-0.422	0.478	**0.832***	**-0.834***
	PIP2C	0.632	0.236	0.472	-0.635	-0.589	-0.626	-0.008	0.356	0.788	**-0.916***
Root hydraulic parameters	Lpr		-0.135	0.433	-0.757	**-0.825***		-0.243	0.259	-0.451	0.377
	Lo	-0.135		-0.032	-0.171	0.301	-0.243		0.126	-0.061	-0.123
	% Apoplastic water flow	0.433	-0.032		**-0.854***	-0.194	0.259	0.126		0.477	-0.675

Under drought stress conditions, Lpr did not correlate with any of the studied parameters (Table 2). In contrast, Lo positively correlated with sap and leaf ABA content (Table 2). In addition, the percentage of water flowing through the apoplastic pathway presented a negative correlation with JA-Ile in leaves. The data also revealed a strong negative correlation of sap IAA with sap JA, as well as with root IAA, ABA, SA and JA-Ile (Table 2). Moreover, this parameter correlated negatively with PIP1 protein abundance and positively with PIP2B protein content. Finally, root IAA content presented a positive correlation with ABA, SA and JA-Ile in roots, and a negative correlation with PIP2B and PIP2C (Table 2).

Discussion

In this study, the beneficial effect of AM colonization on plant physiology was evidenced by the higher dry weight of AM plants compared to non-AM ones (Table 1). This may be the consequence of a better plant hydration and nutrition due to the increased uptake surface of the fungal hyphae. Under drought stress, AM plants also presented lower electrolyte leakage than non-inoculated plants, which suggest a higher membrane stability of these plants (Table 1). This parameter was considered a good indicator of the plant tolerance to water stress (Ortiz *et al.*, 2015).

The information available about the IAA effect on water relations and drought tolerance is scarce. Moreover, to the best of our knowledge, its interactive effect with mycorrhizal inoculation is almost elusive. In our study, exogenous application of IAA negatively affected osmotic root hydraulic conductivity (Lo) in both non-AM and AM plants, although only during drought stress (Figure 1B). The application of 6-FI partially recovered the negative effect of IAA on Lo, but the effect was not enough to be statistically significant (Figure 1B). As this parameter is an estimation of the water flowing by the cell-to-cell pathway, it is thought that its values are largely determined by water channel activity. This result agrees with previous findings from Péret *et al.* (2012), which showed that IAA inhibited Lpr and root aquaporins in Arabidopsis during lateral root formation. Different studies have shown that hormonal treatment can affect hydraulic conductivity and probably aquaporins. In maize roots, exogenous application of ABA enhanced Lpr especially at root cortical cell level, which suggest the implication of aquaporins in this process (Hose *et al.*, 2000; Ruiz-Lozano *et al.*, 2009). In other studies, SA was found to inhibit Lpr and this was related to internalization of PIPs in cell vesicles (Boursiac *et al.*, 2008) or to a fine regulation in roots of the aquaporins ZmPIP2;4 and ZmTIP1;1 (Quiroga *et al.*, 2018).

In this study, hydrostatic root hydraulic conductivity (Lpr) was enhanced when IAA was applied in both non-AM and AM plants during drought (although it was not significant for AM plants). This effect could be explained by a mechanism compensating the drop of Lo in non-AM plants treated with IAA, which enhanced the proportion of water flowing by the apoplastic water pathway (Figure 1C). Nevertheless, in AM plants, such an increase in apoplastic water flow was not observed when treated with IAA, and the high levels of Lpr in these plants (Figure 1A) may be due to a higher water transport by the own AM fungal hyphae, as was evidenced for ectomycorrhizal fungi (Lehto and Zwiazek, 2011). Interestingly, the same opposite response of apoplastic water flow to the hormonal treatment in AM plants compared to non-AM plants was also found in a previous study

after the external application of SA (Quiroga *et al.*, 2018), suggesting that when these hormones are applied exogenously, the fungus differentially modulates the water flowing by the apoplastic pathway (Bárzana *et al.*, 2012). The possible involvement of nitric oxide in this process was discussed (Quiroga *et al.*, 2018), although it remains to be elucidated yet.

There was not a clear effect of the application of IAA or 6-FI on aquaporin accumulation or in the phosphorylation status of PIP2 aquaporins (Figure 2). This may be due to the short time of application of the hormonal treatment (only 6 hours before harvesting). On the other hand, the antibodies used in this study are not isoform-specific, but general for the whole PIP1 and PIP2 subfamilies, which means that changes in specific aquaporins may be diluted by other isoforms and are, thus, not detected.

Aquaporin activity can be regulated by phosphorylation events in different residues, and previous studies have shown that the phosphorylation of PIP2s at Ser-280 and Ser-283 was related to the regulation of plant hydraulic conductivity (Prado *et al.*, 2013). In this case, phosphorylation generally decreased with the AM colonization under WW conditions, but the differences were not significant under drought stress (Figure 2C, D and E). This is in agreement with previous results on mycorrhizal maize plants (Quiroga *et al.*, 2019b, 2019a), and it may suggest a decrease in the activity of these proteins when the mycorrhizal fungus is present in the roots.

Although the external application of IAA was enough for affecting root hydraulic conductivity levels, it is possible that its concentration was not enough to alter internal plant contents in all tested tissues. Indeed, despite the application of IAA, the levels of this hormone enhanced only in roots of AM plants, especially under drought stress conditions and in leaves of non-AM plants under well-watered conditions (Figure 4A, B). In fact, during drought, root IAA levels in AM plants were generally much higher in all treatments, compared to the other plants. Elevated IAA levels in AM roots were also observed in trifoliate orange during drought stress, and they were related to greater root-hair growth, enhancing drought tolerance (Liu *et al.*, 2018). In the case of leaves, the increase of IAA levels was only observed in non-AM plants during drought stress (Figure 4B). Curiously, although IAA sap levels were not significantly affected by the chemical treatments, isopentenyl-adenine (iP, a naturally occurring cytokinin) sap levels were highly diminished with IAA treatment in AM plants under DS conditions (Figure 3E). This can be easily understood, due to the well-known auxin/cytokinin antagonism (Moubayidin *et al.*, 2009; Rowe *et al.*, 2016).

ABA root concentration was increased in AM plants, regardless of the water treatment (Figure 4C). This is not surprising, as ABA is necessary for an efficient AM colonization, being related to arbuscule formation (Herrera-Medina *et al.*, 2007; Martín-Rodríguez *et al.*, 2011; Miransari *et al.*, 2012). Moreover, it was shown that AM fungi could enhance endogenous ABA levels during plant colonization (Ludwig-Müller, 2010). The same pattern of accumulation occurred for SA levels in roots, being increased by the fungal presence (Figure 4E). SA has been suggested to play a role in AM colonization, although mainly at early stages of the symbiosis (Foo *et al.*, 2013).

It is noteworthy, that plant hormones act in synchrony with each other, and an intricate crosstalk is established in order to regulate plant physiology and development (Munné-Bosch and Müller, 2013). During this interaction, hormones can mutually influence their endogenous contents. In our study, this fact is suggested through the strong correlations among root and sap IAA and root ABA, SA and JA-Ile levels during drought stress (Table 2). In a recent study, ABA and SA alleviated drought stress through the maintenance of membrane stability and leaf water status, and had effects on common metabolic pathways (Li *et al.*, 2017). The fact that these hormones were only correlated during water deficit suggests a tight hormonal control of plant physiology under these conditions, and highlights their mutual relationship.

Conclusions

Here, the effect of IAA on the regulation of root radial water transport of AM plants during well-watered and drought conditions was analysed. Exogenous IAA application affected root hydraulic parameters, mainly Lo, during water deficit conditions, which was decreased in both AM and non-AM plants. The alteration of SA, ABA and JA-Ile levels by the IAA application under drought confirms that water transport in roots is regulated by the combined action of different hormones.

Interestingly, under water deficit conditions apoplastic water flow was differentially regulated in non-AM and AM plants by IAA application, which is in line with previous studies. The effect of IAA on the internal cell component of root water conductivity (Lo) suggests that aquaporins are involved in the IAA-dependent inhibition of this internal cell pathway, although this was not reflected at protein level for the analysed antibodies, and further studies are needed to confirm this hypothesis.

