Regulación de las Características Hidráulicas de la Raíz por Jasmonato de Metilo, Especies de Nitrógeno Reactivo y Micorrizas Arbusculares.

Beatriz Sánchez Romera

Tesis Doctoral

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Regulation of Root Hydraulic Properties by Methyl Jasmonate, Reactive Nitrogen Species and Arbuscular Mycorrhizae.

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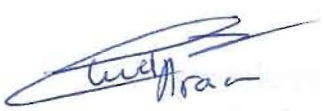
Fdo.: Beatriz Sánchez Romera

Licenciada en Biología por la Universidad de Granada

Granada a 24 de Junio de 2014

V°B° Directores de la Tesis



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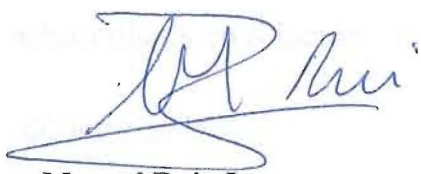

Fdo.: **Juan Manuel Ruiz-Lozano**

Investigador Científico del CSIC

Estación Experimental del Zaidín

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El doctorando Beatriz Sánchez Romera y los directores de la tesis, Ricardo Aroca Álvarez y Juan Manuel Ruiz Lozano. Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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Ricardo Aroca Álvarez **Juan Manuel Ruiz Lozano**

Fdo.:

Doctorando:

Beatriz Sánchez Romera

Fdo.:

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Although the thesis is entirely written in English, I have decided to write the acknowledgments in Spanish to express my gratitude wholeheartedly.

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P.D. Espero que nuestros caminos se vuelvan a cruzar, aunque no sé ni donde ni cuando!!!

‘‘Nadie hable mal del día
hasta que la noche llegue
yo he visto mañanas tristes
tener las tardes alegres’’

(E. Morente)

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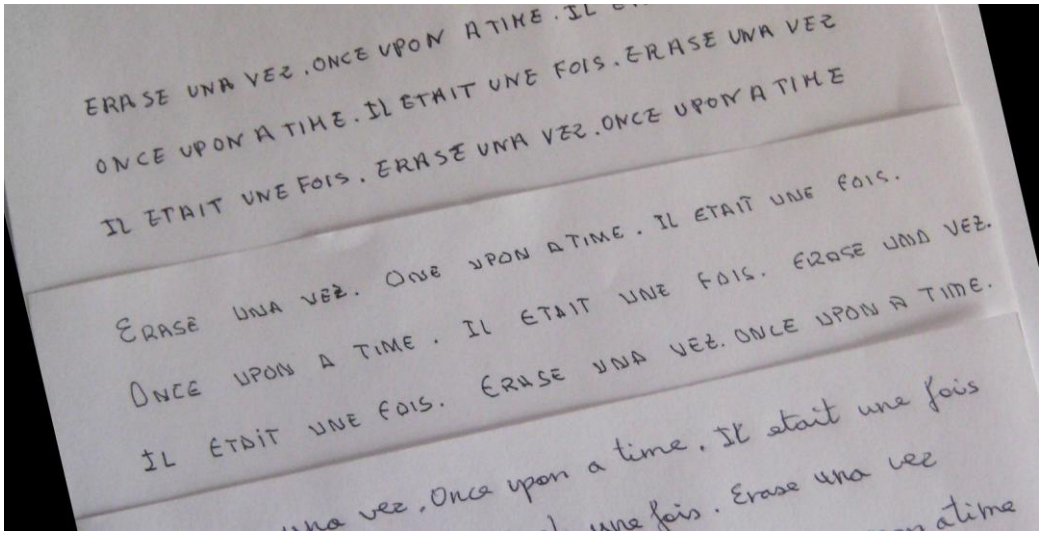
Introduction



Introduction

This work is focused on the study of root hydraulic properties, dealing with the changes that occur at the molecular and physiological levels under both well-watered and drought conditions. Plant response against stress situations is mediated by a dialogue between molecules, which leads to the activation of many stress-responsive genes, activating proteins or relocating certain molecules, in order to withstand the stress (Espunya *et al.* 2012, Sohrabi *et al.* 2012). Jasmonic acid (JA) and nitric oxide (NO) are among these molecules. Both are known to be involved in the defense responses of plants against biotic stresses (Creelman & Mullet 1995, Neill *et al.* 2008, Santino *et al.* 2013) and are known to participate in the defense response against abiotic stresses, such as drought, salinity or chilling (de Ollas *et al.* 2013, Fan,Du & Guo 2013, Ismail,Riemann & Nick 2012, Lee *et al.* 1996b, Liao *et al.* 2012). However, it is still not well known what role they play in regulating hydraulic properties of roots, or how their action may be affected by drought.

On the other hand, it is known that arbuscular mycorrhizal (AM) fungi provide numerous benefits to their host plants, both under well-watered and under drought conditions (Abbaspour,Saeidi-Sar & Afshari 2011, Al-Karaki 1998, Lee *et al.* 2012a, Lee *et al.* 2012b, Porcel & Ruiz-Lozano 2004). Although AM symbiosis have been extensively studied, the information concerning the effects of JA and NO on AM symbiosis is still not clear and the effects of these molecules on root hydraulic properties of AM plants have not been addressed by the earlier studies.

In the following pages, the functions of both molecules and its role against drought stress will be explained. Also several aspects of AM symbiosis and the benefits of this symbiosis to plants will be described in depth. Finally, it will be described how water moves into the roots and how different proteins and other molecules known to-date participate in the processes of water transport.

Jasmonic Acid (JA)

Although hormones play an important role in several plants physiological processes, in this section the functions of JA known to-date will be described. The focus will be on the role of JA under abiotic stresses and how it interacts with other molecules.

JA Biosynthesis

JA is a plant hormone that is synthesized through the octadecanoid pathway. Early studies described its methyl ester in the essential oils of *Jasminum grandiflorum* (Demole,Lederer & Mercier 1962). Since then, numerous studies have been conducted to understand the role of JA in plant development and in plant defense against stress conditions.

Although the leaves are the main place where the synthesis of JA occurs (Creelman & Mullet 1997), it has been demonstrated that its biosynthesis can take place in roots (Pedranzani *et al.* 2003) since expression of genes encoding enzymes involved in its biosynthesis have been found in root tissues (Leon-Morcillo *et al.* 2012a, Lopez-Raez *et al.* 2010).

The first steps in the JA biosynthesis take place in the chloroplast (Fig.1), where α- linoleic acid is released from galactolipids and then is oxidized by 13-lipoxygenase (13-LOX), forming a 13-hydroperoxy, linoleic acid derivative (13-HPOT) (Bannenberg *et al.* 2009, Bell & Mullet 1993, Melan *et al.* 1993, Vick & Zimmerman 1983). This compound is transformed into allene oxide, by means of the enzyme allene oxide syntase (AOS). The last step that takes place in the involves the oxide allene cyclase (AOC) which is responsible for transforming allene oxide into (9S, 13)-cis-(+)-oxophytodienoic acid (OPDA)(Ziegler *et al.* 2000).

OPDA is transported to the peroxisome (Fig.I1), where it is oxidized to 3-oxo-2-(2 '(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC: 8) by the enzyme OPDA reductase (OPR)(Schaller & Weiler 1997). The second step in the peroxisome is carried out by fatty acyl-CoA oxidase (ACX) producing OPC:6 (Schilmiller,Koo & Howe 2007), which in turn is oxidized by multifunctional protein (MFP) to form OPC:4Coa. The last step in the peroxisome is a thiolation by keto-acyl-CoA-thiolase (KAT), being JA-CoA as the end product (Castillo *et al.* 2008).

This product is released into the cytosol, where it can be methylated by jasmonic acid methyl transferase (JMT) and form MeJA, or be conjugated to amino acids such as isoleucine to form JA-Ile through enzyme jasmonate resistant 1 (JAR). For more information see Santino *et al.* (2013) and Wasternack *et al.* (2007, 2013). Currently, it is known that JA and MeJA are the only precursors of the bioactive molecule, which is JA-Ile (Fonseca *et al.* 2009, Staswick & Tiryaki 2004, Suza *et al.* 2010).

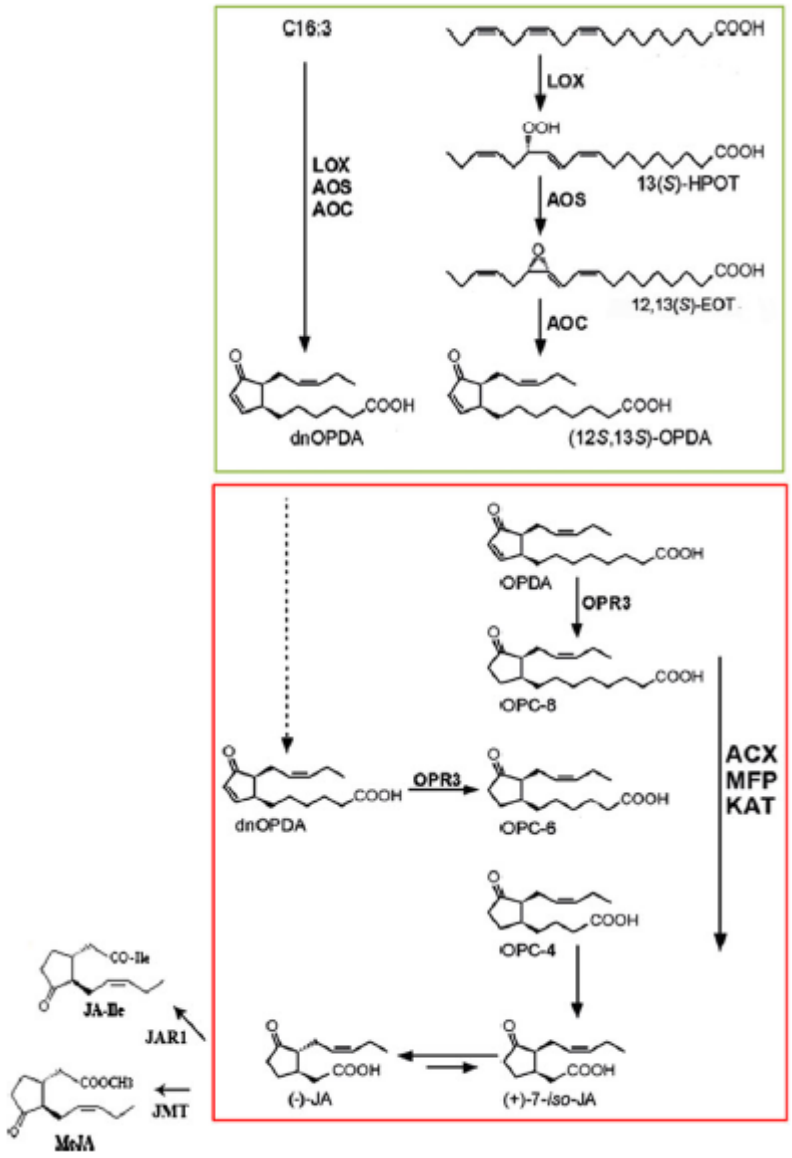


Figure I1. Jasmonate biosynthesis route which is developed in different subcellular compartments: the plastid (green square) where OPDA (cis-(+)-12-oxophytodienoic acid) and dn-OPDA (dinor- OPDA) are synthesized, the peroxisome (red square) where (dn)OPDA is converted to JA (jasmonic acid). Jasmonoyl-isoleucine (Ja-Ile),methyl jasmonate (MeJA), (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT); allene oxide synthase (AOS), allene oxide cyclase (AOC), 12-oxophytodienoate reductase 3 (OPR), 12-oxophytoenoic acid (OPC-8), 3-keto-acyl-CoAthiolase (KAT), fatty acyl-CoA oxidase (ACX), jasmonic acid methyl transferase (JMT), jasmonate resistant 1 (JAR1). Figure taken from Santino *et al.* (2013).