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CHAPTER VI

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

Adapted from:

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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 stablished. 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 analysed. The development of Casparian bands in these roots was also assessed. NaN_3 clearly inhibited osmotic root hydraulic conductivity (Lo). However, the inhibitory effect of sodium azide on Lo was lower in AM plants than in non-AM plants, which together with their higher relative apoplastic water flow values suggest a compensatory mechanism for aquaporin activity inhibition in AM plants, leading to a higher hydrostatic root hydraulic conductivity (Lpr) 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

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; Lpr) 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 contain also the uncharacterized XIPs (X intrinsic proteins) (Gupta and Sankararamakrishnan, 2009), which are not present in maize.

Under adverse environmental conditions, aquaporins appear to have a key role in the regulation of plant water balance (Kapilan *et al.*, 2018), affecting important parameters such as the root hydraulic conductivity (Hachez *et al.*, 2006b). The use of aquaporin inhibitors may provide, thus, information on the relative participation of cell-to-cell and apoplastic paths in the whole-root water uptake. Several inhibitors of aquaporin activity have been used to this purpose, being mercurials the most widely used. Hg causes conformational changes in the protein leading to aquaporin pore blockage and inhibition of water transport (Niemietz and Tyerman, 2002). Nevertheless, Hg has been reported to have a large number of collateral effects that causes indirect inhibition of cellular metabolism (Kamaluddin and Zwiazek, 2001; Niemietz and Tyerman, 2002; Maurel *et al.*, 2008). Sodium azide has also been commonly used to inhibit aquaporin activity and Fitzpatrick and Reid (2009) compared sodium azide (0.5 mM) and butyric acid (10 mM) and observed that sodium azide was more efficient than butyric acid inhibiting the activity of aquaporins. Such an effect was related to the fact that azide has a dual effect: it causes acidification of cytoplasm but also inhibits the phosphorylation process, both effects contributing to close the aquaporin channel (Tournaire-Roux *et al.*, 2003).

Arbuscular mycorrhizal (AM) symbiosis occurring between soil fungi from the subphylum Glomeromycotina and most plant roots enhance water and nutrient uptake from soil due to a vast mycelial network that can access further than the root depletion

zone in the rhizosphere (Smith and Read, 2008). Inside the plant, they provide numerous benefits to plant physiology, the most evident being the stimulation of plant growth and improved mineral nutrition (Azcón-Aguilar and Barea, 2015). In addition, they have the ability to improve plant performance under different abiotic stresses such as drought, salinity, waterlogging or pollution (Lenoir *et al.*, 2016). In the case of water deficit, the enhancement of drought tolerance was reported in different plant species (Ortiz *et al.*, 2015; Chitarra *et al.*, 2016; Ruiz-Lozano *et al.*, 2016; Quiroga *et al.*, 2017). Under these conditions, but also when the plant is well irrigated, AM symbiosis was found to differently regulate root water transport, generally inducing a rise in Lpr (Aroca *et al.*, 2007; Bárzana *et al.*, 2012, 2014, 2015; Quiroga *et al.*, 2017).

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 stablished. Moreover, results by Knipfer and Fricke (2010) in barley emphasize that membranes (and aquaporins) are control points for radial water transport in roots and question the well accepted idea that low-resistance apoplastic pathway of water movement driven by hydrostatic gradients is required in roots to meet the transpirational water demand of the shoot. Furthermore, AM fungi were suggested to modulate the switching between water transport pathways in roots (Bárzana *et al.*, 2012), which would provide higher flexibility to these plants to cope with water stress. This mycorrhizal water regulation could be in part mediated by the regulation of aquaporins, as it was found in several species, including maize (Bárzana *et al.*, 2014; Quiroga *et al.*, 2017). However, this aspect requires a more in deep investigation to elucidate the mechanisms and the conditions under which this could occur.

Therefore, the aim of this investigation was to determine if the AM symbiosis alters the routes of radial water movement in the root of the host plant. We hypothesize that this may be achieved by the regulation of the direct water supply to the plant via fungal hyphae and that this effect may be mediated by changes in the host plant aquaporins activity or amounts, as well as, by changes in apoplastic barriers. Hence, the combined effect of the presence of a mycorrhizal fungus, water deficit and application of the aquaporin inhibitor sodium azide (NaN_3) on radial root water transport and aquaporins accumulation and phosphorylation was studied in maize plants. We also analysed 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.

Materials and methods

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.

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}): 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. 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.

Growing conditions

Plants were grown for eight weeks 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}). 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.

Parameters measured

- 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 ºC for 2 days.

- 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.

- Stomatal conductance

Stomatal conductance (*gs*) 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 manufacter's recommendations. Measurements were taken one day before harvest, thus, before the NaN_3 treatment.

- 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.

- 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.

- Hydrostatic root hydraulic conductivity (Lpr)

Lpr 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. Lpr was determined by dividing L by root dry weight (RDW) and expressed as mg H_2O g RDW^{-1} MPa^{-1} h^{-1}.

- Osmotic root hydraulic conductivity (Lo)

Lo 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 Lo calculation, according to Aroca *et al.* (2007). Lo was calculated as $Lo = Jv/\Delta\Psi$, where *Jv* 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.

- 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 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 µM 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%.

- 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% $FeCl_3$ 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 hemisulfate-stained sections.

- 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 4ºC. 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 KH_2PO_4, 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 4ºC 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.

- Statistical analysis

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

Results

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.

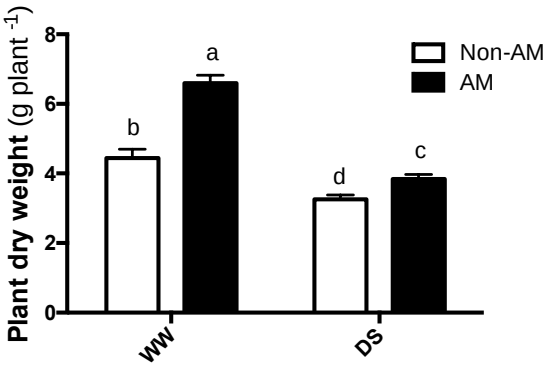

Figure 1. Total dry weight of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Data show the mean ± SE for fifteen plants per treatment. Different letter indicates significant differences between treatments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

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

Table 1. Percentage of mycorrhizal root length, stomatal conductance (*gs*), SPAD values 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 drought stress DS).

	Mycorrhization (%)	*gs* (mmol H_2O m^{-2} s^{-1})	SPAD	ΔFv/Fm'
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 ΔFv/Fm'. 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.

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).

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).

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.

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).

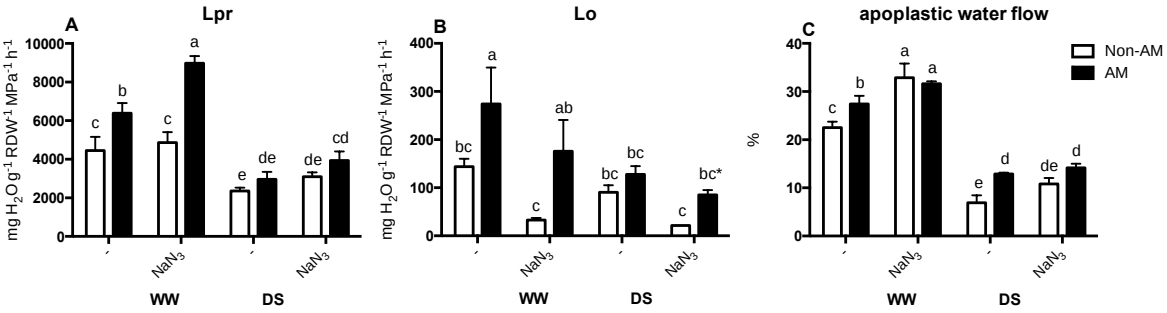

Figure 2. Hydrostatic root hydraulic conductivity (Lpr), osmotic root hydraulic conductivity (Lo) and relative apoplastic water flow of maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). A group of plants from each treatment was treated with NaN_3 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.

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.

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.