In the case of OPDA, which had been believed to be other precursor of JA, has been observed to have a different effects than JA. For instance, OPDA inhibits seed germination, role previously attributed to JA (Dave 2011), and tendril coiling is more promoted by OPDA than by JA (Blechert *et al.* 1999).

Functions of JA

Currently, it is known that JA is involved in physiological processes such as floral development, induction of senescence, fruit ripening, tendril coiling, mechanotransduction, potato tuberization, trichome formation and arbuscular mycorrhizal symbiosis (Balbi & Devoto 2008, Browse 2005, Creelman & Mullet 1995, Creelman & Mullet 1997, Koda 1992, Koda 1997, Parthier 1991, Pauwels *et al.* 2008, Reinbothe *et al.* 2009, Sembdner & Parthier 1993, Wasternack 2007, Wasternack & Hause 2002, Xu *et al.* 2007, Yoshida *et al.* 2009). For more information see the review of Wasternack *et al.* (2013). In addition, JA is an important hormone in plant defense against pathogen attacks (Zhang & Turner 2008). Similarly, it has been recently discovered its involvement as a protectant against abiotic stresses. Some of these functions will be more fully described in this section:

Growth regulation: Root growth inhibition was among the first effects attributed to JA (Dathe *et al.* 1981, Staswick,Su & Howell 1992). This inhibition was observed in JA-treated plants and mutant plants which over-express JA (Ellis *et al.* 2002, Staswick *et al.* 1992). Whereas root growth is reduced less in mutants that have reduced sensitivity to JA (Browse 2005, Wasternack *et al.* 2006).

Auxin (IAA) is involved in root growth and its biosynthesis is affected by JA (Sun *et al.* 2009a). JA causes a redistribution of IAA modulated by endocytosis (Sun *et al.* 2011). Therefore the JA-induced root growth inhibition occurs because JA affects the IAA distribution inside roots.

On the other hand, both hormones, JA and IAA, interact positively in the process of lateral and adventitious root formation (Peret *et al.* 2009, Stenzel *et al.* 2012). It has been observed that JA- insensitive mutants show fewer lateral roots. Moreover, an increase in some activities of JA biosynthesis enzymes were observed in emerging lateral roots (Stenzel *et al.* 2012). Whereas mutant impaired in JA perception and signaling showed more adventitious roots than the wild type (Gutierrez *et al.* 2012).

Trichome formation: Glandular trichomes are important because they are involved in defense against insects and herbivores attack (Tian *et al.* 2012). Li *et al.* (2004) found that JA is involved in glandular trichome formation. They observed abnormal development of glandular trichomes and they analysed the differences in exudates production of trichomes between WT and *jai1* tomato plants, with mutant plants showing a reduction of the exudate compound compared with WT plants. Tian *et al.* (2012) noted that trichomes produced JA-inducible defense compounds against herbivores such as monoterpenes and sesquiterpenes.

Leaf movements: It is known that hyponastic movement, an upward bending of leaves or other plant parts, is stimulated by JA and inhibited by salicylic acid (SA) (van Zanten *et al.* 2012).

Leaf senescence: Senescence was another of the initially reported physiological activities of JA. It is related to down regulation of proteins encoded by photosynthetic genes. Specifically, JA-induced chlorophyll breakdown, where chlorophyllase 1 enzyme, involved in chlorophyll degradation, is strongly induced by JA (Tsuchiya *et al.* 1999) . Whilst the Rubisco activase is down-regulated by JA (Shan *et al.* 2011).

Gravitropism: Gravitropism is a growth movement by a plant in response to gravity. IAA is involved in the regulation of this phenomenon (Benjamins,Malenica & Luschnig 2005, Swarup *et al.* 2005) and now it is known that JA is also implicated in this process. Gutjahr *et al.* (2005) found that a JA and IAA gradients were involved in gravitropism and this process was inhibited when JA and IAA were conjugated with tryptophan (inactive form of the hormones) (Staswick 2009).

Stomatal closure: This function of MeJA has been well studied (Akter *et al.* 2013, Hossain *et al.* 2011, Islam *et al.* 2010, Munemasa *et al.* 2007). Moreover, it is known that MeJA application induces an increase of calcium concentration in guard cells conducting to stomatal closure (Munemasa *et al.* 2011a, Sun *et al.* 2009b). A recent study has shown that this function of MeJA is partially mediated by ABA-enhanced biosynthesis (Hossain et al. 2011).

Reproductive organs: Reproductive organs development is regulated by JA. JA acts differently depending on the kind of plants. In dicotyledonous plants, mutants impaired in JA biosynthesis and perception show sterile male (Browse 2009a, Browse 2009c). They present insufficient filament elongation, non-viable pollen and show anther dehiscence. Only the fertility could be restored by JA applications in mutants impaired in JA biosynthesis (Mandaokar *et al.*

2006). Moreover, JA is involved in final stages of petals growth, since JA regulation of the bHLH TF BIGPETAL gene expression, which limits petal size (Brioudes *et al.* 2009).

On the other hand, monocotyledonous have both sex organs in the same plant, male tassel at the top and female organs. The pistil primordial are aborted in staminate flowers by a *tasselsseed*-mediated cell death process (Acosta *et al.* 2009). There are two genes required for sex determination which are regulated by JA. In maize, they are called *tasselseed1* (*ts1*) and *tasselseed2* (*ts2*) and it is known that *tasselseed* mutations *ts1* and *ts2* cause the conversion of tassel inflorescence from staminate to pistillate and both genes are necessary to eliminate pistil primordia through cell death (Acosta *et al.* 2009). The homozygous *ts1* mutant causes a loss of 13-LOX activity, decreasing JA levels in inflorescences. Similarly *ts1*, *ts2* acts in β-oxidation steps of the carboxylic acid side chain of OPDA biosynthesis pathway (Acosta *et al.* 2009, Browse 2009b).

Jasmonate signaling in response to biotic stresses: Plants have to deal with a complex environment, which is in continuous exposure to biotic stresses caused by different pathogens such as viruses, bacteria, fungi and insects. Responses to biotic stresses are mainly mediated by plant hormones such as SA, JA and ethylene (ET), which act as primary signals in the regulation of plant defenses. In general, local and systemic defense responses, including systemic acquired resistance (SAR) against biotrophic pathogens is mediated by SA (Durrant & Dong 2004), whereas JA and ET mediate responses against necrotrophs (Glazebrook 2005). However, this classification appears to be simplistic since many potential pathogens change their pathogenic style along their life cycle. Even the crosstalk between SA and ET/JA pathways can be either mutually antagonistic or synergistic, and the result is a more complex crosstalk among pathways (Adie *et al.* 2007, Beckers & Spoel 2006, Spoel,Johnson & Dong 2007).

JA Signaling

The plant response to JA-Ile, which is the active molecule of JA signaling, depends on its concentration in the cells. When the plants are not subjected to stress conditions, JA-Ile concentration is low inside the cells. In this situation, basic-helix-loop-helix (bHLH) related transcription factors interact with the target sequence inside the jasmonate-responsive gene (G-box) promoters but their activity is repressed by homo- or heterodimers of JASMONATE-ZIM-DOMAIN (JAZ) proteins (Fig.I2a) (Balbi & Devoto 2008, Browse 2009a, Chico *et al.* 2008, Chini

et al. 2009, Chung & Howe 2009, Fonseca *et al.* 2009, Gfeller *et al.* 2011, Kazan & Manners 2008, Memelink 2009, Staswick 2008).

On the other hand, under stress conditions (Fig.I2b), the plants increase the JA-Ile synthesis and its concentration inside the cells. Hence, JA-Ile joins to its receptor, COI1 (CORONATINE INSENSITIVE1), which is part of the SCF^{COI1} (Skp-Cullin-F-box) complex and E2 ubiquitin-conjugated enzyme. Subsequently, the COI1-JA unit of the SCF^{COI1} complex binds to JAZ proteins, leading to mobilization of JAZ protein to 26S proteasome for its degradation. Therefore, bHLH transcription factor is released, beginning the JA responsive genes activation (Chini *et al.* 2007, Niu,Figueroa & Browse 2011, Thines *et al.* 2007, Toda *et al.* 2013, Yan *et al.* 2007).

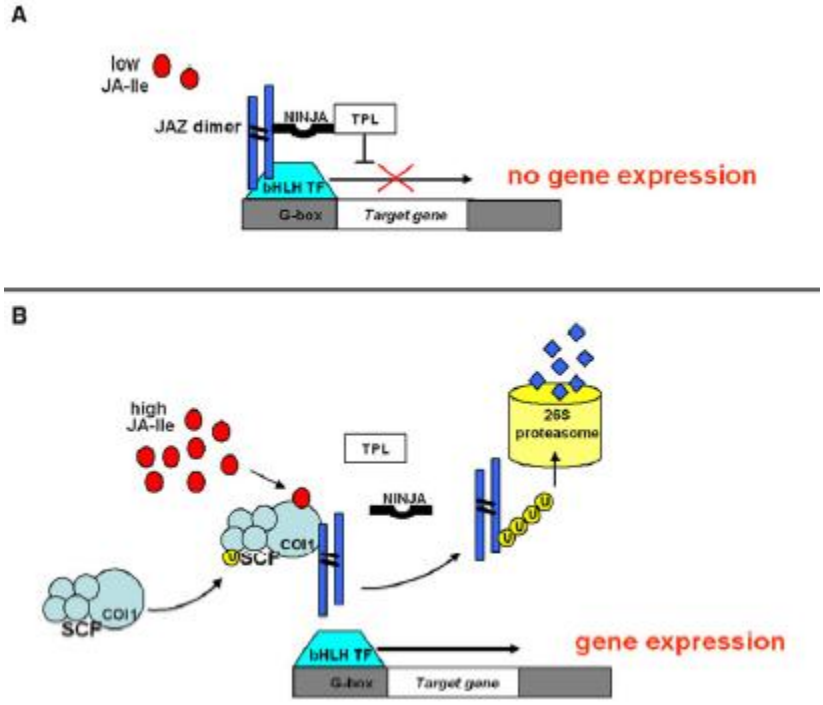


Figure I2. Scheme of jasmonate perception and regulation of expression of jasmonate target genes. **(a)** under non- stress conditions JA-Ile is at low concentration and JA does not induce gen response. **(b)** Under stress or developmental cues JA-Ile level increases and it starts the gene response. Figure taken from Santino *et al.* (2013).