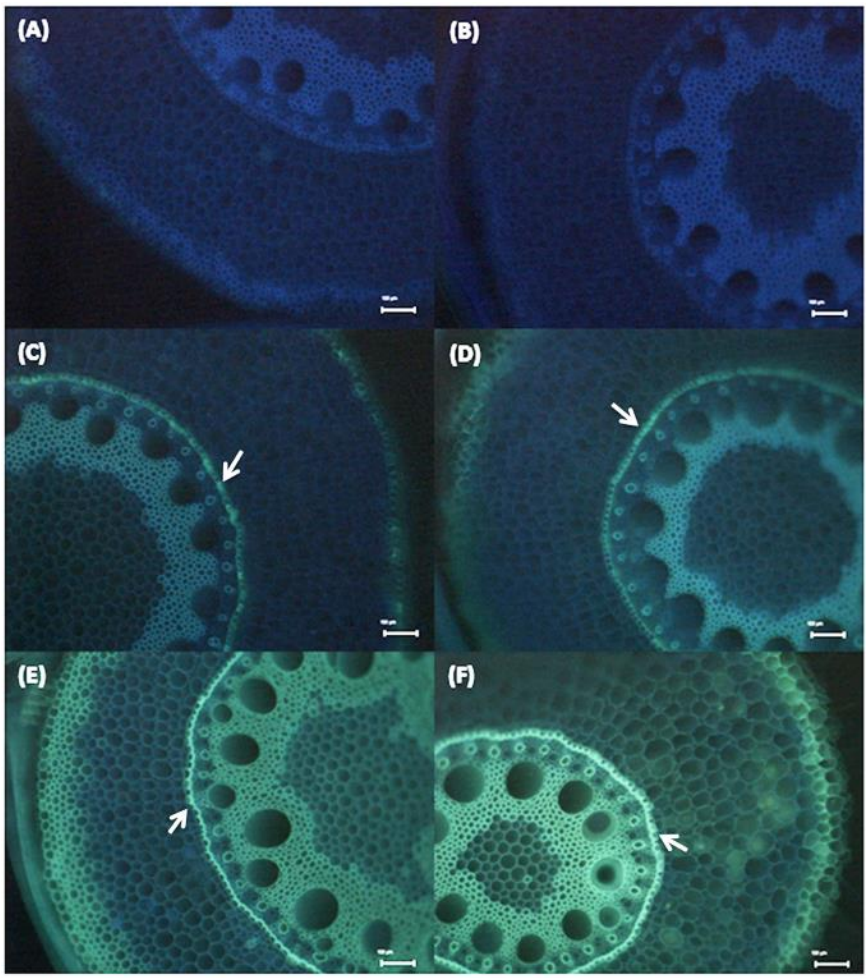

Figure 3. Development of Casparian bands in root sections taken at 50 mm from the root tip. **A-B,** transverse control root sections not stained with berberine hemisulfate. **C-F,** transverse root sections stained with berberine hemisulfate and toluidine blue as described in the materials and methods.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

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.

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 analysed aquaporins or treatments, being more evident when using specific antibodies for different PIP2 isoforms than when using the general antibodies for PIP1 or PIP2.

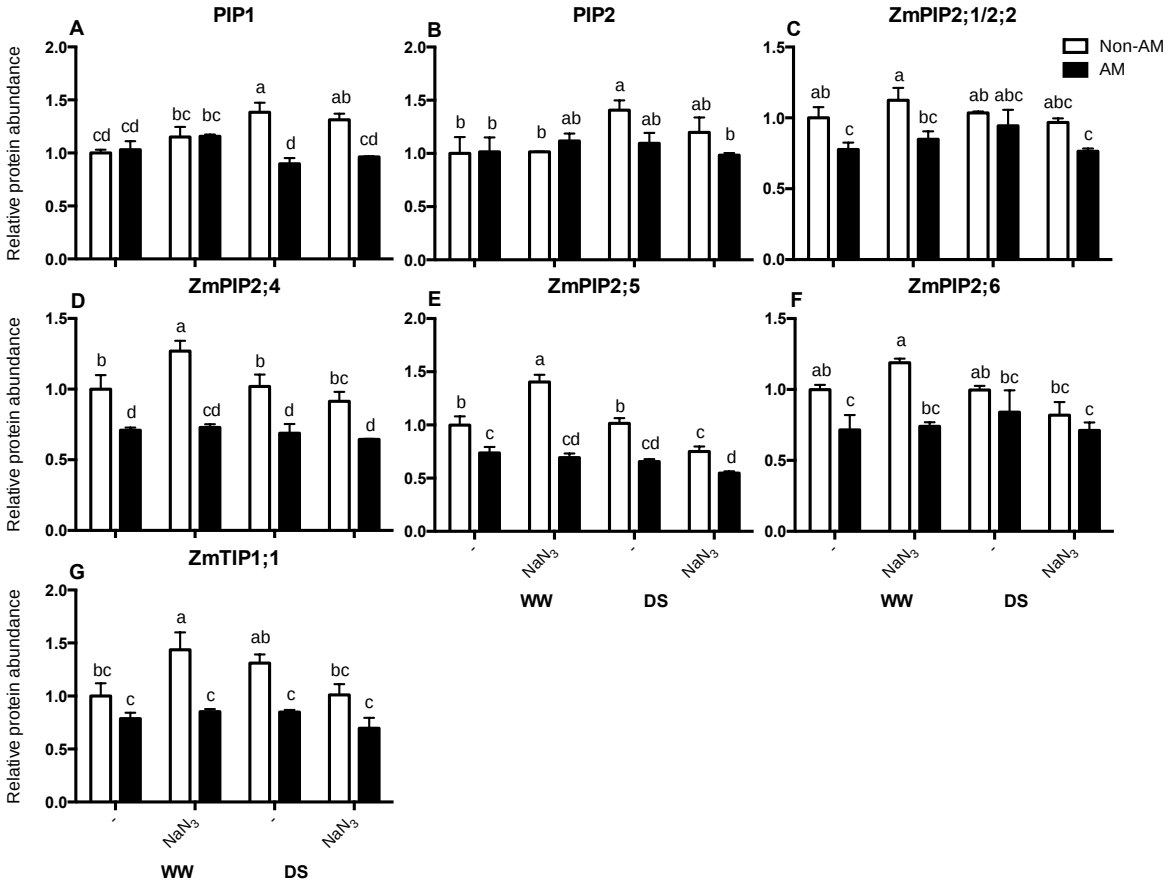

Figure 4. Relative protein abundance in the microsomal fraction of roots from plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). A group of plants from each treatment was treated with NaN_3 for 30 min before 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.

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.

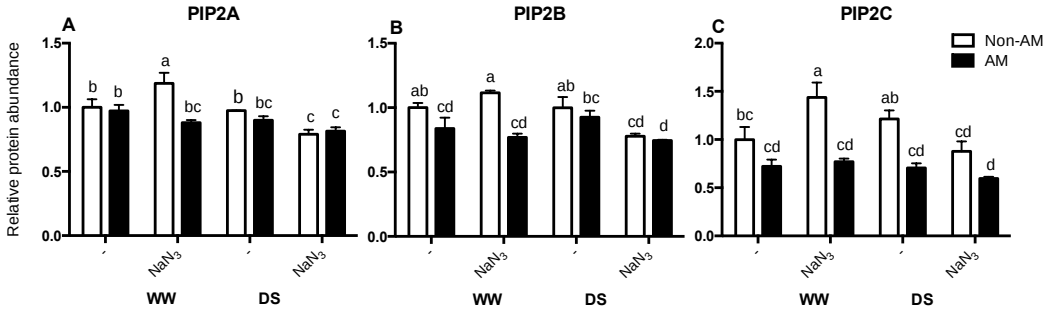

Figure 5. PIP2A (Ph-Ser280), PIP2B (Ph-Ser283) and PIP2C (Ph-Ser280/Ser283) relative protein abundance in the microsomal fraction of roots from plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). A group of plants from each treatment was treated with NaN_3 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.

Discussion

When a mycorrhizal fungus colonizes plant roots, structural changes are produced in cells, affecting root water uptake and transport. The improvement of plant physiology by the AM symbiosis during water-limiting conditions has been extensively studied in different crop species (Ruiz-Lozano *et al.*, 2012; Augé *et al.*, 2015), although studies of their differential effect on root water transport are still elusive. In previous studies, the AM symbiosis was suggested to modulate the switching between apoplastic and cell-to-cell water transport in roots when the water conditions were limiting (Bárzana *et al.*, 2012).

The focus of the present study was to better understand how the arbuscular mycorrhizal symbiosis regulates radial root water transport in maize plants. For that we measured osmotic (Lo) and hydrostatic (Lpr) root hydraulic conductivities and we used sodium azide as inhibitor of aquaporins activity and of cell-to-cell water transport (Tournaire-Roux *et al.*, 2003; Grondin *et al.*, 2016). The development of Casparian bands was also assessed.

The AM symbiosis positively affected plant development and physiology under drought stress

The beneficial effect of mycorrhizal inoculation during drought stress was confirmed by plant growth and physiological data. AM plants exhibited higher dry weight under the two watering conditions. Additionally, AM plants enhanced stomatal conductance (*gs*) both under well-watered or drought stress conditions and the efficiency of photosystem II and chlorophyll content were also significantly higher during water deprivation. The maintenance of a high stomatal conductance allows the plant a better CO_2 uptake for photosynthesis (Sheng *et al.*, 2008). This combined with the improved chlorophyll content and efficiency of photosystem II are surely related with the improved plant growth. These changes have been linked to a higher capacity for CO_2 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 (Vpmax) and CO_2-saturated photosynthetic rate (Vmax) 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

NaN_3 is a metabolic inhibitor that mimics O_2-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, NaN_3 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 (Jozefkowicz *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 (*Jv*) was previously studied (Kamaluddin and Zwiazek, 2001; Tournaire-Roux *et al.*, 2003; Postaire *et al.*, 2010; Sutka *et al.*, 2011). A decrease in *Jv* 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, Lo 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 Lo values were already higher than in non-AM plants. Thus, in AM plants the inhibitory effect of sodium azide on Lo was lower than in non-AM plants, which together with the higher apoplastic water flow values suggest a compensatory mechanism for aquaporin activity inhibition in these plants, leading to a higher Lpr compared to non-AM plants.

The general decrease of Lpr 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 Lpr and Lo values between AM and non-AM plants in response to sodium azide or drought were not statistically significant when all treatments were analysed 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 Lpr, as well as, of the percentage of apoplastic water flow. Lo was not significantly decreased by drought stress, except in AM plants untreated with sodium azide. In general, apoplastic barriers decrease Lpr 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 Lpr and lower or no effect on Lo. 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 Lpr and Lo 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 analyse 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 considered 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 & 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 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 (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.

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, Lpr, Lo 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 Lo 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 Lo 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.

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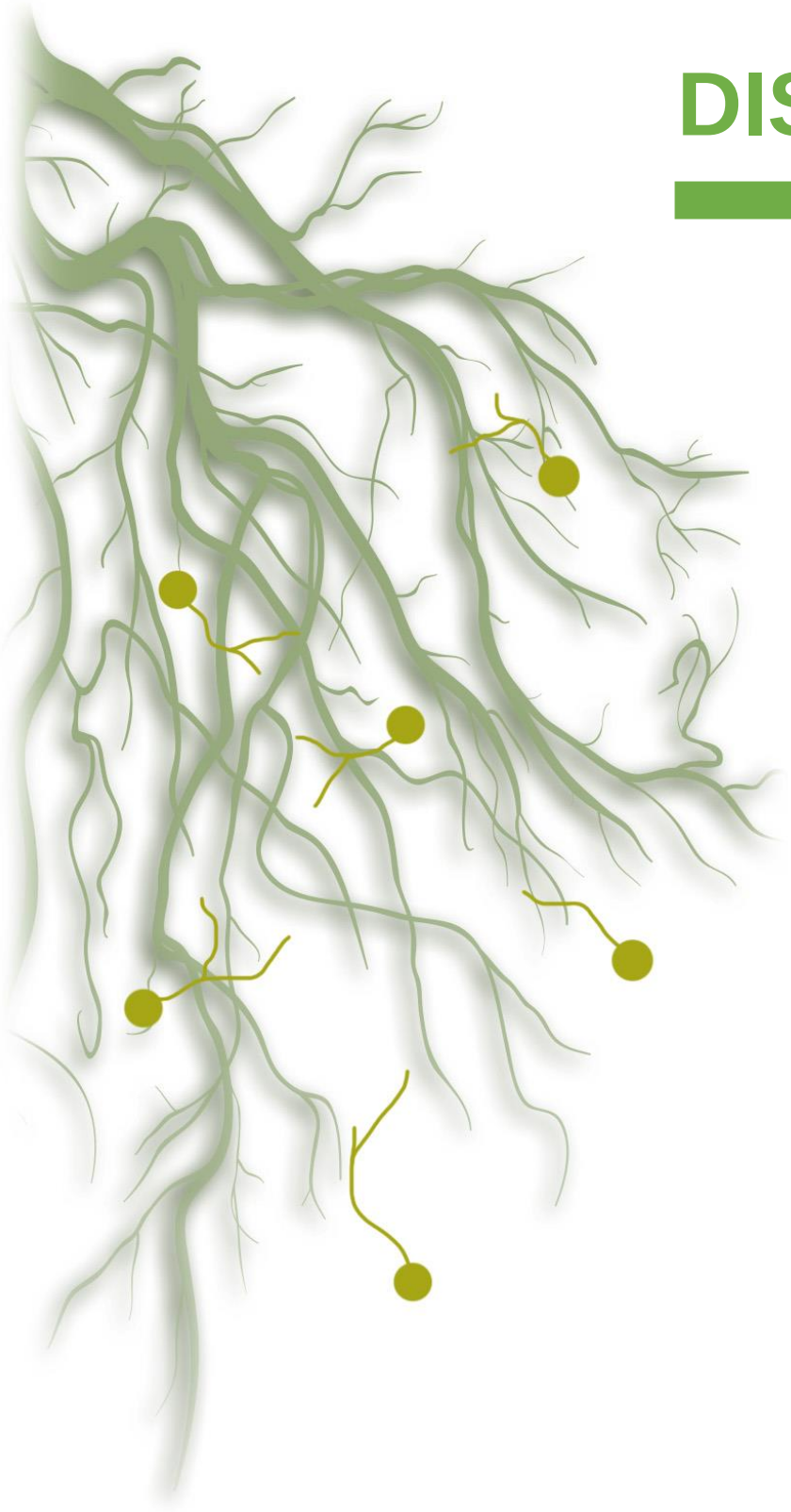
GENERAL DISCUSSION



This section aims to provide a general overview of the investigation undertaken in this PhD Thesis, integrating all the obtained results and discussing the future prospects on this research topic.

Despite their ancient relationship with plant roots, the use of arbuscular mycorrhizal fungi in agriculture as an alternative to conventional fertilization practices is relatively new and mainly motivated by the climate change scenario. Several works in the last few years have been dedicated to understand the AM mechanisms of plant drought tolerance, as the impact of this stress (together with other abiotic factors) on crop production is expected to increase in the next years. However, fundamental knowledge about such mechanism is still limited (Berruti *et al.*, 2016).

This PhD Thesis focuses on understanding how AM fungi regulate host plant aquaporins to enhance plant drought tolerance, by means of the specific combination *R. irregularis-Zea mays* L. It is worthy to highlight that one of the challenges of the use of AM fungi as inoculum is to find the most adequate fungal-plant combination. Generally, mycorrhizal plants can be colonized by most AM isolates or species, but not all provide the same benefits for the plant. Overall, studies indicate that native isolates are more efficient improving plant performance under specific environmental limitations (Estrada *et al.*, 2013). In our case, from the different species analyzed in previous studies, *R. irregularis* strain EEZ58 was the most competent promoting growth under water limited conditions.

The plant species and cultivar considered are also key determinants of the responses to the AM symbiosis. Maize is highly susceptible to drought stress, especially during the reproductive phase, experiencing important decreases in yields under such conditions (Daryanto et al., 2016). In previous studies, it was investigated in which way the AM symbiosis modulates the expression of the whole set of aquaporin genes present in maize, both under optimal water conditions and under different drought stress scenarios. The results showed that the AM symbiosis regulates the expression of a wide number of aquaporin genes in the host plant (16 genes out of 33 existing in maize), comprising members of the different aquaporin subfamilies (Bárzana et al., 2014). The regulation of these genes depends on the watering conditions and on the severity of the drought stress imposed. Moreover, several of these AM-regulated aquaporins where functionally characterized in heterologous systems with *Xenopus laevis* oocytes and by yeast complementation. It was shown that they can transport water, but also other molecules of physiological importance for plant performance under both normal and stress conditions (glycerol, urea, ammonia, boric acid, silicon or hydrogen peroxide).