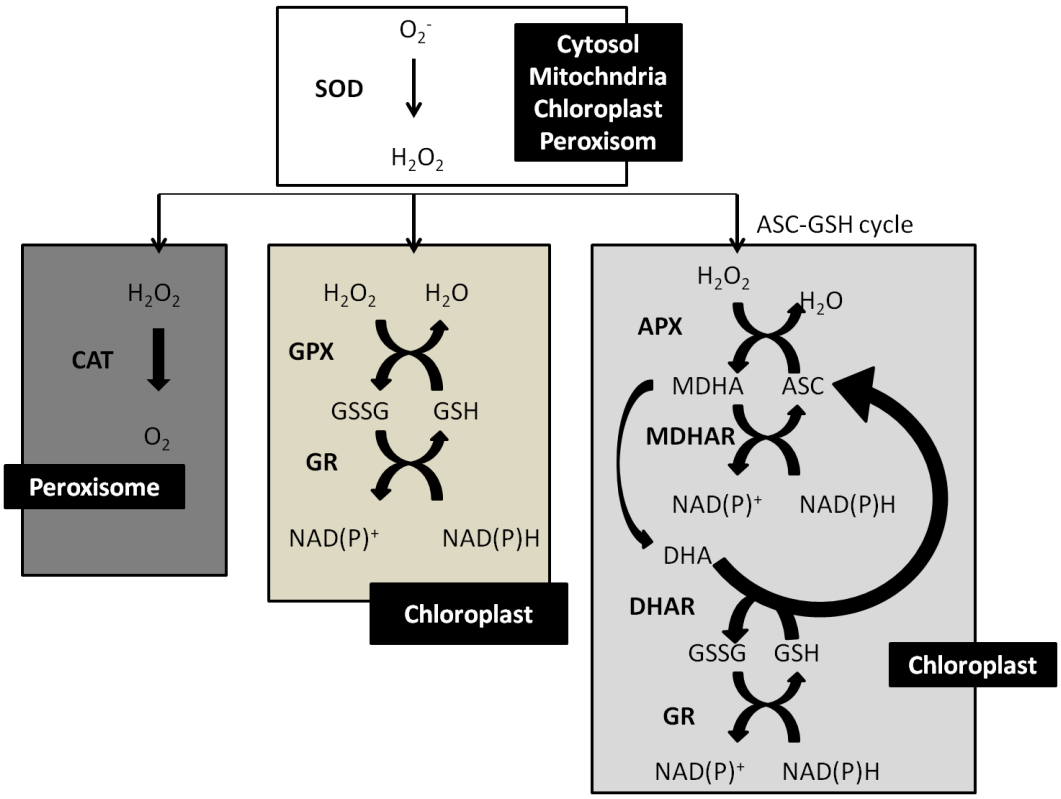


Figure I3. Antioxidant systems by which plants reduce the oxidative damage. The enzymes involves are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Furthermore, oxidized glutathione (GSSG), reduced glutathione (GSH), ascorbate (ASC), monodehydroascorbate (MDHA) and dehydroascorbate (DHA) have also a role in these processes. Within the black box is described their main locations.

JA improves the tolerance of plants against abiotic stress conditions

Stress conditions cause negative effects on plants, increasing the production of reactive oxygen species (ROS), for instance when photosynthesis decreases because of stomatal limitation, the excess light energy cannot be dissipated as fluorescence, heat or consumed in photosynthesis. Thus, mitochondria generate more energy than they can spend. This also happens when the plants try to eliminate pathogens (Gu *et al.* 2014, Lazaro *et al.* 2013, Serrato *et al.* 2013). ROS include, superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O^-$), which are at low concentrations inside the cells under non-stressed conditions, because they are also involved in cell metabolism (Dionisio-Sese & Tobita 1998). These cytotoxic oxygen species are highly reactive and can seriously disrupt normal metabolism through oxidative damage, being responsible of lipid peroxidation and consequently membrane injury, protein degradation,

enzyme inactivation, pigment bleaching and disruption of DNA strands (Scandalios 1993). To prevent and reduce oxidative damage, plants have evolved an antioxidant defense system for scavenging and detoxifying ROS. These include non-enzymatic antioxidants such as glutathione (GSH), ascorbate (ASC) or carotenoids, as well as, ROS-scavenging enzymes such as superoxide dismutase (SOD), peroxidase (POD) catalase (CAT) and ascorbate peroxidase (APX) (Apel & Hirt 2004) and systems that regenerate oxidized antioxidants (glutathione reductase (GR) and mono-and dehydroascorbate reductase (MDHAR and DHAR) (Wang *et al.* 2012 ; Fig.I3).

In relation to JA effects, new studies have emerged focusing on the defensive role of JA in plants against abiotic stresses. To see its protective effects, several experiments added exogenous methyl jasmonate (MeJA) to plants growing under stress conditions. The results showed that MeJA improved resistance against drought in rice (Lee,Parthier & Lobler 1996a), tomato (Ding *et al.* 2001) and strawberry (Wang 1999).Concretely, it was noticed that MeJA-treated plants subjected to drought increased the activities of several antioxidant enzymes such as SOD, POD and CAT, decreasing membrane lipid peroxidation and improving relative water content (RWC) (Anjum *et al.* 2011). Moreover, MeJA-treated plants lost less water in chilled seedlings because they reduced the opening of stomata and increased the root bleeding rate in rice (Lee *et al.* 1996a). Nonetheless, exogenous application of MeJA had negative effect in peanut plants where they triggered lipid peroxidation (Kumari *et al.* 2006).However, Walia *et al.* (2007) observed that pretreatment with JA in salt-stressed plants induced low levels of Na accumulation in the shoots with respect to non-treated barley plants. Abdala *et al.* (2003) reported that salt treatment caused change of concentration of different JA derivatives like JA, OPDA and JA-Ile. Specifically, they obtained an increase of all these compounds in plants treated with salt. In the same way, transgenic over-expression of OsbHLH148, basic helix-loop-helix domain gene, which is a component of the jasmonate signaling pathway in rice, provided tolerance to drought (Seo *et al.* 2011). Wu *et al.* (2012a) observed that plants treated with MeJA under drought conditions decreased the H_2O_2 content and malondialdehyde (MDA, lipid peroxidation levels) content, as well as, increased proline concentration and relative water content, being these plants more tolerant to drought. In the same way, plants treated with JA ameliorated Cd–induced oxidative stress, decreasing H_2O_2 and O_2^- concentration as well as increasing CAT and SOD activities (Noriega *et al.* 2012).

Crosstalk between JA and other molecules

The interaction between different molecules to get a specific answer is important in plant communication. These molecules could be hormones, proteins or ions that act as signaling molecules. JA has been shown to act together with other hormones such as abscisic acid (ABA) and salicylic acid (SA) or ions like calcium.

The most studied hormone because of its interaction with JA has been ABA. ABA is involved in various plant physiological processes including seed dormancy, stomatal movement and responses to abiotic stresses (Wilkinson & William 2002, Zeevart & Creelman 1988). JA interaction with ABA is linked with the stomatal closure regulation. Hossain *et al.* (2011) noted that endogenous ABA accumulation was required for MeJA-induced stomatal closure as well as in MeJA-elicited cytosolic calcium oscillation (Herde *et al.* 1997, Hossain *et al.* 2011, Suhita *et al.* 2004). In addition, de Ollas *et al.* (2013) noticed that an increase of JA and ABA concentration was observed in citrus plants as a consequence of drought. Therefore, their results suggested that JA accumulation was needed for the increase of ABA concentration, despite ABA was not needed to increase JA levels. Moreover, they proposed that "a burst of JA in roots of citrus under severe drought stress conditions leads to a more progressive ABA accumulation that will induce later plant responses" (de Ollas *et al.* 2013).

In the same way, SA is known to participate in several physiological processes, such as vegetative growth, seed production and senescence (Bahrami *et al.* 2013, Belozerova *et al.* 2014, Friedman *et al.* 2003, Hashmi *et al.* 2012, Liu *et al.* 2014, Vanderstraeten *et al.* 1995, Xie *et al.* 2007). However, SA has been extensively studied as a protecting agent against stresses (Borsani,Valpuesta & Botella 2001, Chini *et al.* 2004, Freeman *et al.* 2005, Kang & Saltveit 2002, Larkindale & Huang 2005, Munne-Bosch & Penuelas 2003). The most important role of SA is probably its involvement in systemic acquired resistance (SAR) against pathogens. SA could be converted to methyl salicylate (MeSA) which is considered an important long-distance signal in SAR (Chen *et al.* 2003, Park *et al.* 2007, Vlot *et al.* 2008).

It is well known that SA concentration increases in stressed plants (Munne-Bosch & Penuelas 2003). Munné-Bosh *et al.* (2003) found a positive correlation between SA and α-tocopherol levels in plants grown under drought conditions. In the same way, it is known that JA is involved in the synthesis of tocopherol (Sandorf & Hollander-Czytko 2002). This increase of α-

tocopherol makes the plants more resistant against stresses because it is an effective antioxidant agent which is located in the cellular membranes. Hence, there is an interaction between SA and JA in plant responses to stresses (de Bruxelles & Roberts 2001).

Polyamines (PAs) are also involved in several physiological processes such as seed germination, root growth, cell division, elongation, replication, transcription, translation, and membrane and cell wall stabilization has been studied (Hussain *et al.* 2011). Recently, its protective role in plants under stress conditions. Radhakrishnan *et al.* (2013) observed that adding one kind of polyamines (spermine), changed the concentration of ABA and JA in osmotic stressed plants. Specifically, untreated stressed plants showed an increase of ABA concentration and a decrease of JA concentration in leaves. However, opposite results were obtained after spermine application. Moreover, spermine treatment reduced lipid peroxidation and increased antioxidant enzyme activities. Moreover, Lee *et al.* (1996b) noticed that putrescine and spermidine changed their concentration after MeJA application. In the case of putrescine, its concentration was increased after MeJA application. Contrarily, spermidine concentration was reduced. Therefore, Lee *et al.* (1996b) suggested that putrescine is required for the induction of chilling tolerance of rice seedlings by MeJA.

Nitric oxide

Nitric oxide (NO) is a small size, non-charged and short-lived (3 seconds), molecule, which is highly diffusible across biological membranes and has the ability to react with other molecules. NO was discovered first in animals and then was widely studied in plants (Wu *et al.* 2014, Yoshida *et al.* 1980). One of the most studied NO functions in plants is its role in defense against stress conditions (Arasimowicz-Jelonek,Floryszak-Wieczorek & Kubis 2009a, Arasimowicz-Jelonek,Floryszak-Wieczorek & Kubis 2009b, Fan & Liu 2012, Xiong *et al.* 2012). However, it is known that NO is involved in induction of seed germination and reduction of seed dormancy (Beligni & Lamattina 2000, Bethke,Libourel & Jones 2006a, Bethke *et al.* 2006b, Libourel *et al.* 2006), regulation of plant metabolism and senescence (Guo & Crawford 2005, Leshem,Wills & Ku 1998), induction of cell death (Pedroso & Durzan 2000), regulation of stomatal movement (Bright *et al.* 2006, Garcia-Mata & Lamattina 2001, Garcia-Mata & Lamattina 2007, Neill,Desikan & Hancock 2003), regulation of photosynthesis (Takahashi & Yamasaki 2002), mitochondrial functionality (Zottini *et al.* 2002), gravitropism (Hu *et al.* 2005) and floral regulation (Hu *et al.* 2005). Also, NO was proven to be able of regulating the multiple plant responses towards a variety

of biotic and abiotic stresses and alleviating some consequences caused by oxidative stresses (Beligni & Lamattina 1999, Crawford & Guo 2005). On the other hand, NO can cause different physiological effects in higher plants, depending on its concentrations in plant tissues. At low concentrations, NO can act as a signal molecule in physiological processes. Instead, high NO concentrations can inhibit physiological processes or trigger oxidative damage and cell death (Moreau 2010). Examples of that were found in the study by Xu *et al.* (2005) where the treatment with the NO donor sodium nitroprusside (SNP) at 0.1 and 0.5 mM increased the average growth rate and biomass of *Catharanthus roseus* cells but they were inhibited at 10 and 20 mM. Moreover it is known that SNP treatment at 10, 50, and 100 μM increased the biomass of *Artemisia annua* hairy roots. However doses higher than 200 μM inhibited its growth (Zheng *et al.* 2008). In the same way, the number and length of adventitious roots were improved when the plants were treated with low NO concentrations (10–200 μM) and were suppressed when they were exposed to high concentrations (1 mM) (Liao,Xiao & Zhang 2009).