The obtained results suggested that the effects of the AM symbiosis on plant performance under drought stress are the result of the combined action of the different aquaporins regulated by the AM symbiosis (including PIPs, TIPs, NIPs and SIPs), influencing the transport of water and, most probably, also of other solutes of physiological importance for the plant under drought stress conditions. Hence, results emphasized that additional studies were needed to elucidate the specific function that each aquaporin isoform regulated by the AM symbiosis plays *in planta* in order to enhance the plant tolerance to drought. Thus, the main objective of this Thesis was to identify the key aquaporin isoforms regulated by the AM symbiosis contributing to the better performance of AM plants under drought, and to elucidate their function *in planta*. In the same way, it was necessary to understand if these aquaporins have a key influence on the root water transport capacity of the host plant and if they contribute to the higher flexibility of AM roots for switching between cell-to-cell and apoplastic water pathways.

Most maize genotypes are able to form effective symbioses with AM fungi under drought stress conditions, but the response may differ among them (Boomsma & Vyn, 2008), although some studies point to little relevance of the selection of this combination (Rivera *et al.*, 2007). Contrasting to this last assumption, the first chapter of this Thesis highlights the divergent responses to AM symbiosis of two maize genotypes differing in drought tolerance: PR34G13, a drought-tolerant cultivar, and PR34B39, a drought-sensitive cultivar. It particularly focused on the differential regulation of root aquaporins by the AM symbiosis under well-watered and drought stress conditions and its impact on plant performance. It is clear that the effect of the AM fungus on plant physiology depends on the balance between benefits and costs. Therefore, the tolerant cultivar would need to rely less on the symbiosis than the sensitive one to overcome the stress. Related to this finding, a recent genome-wide association study (GWAS) with 94 wheat genotypes detected differences in the response to mycorrhizae under drought stress conditions. It was suggested that the identification of genomic regions associated with the response to mycorrhization may be used in plant breeding (Lehnert *et al.*, 2018).

The benefits obtained from the AM inoculation in the drought-sensitive maize cultivar were also related to a higher and broader regulation of root aquaporins, which is the main topic of this work. Indeed, in this study, the 16 maize aquaporins previously shown to be regulated by the AM symbiosis under different drought scenarios (Bárzana et al., 2014) were analysed to check a possible differential regulation by the AM symbiosis in the two maize cultivars with contrasting drought sensitivity. Results showed that there were differences in the expression of several of the studied aquaporins between the drought-sensitive and the drought-tolerant genotypes. In the sensitive genotype, a general

down-regulation of aquaporins by the AM symbiosis, under drought and/or well-watered conditions (*ZmPIP1;1, ZmPIP1;3, ZmPIP1;4, ZmPIP1;6, ZmPIP2;2, ZmPIP2;4, ZmTIP1;1* and *ZmTIP2;3*) was featured. However, AM regulation of aquaporins in the drought-tolerant genotype was weaker, and only three aquaporins (*ZmPIP1;6, ZmPIP2;2* and *ZmTIP4;1*) were found to be altered. It is noteworthy that these three aquaporins were even up-regulated under well-watered conditions, which is an opposite behaviour than in the sensitive genotype, similar to results reported by Vinnakota et al. (2016) or by Liu et al. (2013) in two rice varieties and two *Malus* species with contrasting drought sensitivity. The function of plant aquaporins may explain differences in root water uptake and transport between AM and non-AM plants, as well as differences in the solutes that are transported by these proteins, which consequently may be related to resistance to drought stress (Chaumont & Tyerman, 2014). Thus, the broader and contrasting regulation of these maize aquaporins by the AM symbiosis in the drought-sensitive than the drought-tolerant cultivar suggests a role of these aquaporins in water homeostasis or in the transport of solutes of physiological importance in both cultivars under drought stress conditions, which may be important for the AM-induced tolerance to drought stress. From this study, eight maize aquaporins were selected for being regulated by the AM symbiosis or for being putative transporters of solutes with relevance in drought stress tolerance. These aquaporins were analyzed in the subsequent experiments.

The objective of the second chapter was to go further on the mechanisms of AM drought tolerance and to understand if the main effect of the regulation of plant aquaporins was the improvement of root cell water transport capacity. Results demonstrated that the AM symbiosis improved hydraulic conductivity of root cortical cells. 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 Pf levels observed in non-stressed plants, while these levels declined drastically in the absence of the AM fungus. Interestingly, Pf values higher than 12 $\mu m\ s^{-1}$ were found only in protoplasts extracted from AM plants, revealing the higher water permeability of AM root cells, as compared to non-AM ones. The mRNA expression levels of two aquaporins with high water transport capacity, *ZmPIP2;2* and *ZmPIP2;6* and the fungal aquaporin *GintAQPF2* showed similar trends compared with Pf and Lpc values. However, differences were not enough to explain the higher permeability of AM root cells. The use of whole roots could mask cell-type specific differences in mRNA or protein levels, as those roots have different cells and different stages of arbuscule development. Thus, mRNA abundance of the analyzed aquaporins should be verified in cortex cells to better understand the AM regulation. In any case, AM plants under water deficit increased the

phosphorylation status of PIP2s, which is linked to a higher activity of their water channels, and hence, to the regulation of hydraulic conductivity in plants (Prado *et al.*, 2013).

The AM effect on root cells water permeability was also related to a better performance of the shoots, thanks to an increased PEPc activity and CO_2-saturated photosynthetic rate. These results support the idea of the AM symbiosis providing systemic benefits for plant drought tolerance.

Taking into account that the AM symbiosis regulates a wide number of plant aquaporins under drought stress (Bárzana et al., 2014) and that besides water, some of these aquaporins can transport other substrates such as H_2O_2, boron, silicon, ammonium, urea, glycerol, O_2, or CO_2, it is possible that the AM-regulated aquaporins may be involved in the plant mobilization of these compounds, contributing in this way to the enhanced drought tolerance of AM plants. In this context, the third chapter of this Thesis constitutes a first approach to investigate the possible involvement of solutes as substrates of aquaporins and to elucidate if maize aquaporins regulated by the AM symbiosis are involved in the boron transport and homeostasis *in planta* under water deficit conditions (Yoshinari & Takano, 2017; Shireen *et al.*, 2018). For that, AM and non-AM maize plants were cultivated under different boron levels in the growing substrate (0, 25 μM or 100 μM) and subjected to drought stress or maintained under well-watered conditions. The obtained results do not support the idea that the AM symbiosis is affecting the transport of this solute through regulation of the aquaporins with B transport capacity. The regulation of B transport would be probably more related to the movement of water in roots, which would concomitantly increase the passive transport of this micronutrient. However, different approaches should be considered to confirm this hypothesis, such as specific experiments under low and high air relative humidity in order to modify transpiration stream and Lpr (Calvo-Polanco et al., 2017) and determine the effects of these changes in Lpr on plant boron mobilization; or with the use of stable B isotopes (Macho-Rivero *et al.*, 2018) The existence of other uncharacterized B transporters in maize was already suggested (Matthes *et al.*, 2018), and this fact could be related to the lack of response observed in this study. Nevertheless, the regulation of mRNA levels of some aquaporins (*ZmPIP2;2*, *ZmTIP2;3* and *ZmNIP1;1*) and boron efflux transporters (*RTE*, *RTE2* and *RTE3*) by different B concentrations mainly in non-AM plants, points out a role of all these proteins in the *in planta* B transport. In the case of *RTE* genes, the results confirmed their previously proposed role.

As mentioned above, besides boron, there are other aquaporin substrates whose transport could be affected by the AM symbiosis. This research line still needs to be properly addressed in future studies.

The results obtained in the first three chapters of this Thesis suggested that rather than the transport of other solutes, the AM symbiosis would be mainly modifying water transport in the roots through aquaporins. According to this, subsequent experiments were more focused on the study of the modification of root water transport capacity by the AM symbiosis. Thus, chapters four and five studied the implication of some phytohormones in the AM regulation of aquaporins and radial water transport under water deficit conditions. Results previous to this Thesis suggested that the presence of the AM fungus in the roots of the host plant could modulate the switching between cell-to-cell and apoplastic water transport pathways. This fact was understood as a way to provide higher flexibility in the response of AM plants to water shortage according to shoot demands (Bárzana *et al.*, 2012). Additionally, evidences also suggested that the modulation of different phytohormones in the host plant could contribute to the switching between root water transport pathways mediated by the AM fungus (Calvo-Polanco *et al.*, 2014; Sánchez-Romera *et al.*, 2014). Among the different phytohormones, SA was selected as it was found to regulate PIP aquaporins and Lpr by a ROS-mediated mechanism that induced membrane internalization of those proteins (Boursiac *et al.*, 2008). Similarly, IAA reduced Lpr at cell and whole-organ levels, and repressed aquaporins through an auxin response factor (ARF7) in *Arabidopsis* plants (Péret *et al.*, 2012).

In chapter four, exogenous SA application altered root hydraulic parameters, decreasing Lpr and Lo under drought stress. The effect of SA on the osmotic component of root water conductivity (Lo) suggested that aquaporins could be involved in this regulation, as previously stated by Boursiac et al. (2008). Unfortunately, correlation of these parameters with aquaporin gene expression was not evident and it was hypothesized that SA-responsive elements should be analyzed in the promoter regions of maize aquaporin genes in future studies. In any case, a correlation of ZmTIP1;1 and ZmPIP2;4 protein levels with Lo under water deficit suggested their role in water transport under the experimental conditions. Nevertheless, this finding needs further studies to confirm the hypothesis. In addition, SA application differently modulated the percentage of water flowing by the apoplastic pathway under the imposed stress, decreasing its contribution to total root water flow in AM plants and increasing it in non-AM plants. This differential effect of SA may be mediated by altered nitric oxide (NO) content in these plants, since it has been recently shown that SA-induced NO regulates maize water content and hydraulic conductivity under drought (Shan and Wang, 2017). Moreover, Sánchez-Romera et al. (2017) have suggested that NO favours apoplastic water pathway inside roots. Thus, a higher NO content in non-AM plants than in AM ones could explain the SA-induced enhancement of apoplastic water flow in non-AM plants and the opposite

effect in AM plants. The possible role of NO in this effect deserves, thus, future investigations too. The differential effect on apoplastic water flow was consistent with a higher plasticity of AM roots to switch between water transport pathways, suggested by Bárzana et al. (2012).

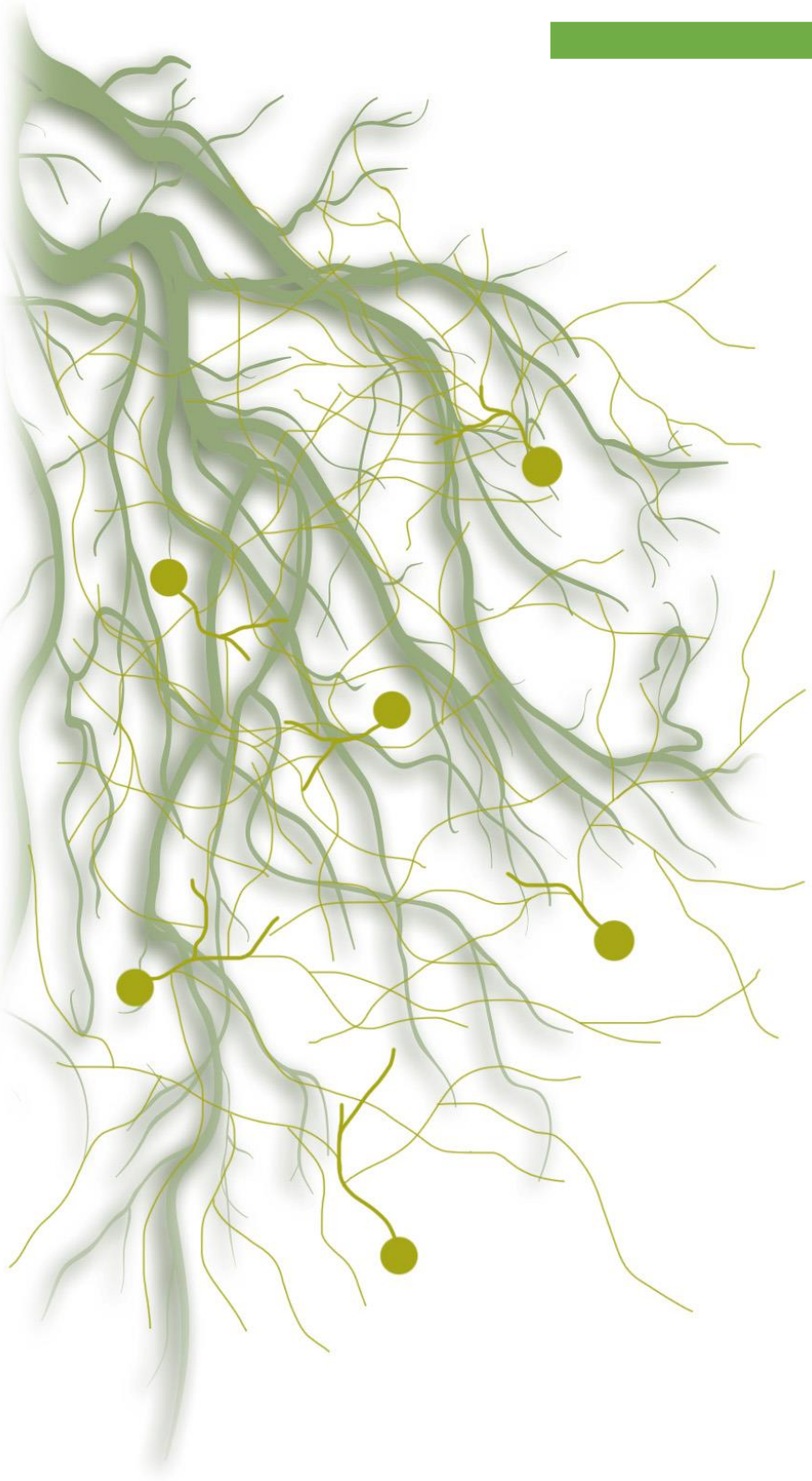
In chapter five the same experimental approach than in the previous chapter was followed, using IAA instead of SA. IAA affected root hydraulic parameters (mainly Lo) during water stress conditions, similarly to SA and in accordance with the study from Péret et al. (2012). The function of IAA in drought stress tolerance has not been studied in detail, but different studies point to a role, especially in the regulation of root arquitecture during osmotic stress (Kazan, 2013). The exogenous IAA application affected root hydraulic parameters, mainly Lo, during water deficit conditions, which was decreased in both AM and non-AM plants. This effect of IAA on the internal cell component of root water conductivity (Lo) suggests that aquaporins are involved in the IAA-dependent inhibition of this internal cell pathway. Interestingly, IAA application also regulated differently apoplastic water flow in non-AM plants and AM plants under water deficit, confirming our previous hypothesis. In both SA and IAA experiments exogenous application of the hormone altered endogenous levels of other phytohormones (such as ABA, SA, JA or JA-Ile). This reveals the complex network that regulates water transport in roots. For instance, it has been shown that MeJA is involved in the regulation of phosphorylation state at Ser-280 in PIP2 aquaporins and that this could be its main function in the regulation of root hydraulic properties (Sánchez-Romera et al., 2014). In addition, ABA has been shown to enhance root hydraulic conductivity in several plant species and the effects of MeJA on Lpr were in part regulated by ABA and in part independent of ABA (Sánchez-Romera et al., 2014). In addition, hormonal balance is also altered by the AM symbiosis, which has been generally associated with enhanced drought tolerance (Pozo *et al.*, 2015), adding complexity to the study.

Finally, from the obtained results an additional experiment was proposed that was addresed in chapter six. This study aimed 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. For that we measured osmotic (Lo) and hydrostatic (Lpr) root hydraulic conductivities and we used sodium azide (NaN_3) as inhibitor of aquaporins activity and of cell-to-cell water transport, in order to understand the role of the AM symbiosis on plant aquaporins activity. Additionally, the study constitutes a first approach to elucidate the role of the AM fungus on the modification of apoplastic barriers. Once more, it was confirmed that the fungus modifies water transport in roots, increasing all hydraulic parameters compared to non-AM

plants. NaN_3 inhibition of Lo was lower in AM plants than in non-AM plants. The former plants also had higher relative apoplastic water flow values, suggesting a compensatory mechanism in these plants for aquaporin activity inhibition and leading to higher Lpr values as compared to non-AM plants. The lower inhibition of Lo in AM plants seems to be related to the regulation of aquaporins activity through posttranslational mechanisms. Surprisingly, Casparian bands increased with drought but also in AM plants, although this did not decrease water flow values in those plants. There is the possibility that apoplastic barriers of AM roots have a different composition, which could explain the different water transport of these roots. It is known that the formation of apoplastic barriers is highly regulated by environmental conditions. This plasticity could be a strategy to regulate water and nutrient transport depending on the circumstances (Barberon 2017). For this reason, the composition of these barriers after AM colonization deserves further attention.