NO Biosynthesis

NO can be produced by several different pathways (Fig.I4). The enzymatic path includes three enzymes such as nitrate reductase (NR), nitrite: NO reductase (NI:NOR) and nitric oxide synthase like (NOS). Nitrite is used as a substrate by NR and Ni:NOR. It is known that NR take NO_2^- and NADH to produce NO (Bright et al 2006; Neill 2008) whereas, Ni:NOR does not use reduced nicotine adenine nucleotides but it uses cytochrome c as an electron donor (Stöhr et al 2001; Meyer and Stöhr 2002). Lastly, in the case of NOS, its activity has been demonstrated, although its gene has not been found yet. NOS acts reducing L-arginine to citrulline to produce NO (Klessig,Ytterberg & van Wijk 2004, Zemojtel *et al.* 2004). The non-enzymatic path is a reduction of nitrite to NO at acid pH in the apoplastic space of seeds and roots (Bethke,Badger & Jones 2004, Neill *et al.* 2003). Finally, other NO sources less studied are mitochondrial electron transport-dependent reductase (Planchet *et al.* 2005) and NO production triggered by polyamines (Gaupels *et al.* 2008).

Due to high reactivity, NO may react with other molecules resulting in new products (Fig.I5). The S-nitrosylation or S-nitrosation is caused when NO is transferred from a nitrosothiol to a sulfhydryl group of a cysteine residues. They are known to be involved in the transport, storage and release of NO, as well as, in post-translational modifications and in cell signaling to overcome

stress conditions. Therefore, S-nitrosylation is an important redox-dependent regulation mechanism (Benhar,Forrester & Stamler 2006, Foster,McMahon & Stamler 2003, Moreau *et al.* 2010).

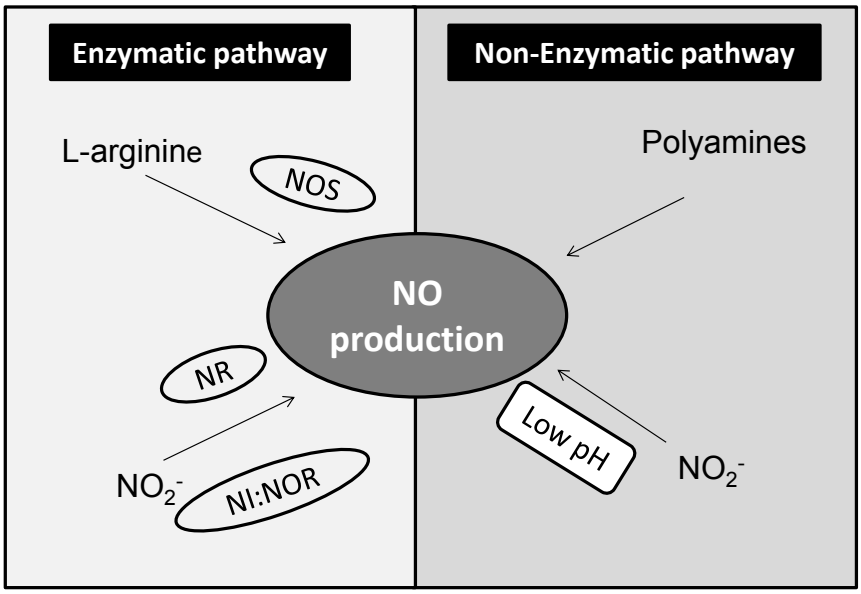


Figure I4. Main routes of nitric oxide (NO) production in plants cells. NO can be synthesized by an enzymac way (nitrate reductase (NR), nitrite NO reductase (NI.NOR) and l-arginine-dependent NO synthase (NOS) and by a non- enzymatic way. Figure adapted from Wilson *et al.* (2008).

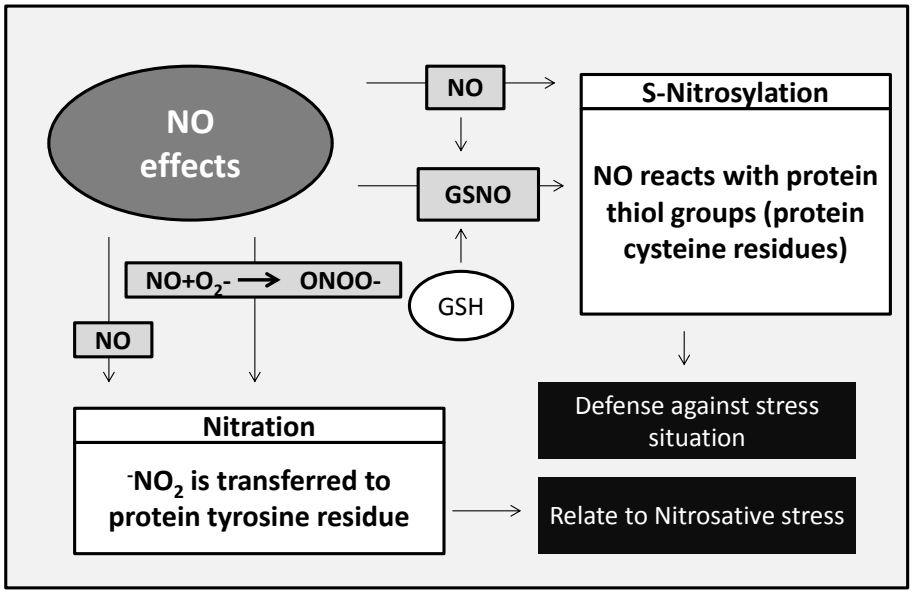


Figure I5. Main reactions between proteins and NO. Although NO can react directly with other proteins, it can link to glutathione to form nitro glutathione (GSNO) or to react with O_2^- to form peroxynitrite ($ONOO^-$).

One example is S-nitrosoglutathione (GSNO) where NO reacts with reduced glutathione (GSH). GSNO functions as a mobile reserve of NO (Diaz *et al.* 2003, Durner & Klessig 1999). Intracellular GSNO concentration is regulated by the activity of GSNO reductase (GSNOR) which catalyzes the reduction of GSNO to oxidized glutathione (GSSG) and NH_3. So, it also controls the NO effects in the cells (Fig.I6) (Barroso *et al.* 2006, Lamotte *et al.* 2005, Malik *et al.* 2011).

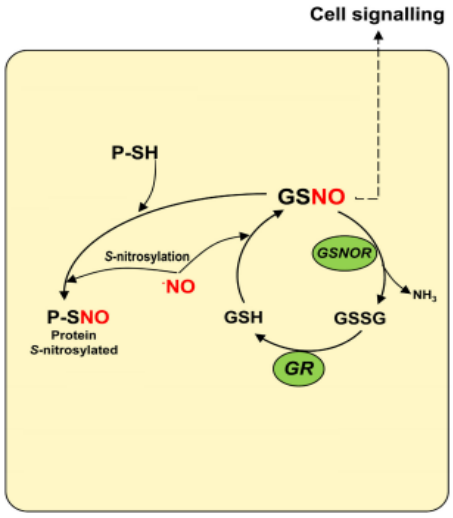


Figure I6. GSNO role in S-nitrosylation process. Figure taken from Corpas *et al.* (2013).

On the other hand, nitration consists in the addition of a nitro group ($^{-}NO_2$) into a protein in a tyrosine residue (Gow *et al.* 2004). This modification can alter protein function and can result in toxic physiological consequences. This situation is known as nitrosative stress (Radi 2004). The most known example is when superoxide radical ($O_2^{\cdot-}$) generates peroxynitrite ($ONOO^-$) when it reacts with NO. $ONOO^-$ also participates in the nitration of tyrosine residues in proteins. Increasing proteins with tyrosine nitration has been considered a marker of nitrosative stress (Bartesaghi *et al.* 2007, Bechtold *et al.* 2009, Corpas,Del Rio & Barroso 2007, Chaki *et al.* 2009, Ischiropoulos 2003, Jasid *et al.* 2009, Radi 2004). Among the proteins susceptible to modification by nitration are rubisco activase and ATP synthase subunit a (Cecconi *et al.* 2009, Lozano-Juste & Leon 2011). In chloroplasts, the main tyrosine nitration sites occur in PSI, PSII, cytochrome b6/f and ATP synthase complex (Galetskiy *et al.* 2011).

Several reports have demonstrated that NO is straightly linked with ROS, for instance, some studies have observed that NO stimulated ROS generation or cooperated with ROS to promote host cell death (de Pinto,Tommasi & De Gara 2002, Delledonne *et al.* 2001). Contrary,

there are other reports where NO acts scavenging ROS to avoid cell damage (Beligni & Lamattina 2002). The first option was amply studied in plants attacked by pathogens, where an increase of NO and ROS killed the pathogen (Lin *et al.* 2011). Secondly, NO regulates the activity of several antioxidant enzymes, such as CAT and APX, to reduce ROS, thereby avoiding cellular injury (Lamattina *et al.* 2003, Shi *et al.* 2007, Tewari,Hahn & Paek 2008, Zheng *et al.* 2009).

Interaction of NO with other hormonal pathways

NO is involved in regulating octadecanoid pathway (Xu,Dong & Zhu 2005). Thus, NO mediates the up-regulation of lipoxygenase (LOX) activity (Arasimowicz-Jelonek *et al.* 2009a), which catalyses the bioxygenation of polyunsaturated fatty acids (PUFA) to form fatty acid hydroperoxides and is involved in plant resistance against abiotic and biotic stresses (Arasimowicz-Jelonek *et al.* 2009a, Royo *et al.* 1999). Some examples of interaction between NO and JA were found by Wang & Wu (2005) who observed that MeJA application increased NO production in *Taxus chinensis* cells. Also, Xu & Dong (2005) found an increase of NOS activity after JA application in *Sophora flavescens* plants, and Dombrowski *et al.* (2007) reported that the joint action of JA and NO caused stomatal closure in *Vicia faba*. Continuing with the role of NO in stomatal regulation, it is known that exogenous NO application induces stomatal closure and reduces transpiration rates (Garcia-Mata & Lamattina 2002). Stomatal closure induced by ABA was inhibited by NOS inhibitors (L-NAME) and NO scavenger (cPTIO) (Garcia-Mata & Lamattina 2002, Neill *et al.* 2002). In the process of ABA-dependent stomatal closure it has been found that ROS acts upstream of NO because ROS production has been recognized as an early event in ABA-signaling networks (Bright *et al.* 2006). In spite of SA can induce NO production (Klepper et al. 1991) and vice versa (Durner,Wendehenne & Klessig 1998), the main role is their interaction for activation of systemic acquired resistance (SAR). It has been showed that GSNO acts synergistically with SA and JA in defense of plants, activating gene responses at local and distant sites (Espunya *et al.* 2012). Espunya *et al.* (2012) reported that GSNO is also necessary to activate the defense response caused by wound, which is dependent on ET and JA.

There is little information about the involvement of gibberellins in NO signaling. The main interaction is due to NO role in DELLAs proteins regulation, which act like repressors GA-responsive for growth and development (Alvey & Harberd 2005, Itoh *et al.* 2005). It has been observed that exogenous NO application led to an increase in the accumulation of DELLAs proteins (Lozano-Juste & Leon 2011). Moreover, some studies indicated that exogenous NO application

inhibited Arabidopsis root elongation, but this effect was partially reverted by the addition of GAs (Fernandez-Marcos,Sanz & Lorenzo 2012). However, in other study it was observed that the addition of SNP stimulates GAs accumulation and subsequently apical root growth (He *et al.* 2012). This apparently opposite effects may be caused by different final concentration of NO, or different sensitivity to NO of each plant species.

On the other hand, some works reported that NO reduced ethylene production (Manjunatha *et al.* 2012, Zhu & Zhou 2007) because the activity of enzyme 1-aminocyclopropane carboxylic acid oxidase, involved in its synthesis, is down regulated by NO (Zaharah & Singh 2011, Zhu,Liu & Zhou 2006). In the case of auxins, their relation with NO is related with the growth of the plant. For instance, Correa-Aragunde *et al.* (2004) noted that NO is involved in the lateral root development in tomato and they suggested a relation of NO with auxin signaling transduction pathway. Other recent studies described that C-PTIO (an scavenger of NO) treatment caused root growth reduction of *Panax ginseng* (Tewari *et al.* 2007) and treatment with a NO donor promoted rooting of juvenile and mature cuttings of woody plants (Abu-Abied *et al.* 2012). Hence, NO is involved in root development.