Overall, the work carried out in this PhD Thesis increases the general knowledge about the plant drought tolerance induced by the AM symbiosis. It is evidenced that the AM symbiosis has a role in the modulation of cell water permeability in roots, which is probably related to aquaporins activity. Moreover, the higher flexibility of AM roots to modulate water transport is confirmed in independent experiments, which is translated into the better performance of these plants under water scarcity. In any case, future studies should deal with the exact role of the different phytohormones involved in this effect, as well as, with a possible change in composition of apoplastic barriers induced by the AM symbiosis. Moreover, it is also necessary to elucidate whether the effect of the AM symbiosis on the plant aquaporins and the water permeability of the root cell membranes is systemic or localized only in the cells colonized by the AM fungus. Thus, laser microdissection technique (Giovannetti et al., 2012; Belmondo et al., 2016) could be used to isolate maize root cells colonized by the AM fungus or non-colonized for subsequent analysis of aquaporins gene expression (RT-qPCR) and for studies of aquaporins immunolocalization in these cells by confocal microscopy.

CONCLUSIONS



1. The beneficial effects of the AM symbiosis on plant performance under drought stress are genotype-dependent. Thus, when plants were subjected to drought stress the AM symbiosis induced a higher improvement of physiological parameters in drought-sensitive plants than in drought-tolerant plants. Moreover, the broader and contrasting regulation of some plant aquaporins by the AM symbiosis in the drought-sensitive than in the drought-tolerant cultivar suggests a key role *ZmPIP1;1*, *ZmPIP1;3*, *ZmPIP2;2*, *ZmPIP2;4*, *ZmTIP1;1*, *ZmTIP2;3*, *ZmTIP4;1* and *ZmNIP2;1* in the AM-induced drought stress tolerance.

2. The AM symbiosis enhances root cell water permeability under water deficit, increasing root cell water conductivity (Lpc) and osmotic water permeability coefficient (Pf) in cortical cells. Under these conditions, *ZmPIP2;2*, *ZmPIP2;6* and the fungal aquaporin *GintAQPF2* were induced in AM plants, as well as, the phosphorylation status of PIP2 aquaporins, which is related to a higher activity of their water channels. Altogether, this explains the higher water permeability of AM root cells. AM plants also display higher photosynthetic capacity thanks to an increased PEPc activity and CO_2-saturated photosynthetic rate, evidencing that the better performance of AM root cells in water transport is connected to the shoot physiological performance in terms of photosynthetic capacity.

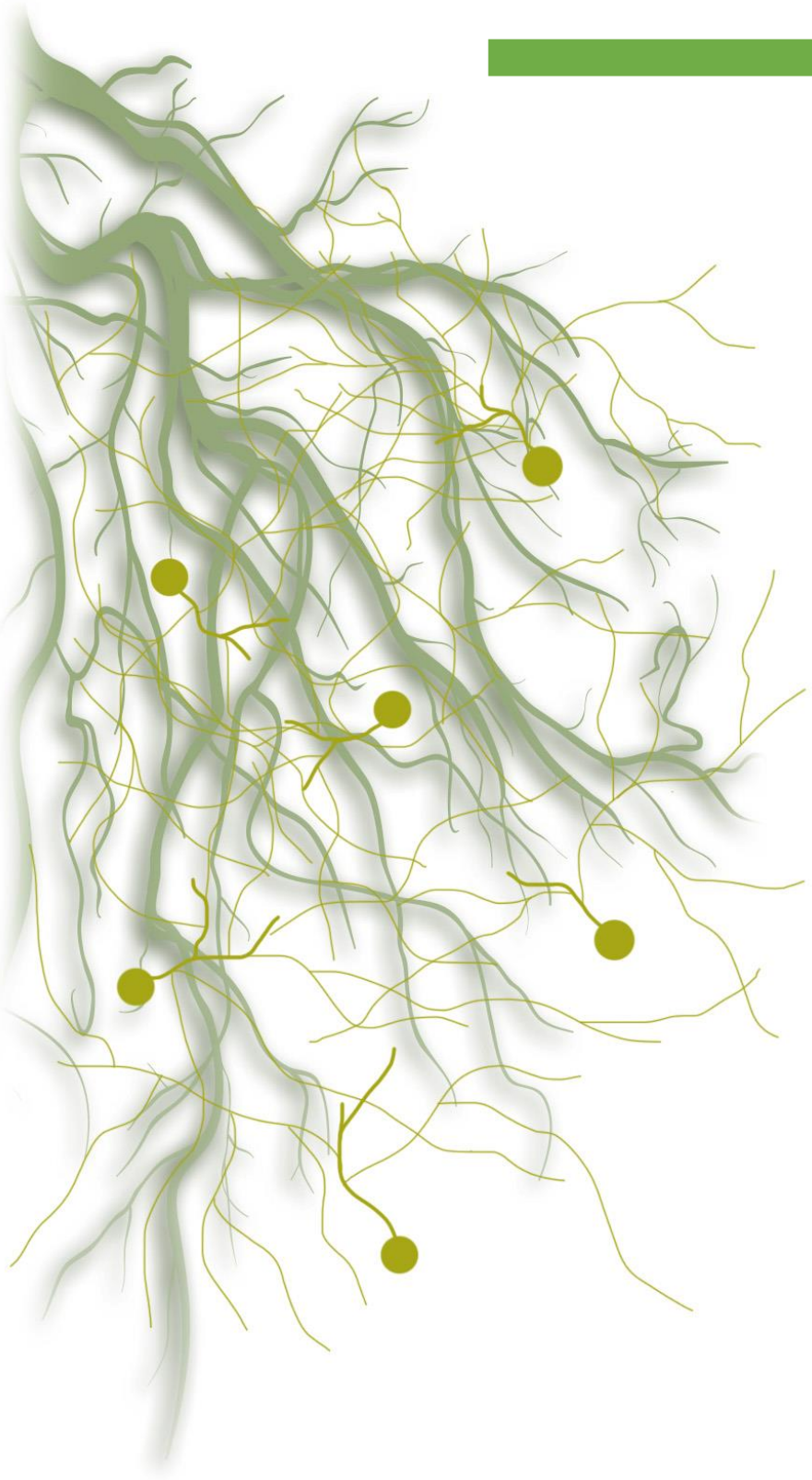
3. A general down-regulation of aquaporins and B transporters occurs in AM plants, suggesting that other mechanisms contribute to B homeostasis in these plants, probably more related to the enhancement of water transport, which would concomitantly increase the passive transport of this micronutrient. Some aquaporins (*ZmPIP2;2*, *ZmTIP2;3* and *ZmNIP1;1*) and boron efflux transporters (*RTE*, *RTE2* and *RTE3*) are transcriptionally regulated by B levels, which confirms their previously proposed role in B transport.

4. Under drought stress the AM symbiosis increases hydrostatic root hydraulic conductivity (Lpr) and osmotic root hydraulic conductivity (Lo). The exogenous application of SA modulates root hydraulic parameters, decreasing Lpr and Lo under drought. This fact is probably due to the fine regulation of the aquaporins ZmPIP2;4 and ZmTIP1;1. Moreover, under drought stress SA differentially modulates the percentage of water flowing by the apoplastic pathway, decreasing its contribution to total root water flow in AM plants and increasing it in non-AM plants.

5. Under water deficit the exogenous IAA application affects root hydraulic parameters, especially apoplastic water flow that is differentially regulated in AM and non-AM plants, and Lo, which is decreased in both AM and non-AM plants. The effect of IAA on the internal cell component of root water conductivity (Lo) suggests that aquaporins are involved in the IAA-dependent inhibition of this water pathway, although the specific isoforms involved could not be identified. In any case, the IAA regulation of root water transport seems to be mediated by the combined action of several phytohormones, such as ABA, SA and JA-Ile.

6. The AM symbiosis modifies the radial root water transport, enhancing Lpr, Lo and the percentage of apoplastic water flow. The application of sodium azide as inhibitor of aquaporins activity decreased water flow through the cell-to-cell pathway (Lo) to a lower extent in AM plants than in non-AM plants. AM plants also had higher relative apoplastic water flow values, suggesting a compensatory mechanism in these plants for aquaporin activity inhibition and leading to higher Lpr values. The lower inhibition of Lo in AM plants seems to be related to posttranslational mechanisms regulating aquaporins activity.

CONCLUSIONES



1. El beneficio de la simbiosis MA sobre el rendimiento de la planta bajo condiciones de estrés por sequía es dependiente del genotipo. Así, cuando las plantas se sometieron a sequía la simbiosis MA indujo una mayor mejora de los parámetros fisiológicos en las plantas sensibles a sequía que en las tolerantes. Además, la regulación más amplia y en sentido opuesto de algunas acuaporinas por parte de la simbiosis MA en aquellas plantas sensibles a sequía que en las tolerantes, sugiere un papel clave de *ZmPIP1;1*, *ZmPIP1;3*, *ZmPIP2;2*, *ZmPIP2;4*, *ZmTIP1;1*, *ZmTIP2;3*, *ZmTIP4;1* y *ZmNIP2;1* en la tolerancia a sequía inducia por MA.

2. Bajo condiciones de déficit hídrico, la simbiosis MA aumenta la permeabilidad hídrica de las células corticales de la raíz, incrementando su conductividad hidráulica (Lpc) y su coeficiente de permeabilidad hídrica (Pf). Bajo esas condiciones *ZmPIP2;2*, *ZmPIP2;6* y la acuaporina fúngica *GintAQPF2* resultaron inducidas en plantas MA, así como el estado de fosforilación de las acuaporinas PIP2s, lo que está relacionado con una mayor actividad como canales de agua. En conjunto, todo ello explica la mayor permeabilidad hídrica de las células radicales MA. Las plantas MA también mostraron una mayor capacidad fotosintética gracias a un aumento de la actividad PEPc y de la tasa de fotosíntesis bajo saturación de CO_2, evidenciando que el mayor rendimiento de las células MA de la raíz en el transporte de agua está relacionado con un mayor rendimiento fisiológico de la parte aérea en términos de capacidad fotosintética.

3. En las plantas MA se observó una inhibición general de acuaporinas y de transportadores de B, lo que sugiere que la homeostasis de B en estas plantas está regulada por otros mecanismos, probablemente más relacionados con el aumento del transporte de agua, que a su vez incrementaría el transporte pasivo de este micronutriente. Algunas acuaporinas (*ZmPIP2;2*, *ZmTIP2;3* y *ZmNIP1;1*) y transportadores de boro (*RTE*, *RTE2* y RTE3) están regulados transcripcionalmente por los niveles de B, lo que confirma su papel en el transporte de B, propuesto previamente.

4. Bajo condiciones de sequía, la simbiosis MA incrementa la conductividad hidráulica hidrostática (Lpr) y la conductividad hidráulica osmótica (Lo). La aplicación exógena de SA modula los parámetros hídricos, disminuyendo Lpr y Lo bajo sequía. Este hecho se debe, probablemente, a una regulación ajustada de las acuaporinas ZmPIP2;4 y ZmTIP1;1. Además, en condiciones de sequía, el SA modula diferencialmente el porcentaje de agua que fluye por la ruta apoplástica,

disminuyendo este porcentaje en plantas MA e incrementándolo en las plantas no MA.

5. Bajo condiciones de déficit hídrico, la aplicación exógena de IAA afecta a los parámetros hídricos, especialmente al flujo de agua apoplástico, que es regulado diferencialmente en plantas MA y no MA, y a Lo, que disminuye en ambos tipos de plantas. El efecto del IAA sobre el componente interno celular de la conductividad hidráulica (Lo) sugiere que las acuaporinas están implicadas en la inhibición dependiente de IAA de esta ruta, aunque las isoformas específicas no han podido ser identificadas. En cualquier caso, el efecto del IAA en la regulación del transporte de agua en la raíz parece mediado por la acción combinada de varias fitohormonas como ABA, SA y JA-Ile.

6. LA simbiosis MA modifica el transporte radial de agua en la raíz, incrementando Lpr, Lo y el porcentaje de agua apoplástica. La aplicación de azida sódica como inhibidor de la actividad de las acuaporinas disminuyó en menor medida el flujo de agua por la ruta célula a célula (Lo) en las plantas MA que en las no MA. Las plantas MA también mostraron valores más altos de flujo de agua apoplástico, lo que sugiere un mecanismo de compensación en estas plantas frente a la inhibición de la actividad de las acuaporinas, que se traduce en valores más altos de Lpr. La menor inhibición de Lo en plantas MA parece deberse a mecanismos postranscripcionales de regulación de la actividad de las acuaporinas.

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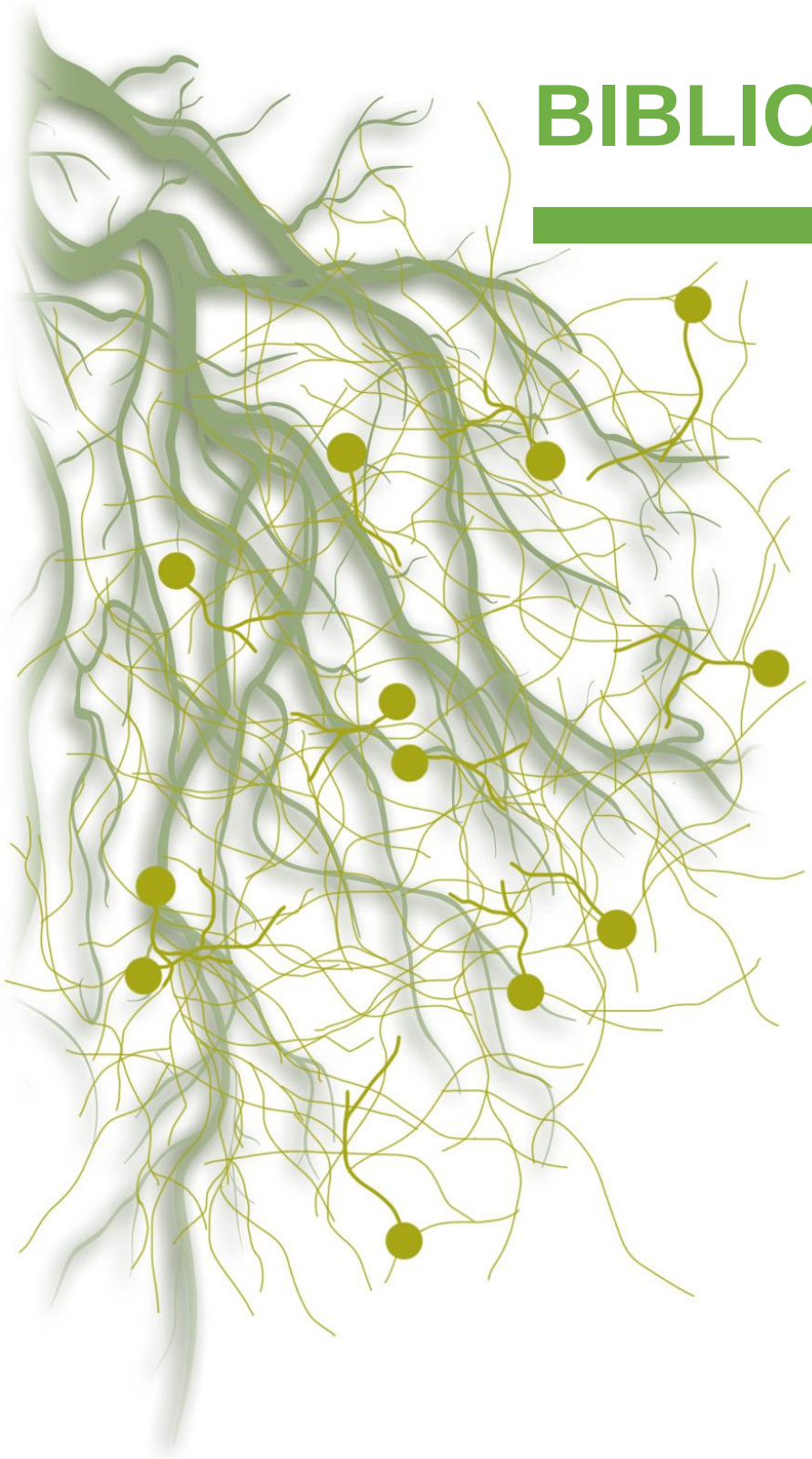

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