Calcium ions also interact with NO signaling, as shown for their implication in SA-induced NO synthesis (Zottini *et al.* 2007), or increase of calcium concentration inside guard cells caused by NO, which is necessary to cause stomatal closure (Garcia-Mata *et al.* 2003, Sokolovski *et al.* 2005).

The interaction between JA and NO has been extensively studied in response to wounding. Orozco-Cardenas *et al.* (2002) observed that NO treatment (using SNP) blocked H_2O_2 synthesis, as well as, the expression of proteinase inhibitor genes in response to JA. Hu *et al.* (2009) observed that NO and JA act together in hypericin biosynthesis (compound that acts as an antibiotic) induced by fungal elicitors from *Aspergillum niger* in *Hypericum perforatum* plants. These results indicated that NO regulated JA biosynthesis and this biosynthesis was necessary for NO-induced hypericin production.

Role of NO in plants under stress conditions

In the last years, many studies have been carried out to examine the role of NO and its derivatives in plants under abiotic stresses. Corpas *et al.* (2008) noticed that pea plants subjected to different types of stress, such as high and low temperature, high light intensity and continuous light, increased the concentration of S-nitrosylated proteins. They also identified an increase in NOS

activity in stressed plants, a high concentration of GSNO and different nitrosylated proteins. Instead, Barroso *et al.* (2006) found that pea leaves of plant subjected to cadmium showed a reduction in GSH and NO concentration, as well as, a decrease of GSNO concentration due to low GSNOR activity.

Focusing on drought, several studies have demonstrated that drought conditions induce an increase of endogenous NO (Arasimowicz-Jelonek *et al.* 2009a, Gould *et al.* 2003, Kolbert,Ortega & Erdei 2010, Xiong *et al.* 2012). Moreover, Fan *et al.* (2012) observed that treatment with SNP (a NO donor) enhanced endogenous NO concentration and contrarily, L-NAME application (a NOS inhibitor) decreased it. Under drought conditions, exogenous NO applications resulted in improvement of plant growth and maintenance of a higher RWC, reduction of LOX activity, stomatal conductance and membrane ion leakage, increment of chlorophyll content and higher photosynthetic rate (Arasimowicz-Jelonek *et al.* 2009a, Arasimowicz-Jelonek *et al.* 2009b, Garcia-Mata & Lamattina 2001, Lei,Yin & Li 2007b, Tian & Lei 2006, Xiong *et al.* 2012). In addition, Xiong *et al.* (2012) proposed that SNP reduced the transpiration rate under well-watered conditions, as does drought stress. Liu *et al.* (2010) observed that SNP reduced oxidative damage under chilling conditions, whereas NAME- and PTIO-treated plants showed lower activity of SOD, CAT, APX and GR, as well as, high content of O^{-}_{2} and H_2O_2.

Arbuscular mycorrhizal symbiosis

Mycorrhiza is the symbiosis formed between a group of soil fungi and plant roots. Its etymology comes from the Greek “mycos” meaning fungus and “rhiza” meaning root. There are a variety of fungi that establish symbiosis with plants, but we will focus on the arbuscular mycorrhizal fungi (AMF).

It is known that AMF have existed for over 400 million years (Brundrett 2002) and currently colonize up to 90% of land plants (Denison & Kiers 2011). These fungi are so named because they form specialized structures called arbuscules (Fig.I7), which are the sites where nutrient exchange occurs between the two symbionts. Specifically, the fungus provides the plant with an extra supply of water and nutrients, especially P. The plant, likewise, provides the fungus with a protected ecological niche to complete its life cycle, as well as, with photoassimilate compounds (Pfeffer *et al.* 2004). It is estimated that 20% of products photo-assimilated by plants are consumed by the AMF (Allen & Shachar-Hill 2009, Smith & Read 2008). Other important

characteristic of AMF is that their hyphae have no septa, and therefore, they are called aseptate or coenocytic hyphae, with hundreds of nuclei in the same cytoplasm. Consequently their spores also contain hundreds of nuclei. Although the life cycle of AM fungi is considered to be asexual, it is possible that genetic material would be exchanged and recombined due to anastomosis among hyphae (de la Providencia *et al.* 2005, Giovannetti *et al.* 2004).

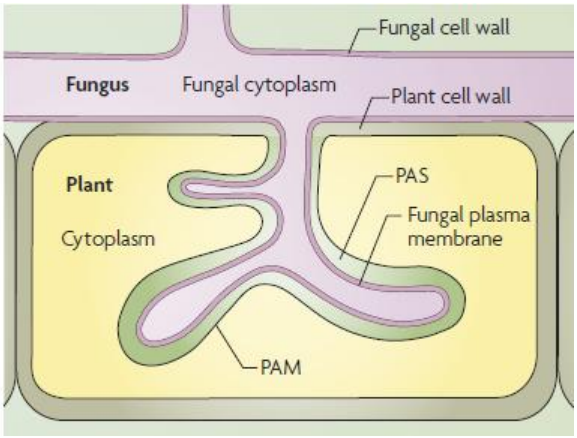


Figure I7 . This picture showing the main structures formed by asbuscular mycorrhizal fungi. The arbuscule, which looks like a tree is the place where the exchange of nutrients between both symbionts take place**.** The arbuscule is surrounded by a plant-derived periarbuscular membrane (PAM) and, between PAM and fungal plasma membrane there is a periarbuscular space (PAS). Figure taken from Parniske (2008).

Steps for the establishment of symbiosis

The arbuscular mycorrhizal symbiosis is established between soil fungi and higher plants. This symbiosis begins through a molecular dialogue between the two symbionts. This process of establishment of the symbiosis has been divided into three stages (Fig.I8):

The pre-symbiotic phase. Initially, strigolactones are hormones produced by plants and released into the medium in order to be recognized by the fungus. Strigolactones stimulate the germination of spores in contact with them, and induce the formation of exploration hyphae. Hyphae follow the concentration gradient of strigolactones to find the plant roots. Moreover, strigolactones are responsible for the physiological changes of hyphae, thus they increase their mitochondrial activity and cause a branched structure (Akiyama 2005, Besserer *et al.* 2006).

The recognition phase. AMF produce and release mycorrhizal factors (Myc) which cause changes in the calcium concentration in the epidermal cells of the root (Kosuta *et al.* 2008), which

in turn induce activation of genes related to the symbiosis (Kosuta *et al.* 2003). The fungus forms a special type of appressoria, called hyphopodium, which is formed from mature hyphae.

The colonization phase. The prepenetration apparatus (PPA) is a transcellular apoplastic compartment produced by the plant to accommodate the fungus in response to chemical and mechanical stimuli. It is formed after 4-5 hours of hyphopodium formation. For the formation of PPA several processes in root cells are activated. First, the nucleus of the cells are directed toward the point of entry of the fungus, from which they create a transcellular tunnel surrounded by a membrane, called perifungal membrane. PPA allows fungal hyphae to penetrate from hyphopodium to the cortex, where hyphae will branch to form arbuscules or vesicles (fungal storage organs) (Genre *et al.* 2008).

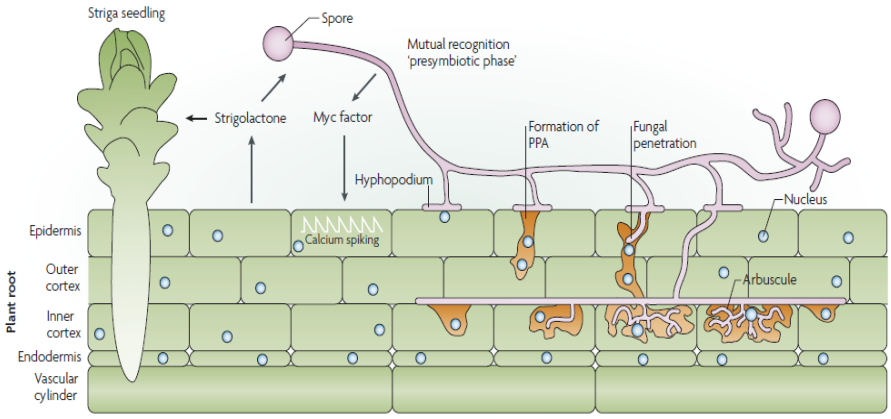


Figure I8. This figure summarizes the different steps that are performed during the establishment of symbiosis, from spore germination to the formation of arbuscules. Figure taken from Parniske (2008).

Benefits provided by the fungus to the host plant

Nutrition

AMF are able to absorb almost all nutrients at different rates. However the fungal efficiency can change depending on nutrients concentration in the soil. Under high concentration of nutrients in the soil, the fungus efficiency to take up nutrients is low. In contrast, under low and medium nutrient concentration, AMF are able to significantly increase the uptake of nutrients by the plant (Smith & Smith 2011).

Improvement of phosphate uptake is the main benefit of the AM symbiosis, which is due to production of enzymes such as phosphatase (Bucher 2007, Javot *et al.* 2007a, Javot,Pumplin & Harrison 2007b). The extensive hyphal network of AMF influences the physicochemical properties of the soil and directly or indirectly contributes to the release of phosphate from inorganic complexes of low solubility. High affinity fungal phosphate transporters, that are expressed in the extraradical mycelium, are probably involved in the uptake of phosphate from the soil (Harrison & Vanbuuren 1995, Maldonado-Mendoza,Dewbre & Harrison 2001). The P absorbed by the fungal hyphae is translocated to the arbuscules and hyphal coils (Smith *et al.* 2011). Moreover, high P concentration in soil causes a decrease in the hyphal colonization of the host roots and under low and medium level of P, the fungi are able to colonize the host plant roots significantly and increase P uptake by the host plants (Smith & Read 2008).

Unlike phosphorus, nitrogen is a mobile macronutrient and is part of proteins and chlorophyll molecules. It has been observed that mycorrhizal fungi are able to provide 50% of plant N requirement (McFarland *et al.* 2010). AM fungi can accelerate decomposition and directly acquire nitrogen from organic material in order to assimilate them (Ho & Trappe 1975, Hodge,Campbell & Fitter 2001, Kaldorf,Zimmer & Bothe 1994). Moreover, *Rhizophagus irregularis* is able to improve plant access to mineral N by absorbing NO_3^- and NH_4^+ (Johansen,Jakobsen & Jensen 1993, Johansen,Jakobsen & Jensen 1994) and Kaldorf *et al.* (1998) found NR activity in arbuscule-containing cells. In addition, mycorrihzal fungi can synthesize arginine and transfer it to host plants (Tian *et al.* 2010). In the same way, Cimen *et al.* (2010) observed an increase of K, Mg and Ca uptake in tomato plants by AMF.

Micronutrients such as Zn, Fe and Cu are also efficiently taken up by mycorrizal fungi as described by Miransari *et al.* (2010 and 2013). Zaefarian *et al.* (2013) investigated what different nutrients were improved in plants by AMF. The results showed that N, P, K, Zn and Cu uptake was improved; however Fe uptake was not changed by the mycorrhizal fungi.

Improved plant water status

AMF improve plant water status due to their morphological attributes. In particular, fungal hyphae have a diameter between 2-5µm, tenfold smaller than root hairs (Allen,Moore & Christensen 1982, Hardie 1985), allowing them to reach soil pores inaccessible to plant roots. Several studies have estimated the rate of water transport by extraradical hyphae to the root, Allen

(1991) quantified 0.1 µl h^{-1} per hyphae entry point and Faber *et al.* (1991) measured 0.37 to 0.76 µl h^{-1} per hyphae. However, in other works could not be able to quantify the rates of water uptake by hyphae (George *et al.* 1992, Koide 1993). Recently, Ruth *et al.* (2011) estimated that hyphae contribution to the total plant water uptake was about 20%.

It is also known that mycorrhizal exudates released to the soil contribute to the formation of stable aggregates that enhance the structural properties of the soil in addition to its moisture retention properties (Auge,Kubikova & Moore 2001, Hallet *et al.* 2009). For instance, the secretion of hydrophobic proteinaceous by fungi of substances as glomalin (Rilling *et al.* 2002) improves to water soil water retention as well as soil stability (Bedini *et al.* 2009).

Enhancement of plant gas exchange

Another effect of AM symbiosis is the change of stomatal behavior in plants under drought conditions (Auge *et al.* 2001, Ruiz-Lozano & Aroca 2010). In spite of there are a study in Citrus species which stomatal conductance (*gs*) are not affected by AM symbiosis, other plants like soybean, sunflower, lettuce or rose showed changes in *gs* by AMF (reviewed by Auge *et al.* 2001). The rates of gs are usually increased in AM plants than non-AM plants (Ebel *et al.* 1997, Ruiz-Lozano & Aroca 2010) and this could be related to altered ABA/cytokinins ratios in leaves of mycorrhizal alfalfa plants (Goicoechea,Antolin & Sanchez-Diaz 1997). On the other hand, Goicoechea *et al.* (2004) observed an decreased gs in AM *Anthyllis cytisoides* under drought conditions. Hence, after several studies it has been concluded that AMF regulates gs and transpiration rates depending on kind of plants and its lifestyle (Caravaca *et al.* 2003, Querejeta *et al.* 2003)

Leaf relative water content (RWC)

Recent research confirmed that AMF regulated stomatal conductance and transpiration rate. However, the involvement of AMF in regulating RWC is unclear. For example, some studies show that RWC is not affected by AMF under well-watered or drought conditions (Aroca *et al.* 2008a, Aroca,Vernieri & Ruiz-Lozano 2008b, Barzana *et al.* 2012, Ruiz-Lozano *et al.* 2009) while in others it increased under well-watered (Barzana *et al.* 2012, Jahromi *et al.* 2008) or drought conditions (El-Mesbahi *et al.* 2012, Porcel *et al.* 2006b). Finally, some studies observed a decrease under drought conditions (Allen & Boosalis 1983, Auge,Schekel & Wample 1986). Most probably these contradictory results are caused by the way that drought was imposed, the volume of the pots

and the size of the plants. In fact, if AM plants are bigger than non-AM plants, they may deplete faster the soil water content of a pot and suffer more stress (Ruiz-Sanchez *et al.* 2011).

Solute accumulation and protection against oxidative damage

Oxidative stress reflects an imbalance between the production of ROS and the ability of the plant to remove them. It is known that under drought conditions, plants must maintain its water potential more negative than the soil to continue a gradient favorable to water movement from the soil to the plant. For this purpose, the plants accumulate specific solutes to decrease their osmotic potential which can be inorganic ions (mainly K^+ and Cl^-) or uncharged organic compounds such as proline or glycine betaine, in addition to carbohydrates (sucrose, pinitol or mannitol) (Hoekstra,Golovina & Buitink 2001, Morgan 1984). For instance, proline, which is accumulated in tissues subjected to water stress to act as an osmoregulator (Kameli & Losel 1993, Singh *et al.* 2000), seems also to play an important role decreasing hydroxyl radicals and regulating redox potentials to protect the cell, as well as, reduce the acidity in the cell and protect against denaturation of macromolecules (Kishor *et al.* 1995). However, the role of AM symbiosis on proline accumulation is not clear. There are studies where an increase of proline has been found in AM plants under drought conditions, but this proline concentration could vary depending on the fungus involved (Azcon,Gomez & Tobar 1996, Garmendia,Goicoechea & Aguirreolea 2004, Goicoechea *et al.* 1998, Ruiz-Lozano,Gomez & Azcon 1995). For example, *Glomus deserticola* accumulated 120 nmol of proline per g fresh weight, while *Rhizophagus irregularis* contained 41nmol proline per g fresh weight (Ruiz-Lozano *et al.* 1995). Instead, others studies showed low proline concentration in AM plants under stress conditions (Aroca *et al.* 2008a, Jahromi *et al.* 2008, Porcel & Ruiz-Lozano 2004, Ramakrishnan,Johri & Gupta 1988, Ruiz-Lozano & Azcon 1996).

On the other hand, plants have several enzymes to protect cells against oxidative damage. For example, in relation to three isoforms of SOD, it has been observed an increase in the SOD acvitity and in the expression of several plant genes encoding for SODs. CuZn-SOD activity has been found in *Funneliformis moseae* and the expression of of a Mn-SOD and a Fe-SOD genes was higher in AM plants than in non-AM lettuce under drought conditions (Palma *et al.* 1993, Ruiz-Lozano,Azcon & Palma 1996, Ruiz-Lozano *et al.* 2001). Also, it has been observed an increase of GR, POD, SOD and CAT activities and an reduction of membrane lipid peroxidation in AM plants under drought conditions (Porcel,Barea & Ruiz-Lozano 2003, Sohrabi *et al.* 2012, Wu & Zou

2009). In the same way, AM plants showed high ASC and GSH contents under drought stress (Ruiz-Sanchez *et al.* 2011, Wu & Zou 2009).

Hormonal implication in AMF colonization

Hormones are molecules involved in most plant physiological processes, including establishment and functioning of AM symbiosis (Fig.I9). It is known that auxins, which are involved in growth and gravitropism, are also involved in the colonization of plant roots by AMF. Specifically, auxins are required to induce genes that encode enzymes involved in the synthesis of strigolactones (Hayward *et al.* 2009, Koltai & Kapulnik 2013, Ongaro & Leyser 2008), which are involved in the recognition between symbionts, growth, production of adventitious roots and root hairs (Kapulnik *et al.* 2011, Kohlen *et al.* 2012, Rasmussen,Beveridge & Geelen 2012a, Rasmussen *et al.* 2012b). The role of JA in the establishment and development of AM symbiosis is also unclear. There are studies where an increase in the concentration of JA in AM plants and, specifically, into the cells containing arbuscules has been observed (Hause *et al.* 2002, Mandal *et al.* 2013, Stumpe *et al.* 2005, Vierheilig & Piche 2002). This increase of JA was accompanied by an increase of expression of genes encoding enzymes involved in the biosynthesis of JA (Hause *et al.* 2002). Isayenkov *et al.* (2005) found that low levels of JA decreased mycorrhization rate and arbuscules formation, as well as, suppressed the expression of the enzyme above mentioned. On the other hand, there are studies that have shown a negative effect of JA on the root colonization by the fungus. For instance, exogenous application of MeJA caused a reduction of fungal colonization in several plants (Ludwig-Muller *et al.* 2002) and fewer arbuscule number (Herrera-Medina *et al.* 2008). Similarly, plants insensitive to MeJA (*jai-1*) increased the level of infection caused by the fungus (Herrera-Medina *et al.* 2008) or plants affected in JA synthesis pathway (*def-1* and *spr-2*) also increased the percentage of AMF root colonization (Leon-Morcillo *et al.* 2012a). Instead, MeJA treated WT plants decreased mycorrhizal colonization and arbuscule formation (Herrera-Medina *et al.* 2008). In conclusion, we can say that JA is involved in the establishment and development of symbiosis, but its effect can vary depending on the type of plant and other environmental cues.

Ethylene (ET) is another hormone involved in the AM symbiosis. Several studies have shown that during symbiosis plant, ET levels are reduced regardless of the inoculated fungus (Barazani,von Dahl & Baldwin 2007, Lopez-Raez *et al.* 2010, Riedel,Groten & Baldwin 2008). Likewise, SA is a hormone whose concentration is decreased in AM roots of pea and tobacco

(Blilou,Ocampo & Garcia-Garrido 1999, Herrera-Medina *et al.* 2003), although in AM barley plants an increase of SA concentration was also reported (Khaosaad *et al.* 2007). Finally, it is known that ABA concentration increases in mycorrhizal roots and that ABA is involved in the formation of arbuscules, as well as it is required for a proper functioning of the symbiosis (Herrera-Medina *et al.* 2007, Martin-Rodriguez *et al.* 2010).

On the other hand, we must consider the type of fungus that is being studied. For example, it has been found that *Funneliformis moseae* and *R. irregularis* have different infectivity, with *F. moseae* having lower mycorrhizal colonization potential than *R. irregularis* (Lopez-Raez *et al.* 2010). However, roots with *F. moseae* showed a greater concentration of SA and JA-Ile (bioactive derivative of JA) (Lopez-Raez *et al.* 2010) than root inoculated with *R. irregularis*. These interactions ere schematically presented in Figure I9.

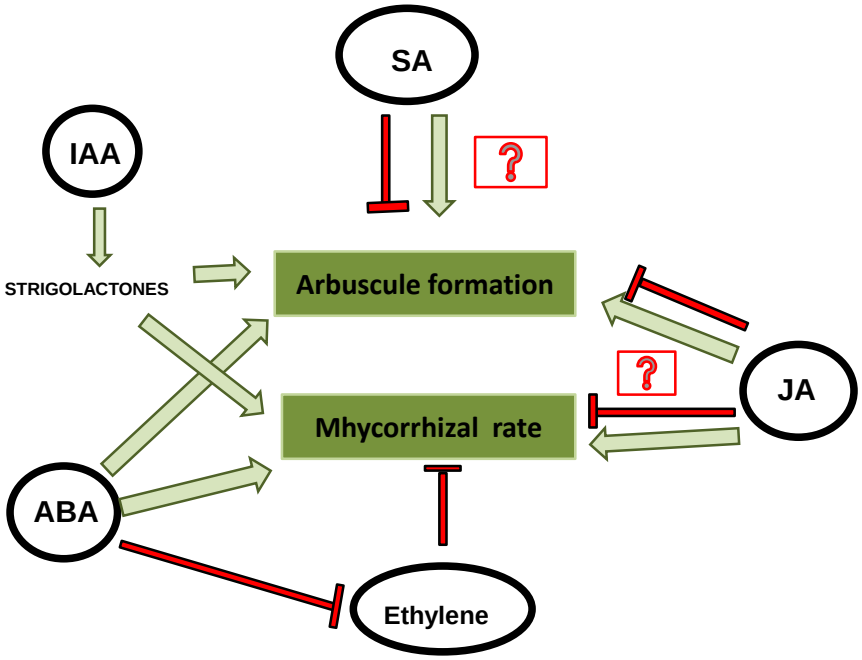


Figure I9. Schematic representation of possible interactions between plant hormones and mycorrhizal fungi. Red lines show inhibition pathways, and green arrows positive effects.

AMF and NO

Some studies have identified an increase of NR and nitrite reductase NiR transcripts in WT plants which contacted with germinated AM spores and during hyphopodium formation (Yamasaki & Sakihama 2000), then both enzymes could be potential sources of NO. Calcagno *et al.* (2012)

observed that after fungal exudates treatment, an increase of NO was detected in the roots of *Medicago truncatula* during the first minutes and this increase of NO depended on the NR activity, as it had been observed by Weidmann *et al.* (2004) and Horchani *et al.* (2011) previously. However, Besson-Bard *et al.* (2009) attributed the NO increase to NOS activity. Recently, Li *et al.* (2013c) reported that AMF decreased the H_2O_2 content and increased the NO and JA content in soybean roots, NO increase was caused by NR. However, the role of NO in AM symbiosis establishment is still unknown.

Water in plants

Water is a major component in plants, accounting for 80-90% of the fresh weight in herbaceous plants and more than 50% in woody plants. Therefore, the study of water in plants is very important. Physical characteristics of water to life include its great versatility as a solvent for inorganic salts, sugars and organic anions, as it constitutes an optimal environment in which to carry out the biochemical reactions. Water facilitates the transport (active and passive) of solutes, acting as a vehicle for movement of solutes and metabolites throughout the plant. It is also responsible for maintenance of tissue turgor and cell expansion. It is also responsible for stomatal opening in order to capture CO_2 and facilitate transpiration, the most effective cooling mechanism in plants (Azcon-Bieto 2008).

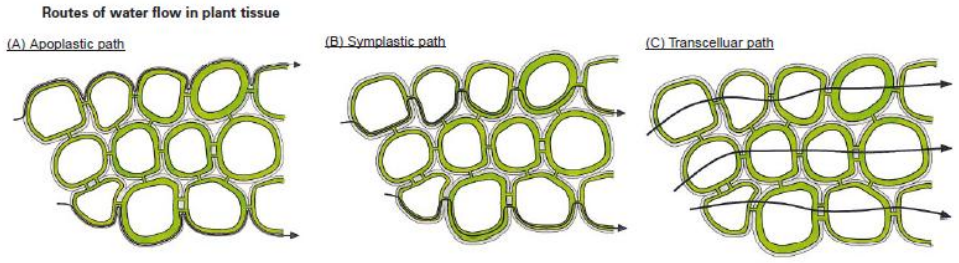


Figure I 10. Routes of water flow in plant tissue. (a) apoplastic path, (b) symplastic path and (c) transcellular path. Figure taken from Steudle *et al.* (1998).

Water movement inside plants

The Soil-Plant-Atmosphere Continuum (SPAC) is the pathway for water moving from soil through plants to the atmosphere. Water movement in the plant is due to a gradient of water potential (Ψ) between the soil and the leaves. Water potential depends on osmotic concentration,

capillary gravity and mechanic pressure among other. Being the most negative water potential in the leaves and less negative at the root surface where it approaches the ground (Azcon-Bieto 2008).

The water absorption across roots is due to two main forces. The hydrostatic force created by the transpiration stream, and the osmotic force produced by root pressure. The water moves from soil solution to the root xylem vessels (radial transport) and then, from the xylem vessels to the aerial parts (axial transport) (Doussan,Pages & Vercambre 1998, Knipfer & Fricke 2011, Steudle & Peterson 1998). The radial transport is the sum of three different pathways which water can follow inside roots. The apoplastic path (Fig. I10a), water flows through the pores of the cell walls and through intercellular spaces. The symplastic path (Fig. I10b), consists of water moving between the adjacent cells through the plasmodesmata. In the transcellular path (Fig. I10c), the water flows across the cellular membranes is largely through aquaporins, which are intrinsic membrane protein that act as water channels. Because the symplastic and transcellular paths cannot be distinguished experimentally, the sum of these two paths is called cell-to-cell path (Steudle & Peterson 1998). These three pathways act simultaneously, however, depending on environmental conditions some pathways are enhanced more than others. For example, when transpirations rate is low, cell-to-cell pathway is predominant (Steudle 2000).

Many studies have measured root hydraulic conductivity (L) to estimate root water transport capacity. L depends on the root architecture, the predominant radial water pathway and on the intrinsic membrane permeability to water (Sutka et al. 2011).

Aquaporins

Aquaporins (AQPs) are membrane protein channels that belong to the ancient family of major intrinsic proteins (*MIPs*). They facilitate the flow of water and uncharged solutes driven by water potential and solute concentration gradients, respectively.

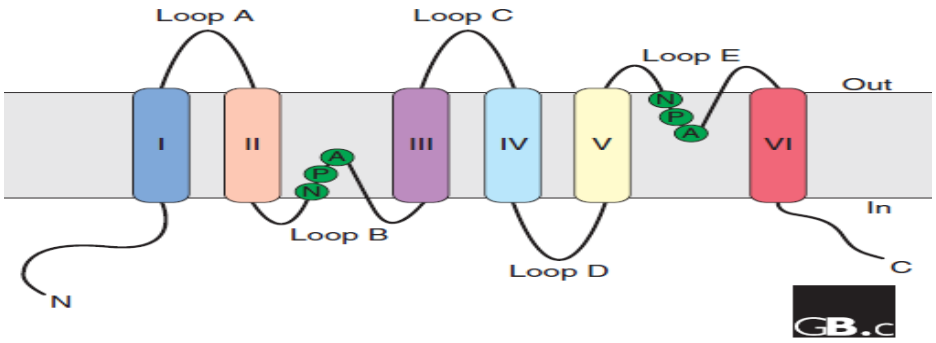


Figure I11. Topology of an aquaporin protein within the membrane. Figure taken by Kruse *et al.* (2006).

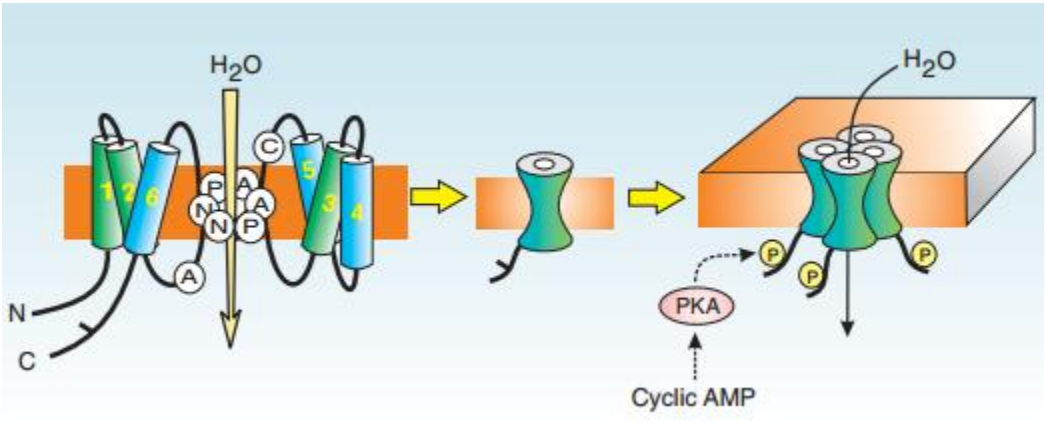


Figure I12. Structure of an aquaporin and assembling process of a tetramer. Figure taken from Berridge (2012).

Aquaporins are 23–31 kDa proteins formed by six transmembrane α- helices interrupted by five connecting loops (A-E) (Fujiyoshi *et al.* 2002, Törnroth-Horsefield *et al.* 2006) Loop B and D as well as N- and C- terminal tails are exposed in the cytosol, whereas loops A, C and E face the cell wall or internal membrane when aquaporins are located in organelles (Fig.I11). AQPs form tetramers where each monomer is an independent water channel (Fig. I12).

All MIPs present a conserved asparagines-proline-alanine (NPA) motif in loops B and E (Fig. I12) (Park & Saier 1996, Reizer,Reizer & Saier 1993). NPA motifs are thought to be essential part of the pore region creating the selectivity for water. In addition to water, some plant aquaporins can transport small neutral solutes like glycerol (Biela *et al.* 1999), urea (Gerbeau *et al.* 1999), formamide, acetamide (Rivers *et al.* 1997), methylammonium (Holm *et al.* 2005), boric acid (Takano et al. 2006), silicic acid (Ma et al. 2004), lactic acid (Choi & Roberts 2007), ammonia (NH_3) (Niemietz & Tyerman 2000), CO_2 (Uehlein *et al.* 2008) and H_2O_2 (Reuscher *et al.* 2013). In

the same way, substrate specificity of aquaporins can be also explained by an aromatic/ Arg (ar/R) motif (Bansal & Sankararamakrishnan 2007, Wallace & Roberts 2004).

Subfamily and localization

In higher plants, aquaporins can be divided into several groups depending on their amino acid sequence (Johanson *et al.* 2001, Sade *et al.* 2009).

Plasma membrane intrinsic proteins (PIP) are divided into two phylogenic groups, PIP1s and PIP2s. The PIP2 proteins have shorter N- terminal extension and a longer C- terminal tail containing phosphorylation sites (Chaumont *et al.* 2000, Chaumont *et al.* 2001, Johanson *et al.* 2001, Johansson *et al.* 2000, Prak *et al.* 2008). Fetter *et al.* (2004) discovered that plant PIP2 proteins have a higher water transport capacity than PIP1 proteins. However, co-expression of PIP1 and PIP2 proteins increased further the membrane water permeability compared when only PIP2 proteins are expressed (Fetter *et al.* 2004). Although they are mainly located in the plasma membrane (PM), they can be also localized in the endoplasmatic reticulum (ER) (Fig.I13) (Sorieul *et al.* 2011) and mitochondrial membranes (Brugiere *et al.* 2004).

Tonoplast intrinsic proteins (TIP) are located in the plasma membrane and tonoplast (Fig. I13), and they are classified in five isoforms (Johanson *et al.* 2001, Quigley *et al.* 2002, Reuscher *et al.* 2013). They are involved in several physiological functions like cell elongation, transport of solutes and osmoregulation processes (Maurel *et al.* 2008). It has been found that their over-expression confers tolerance to several abiotic stresses (Peng *et al.* 2007).

Nodulin- 26–like membrane intrinsic proteins (NIPs) which, in spite of being mainly expressed in the peribacteroid membrane of N_2-fixing symbiotic root nodules, are also present in non-legume plant species (Wallace,Choi & Roberts 2006). Usually, they are located in the plasma membrane (Ma 2006, Takano *et al.* 2006) and ER (Mizutani *et al.* 2006) (Fig. I13). It is known that *Gm*NOD26, the most studied NIP, is an aquaglyceroporin which transports water, glycerol and formamide in a mercury-sensitive manner (Dean *et al.* 1999, Rivers *et al.* 1997), as well as it could be involved in the delivery of reduced nitrogen compounds into the host plant cells by NH_3 transport (Niemietz & Tyerman 2000).

Small basic intrinsic proteins (SIPs). In *Arabidopsis*, there are three SIP homologs (Johanson *et al.* 2001, Quigley *et al.* 2002), mostly located in the ER (Ishikawa *et al.* 2005, Mizutani *et al.* 2006). SIPs are conserved among all plant species. However, the aquaporin gene

family shows signs of rapid and recent evolution (Sakurai *et al.* 2005), and their function is still not well known.

X (for unrecognized) **intrinsic membrane proteins** (XIPs). XIPs have been identified first by Danielson and Johanson (2008). XIPs have been described in the moss *Physcomitrella patens* and in higher plants like poplar, tomato and tobacco (Danielson & Johanson 2008, Lopez *et al.* 2012) and are usually located in the PM (Fig. 11 (Bienert *et al.* 2011). XIPs show reduce water transport capacity (Reuscher *et al.* 2013), nevertheless they are able to transport other compounds such as glycerol, urea and boric acid (Bienert *et al.* 2011, Danielson & Johanson 2008, Gupta & Sankararamakrishnan 2009, Reuscher *et al.* 2013). Moreover, XIPs have modified both NPA motifs, being the first forms by (N/S, P, V/I) and the second one by NPARC which has a cysteine residue, highly conserved in this group (Danielson & Johanson 2008).

GlpF-like intrinsic protein (GIP) which are homologues of *Escherichia coli* GlpF and **hybrid intrinsic protein** (HIP) have only been found in *P. patens* (Danielson & Johanson 2008, Gustavsson *et al.* 2005).

In the same way, three AM fungal AQPs have been found in *Rhizophagus irregularis*. The first was called GintAQP1, its expression has been studied in different hosts and against various stresses, and in spite of not transporting water, its function seems to be related with compensating for the AQP expression of the host plant, or in unstressed mycelium (Aroca *et al.* 2009, El-Mesbahi *et al.* 2012). Most recently, GintAQPF1 and GintAQPF2 were isolated by Li *et al.* (2013b). These AQPs increased their expression under drought stress. Apparently, all of these AQPs could explain why usually AM plants improve water uptake capacity of the host plant under drought conditions.

In brief, there is trafficking of aquaporins inside the cells, and then there is not a determinate location for each aquaporin. As a result of that aquaporins move between different cellular compartments as shown in Figure I13.

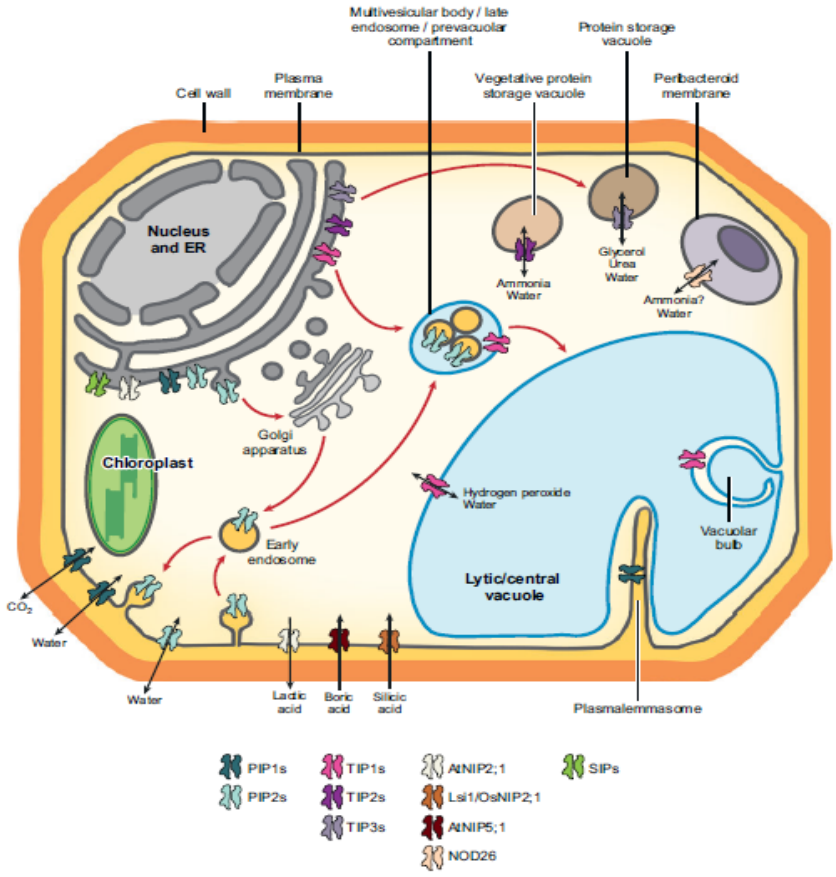


Figure I13. Scheme aquaporin trafficking between different subcellular compartments. Figure taken by Maurel *et al.* (2008).

Aquaporin Regulation

The aquaporins activity is regulated by several mechanisms like intracellular pH, phosphorylation state and divalent cations (Azad et al. 2008, Gerbeau et al. 2002, Maurel et al. 1995, Törnroth-Horsefield et al. 2006, Tournaire-Roux et al. 2003, Wu et al. 2012b). In this section, different aquaporin regulation mechanisms will be described.

Phosphorylation / dephosphorylation states

Protein phosphorylation is one of the most important and best characterized post-translational modifications which regulate the activity of proteins including aquaporins. Several studies have identified several phosphorilation sites in the aquaporins of different plants. For instance Johansson *et al.* (1998) found six serine (Ser) residues which were potential phosphorylation sites in PIP2 spinach aquaporins (Fig. I14). However, only Ser-115 in the first

cytoplasmic loop and Ser-274 located in the C-terminal region were involved in enhancing water transport. There have also been identified several sites of phosphorylation in Arabidopsis, highlighting the role of Ser-280 and Ser-283 in several isoforms of PIP2 (Prak *et al.* 2008). Prak *et al.* (2008) identified the presence of singly and diphosphorylated peptides of AtPIP2;1, AtPIP2;2, AtPIP2;3, AtPIP2;4, and AtPIP2;7, and a triphosphorylated forms of AtPIP2;4 and AtPIP2;7. In the case of AtPIP2;7, its triphosphorylated form was due to the presence of a phosphorylation in Thr-279 aside from of Ser-280 and Ser-283. In addition, they observed that these phosphorylation sites were regulated by stress conditions. For example, they noted that *L* reduction by NaCl stress was correlated to a decrease in the phoshorylation of Ser-283. In the same way, Prado *et al.* (2013) observed an increase arabidopsis rosette hydraulic conductivity under darkness conditions which was related to diphosphorylation of PIP2;1 in two serine positions (Ser-280 and Ser-283).

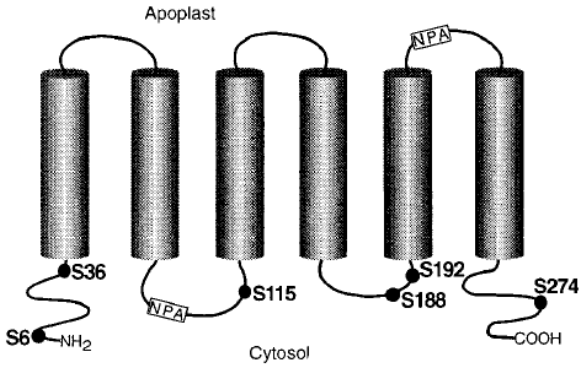


Figure I14. Model of *SoPIP2* showing potential phosphorylation sites on the cytoplasmic side of the membrane. Figure taken from Johansson *et al.* (1998).

Divalent cations

Several studies have been made to demonstrate that divalent cations can be involved in the regulation of aquaporins. Gerbeau *et al.* (2002) checked different cations, such as calcium (Ca^{2+}) barium (Ba^{2+}), strontium (Sr^{2+}) or magnesium (Mg^{2+}), and they observed that Ca^{2+} induced a 60% of reduction in Kexp (a fitted exponential rate constant involved in the calculation of osmotic water permeability of a membrane; Pf) whereas the rest of divalent cations reduced about 25% Kexp. In the same study, Mg^{2+} treatment reduced 35% *L* and Ca^{2+} also reduced 69% *L* in Arabidopsis plants. After that, Alleva *et al.* (2006) observed that Mg^{2+} inhibited Pf, however Ba^{2+} increased Pf about 30%, and Ca^{2+} increased it about 95% in beet root plasma membrane vesicles. In addition, they

found out that highest Pf was obtained when calcium was not present on both sides of the membrane and the most Pf inhibition was recorded when calcium was located on the cytoplasmic face. Subsequently, Törnroth-Horsefield *et al.* (2006) proposed that divalent cations serve to anchor loop D, through a network involving ionic interactions and hydrogen bonds, onto a short α-helix of the N-terminus and is critical for defending the close conformation of SoPIP2;1. On the other hand, Ca^{2+} application in plants subjected to salt stress improve water permeability of whole roots, root cortical cells and protoplasts (Azaizeh,Gunse & Steudle 1992, Carvajal,Cerda & Martinez 2000, Martinez-Ballesta,Martinez & Carvajal 2000). Therefore, more studies are needed to understand these effects.

Protonation

Medium acidification has also been shown to reduce membrane water permeability (Gerbeau *et al.* 2002, Sutka *et al.* 2005, Tournaire-Roux *et al.* 2003), and this inhibition is caused by an inactivation of aquaporins due to a histidine residue (His-199 in Arabidopsis and His-193 in spinach) in plasma membrane intrinsic proteins (PIPs) (Verdoucq,Grondin & Maurel 2008).

It has been demonstrated that lowering the cytoplasmic pH causes an inhibition of aquaporin water transport activity. Half-reduction of Kexp was observed at pH 7.2 - 7.5 and for pH < 6.0, water transport was maximally reduced to 20% of the level recorded at alkaline pH (Gerbeau *et al.* 2002). Alleva *et al.* (2006) observed that Pf was reduced when the pH was lower than 7, with a half reduction obtained at pH 6.6 and maximal inhibition at pH 5.5. Similarly to divalent cation effects, when pH of the interior of the vesicles was acid, Pf was markedly reduced. However, when the internal pH was basic, Pf was maintained at a high value. These results indicated that Pf changed to low pH only on the cytoplasmic face of the plasma membrane. This is because under low pH conditions, caused the rotation of the histidine side chain, which resulted in a salt bridge formation to Asp-28. Therefore, the hydrogen-bond-mediated anchor for loop D onto the N-terminus, which is lost when Ser-115 is phosphorylated (Törnroth-Horsefield *et al.* 2006).

Interaction between aquaporins

As it was explained above, the aquaporins are present in the membranes as tetramers. Fetter *et al.* (2004) discovered that different isoforms of PIPs can interact between them in order to form heterotetramers, which can improve the water transport capacity of a membrane. Some of their results showed that ZmPIP1;2 can interact with several forms of PIP2s like ZmPIP2;1, ZmPIP2;4 and ZmPIP2;5 in *Xenopus* oocytes resulted in an increase in Pf compared with the water

measurements obtained in each of them when they were expressed separately. Moreover, the co-expression between ZmPIP1;1 and ZmPIP1;2 was analysed and showed an increase in Pf whereas the co-expression between ZmPIP1;1 with ZmPIP2;5 did not work. The autors suggested that the two PIP1 isoforms could act in synergy to form functional water channels. Several studies have identified loop E like the responsible in the interaction between monomers to form the tetramer (Duchesne *et al.* 2002, Jung *et al.* 1994). The results of Fetter *et al.* (2004) have been confirmed later by other researchers (Temmei *et al.* 2005, Zelazny *et al.* 2007).

Internalization of aquaporins

Zelazny *et al.* (2007) observed that when ZmPIP1s (ZmPIP1;1, ZmPIP1;2 and ZmPIP1;6) were expressed alone, they were retained in the ER, whereas ZmPIP2s (ZmPIP2;1 and ZmPIP2;5) were located in the PM. Instead, when both aquaporins were co-expressed, ZmPIP1s were re-localized in the PM. As a result, it has been suggested that PIP1–PIP2 interaction is necessary to PIP1 trafficking to the PM. Then, Zelazny *et al.* (2009) identified the N- terminal diacidic motif (Asp-Ile-Glu) in ZmPIP2;4 and ZmPIP2;5 in maize plants as responsible of the trafficking of PIP aquaporins from ER to the PM and the mobilization of PIP1 isoforms to the PM. Then, Sorieul *et al.* (2011) identified an diacidic export motif (Asp-Val–Glu) in the N-terminal tail of AtPIP2;1 and noted that it was responsible of ER localization of PIPs. The PIPs with truncated motif only were located in ER, but not in the Golgi apparatus.

On the other hand, Boursiac *et al.* (2008a) noted that PIPs were accumulated in intracellular membranes after SA, H_2O_2 or salt treatment and this fact was correlated with a descent of *L*. Prak *et al.* (2008) observed that after salt treatment PIPs lacked phosphorylation of Ser-283, indicating that this phosphorylation also plays a role in regulating the trafficking of aquaporins. However, H_2O_2 treatment did not caused a decrease of Ser-283 phosphorylation place in spite of it induced the internalization of PIPs (Boursiac *et al.* 2008b, Prak *et al.* 2008). Hence, it is possible that H_2O_2 can regulate PIP trafficking by diacidic motifs.

Effect of different factors on *L* and aquaporins

Root anatomy and water transport pathways

Each plant has different root anatomy and can follow different strategies to uptake water. In this section, the anatomical properties of the roots will be highlighted, for example deposits of

suberin, which increase according to environmental conditions and the age of the plants (Carvajal,Cooke & Clarkson 1996, Peterson & Enstone 1996, Steudle,Murrmann & Peterson 1993). It was proposed that under non-stressed conditions, the water preferentially flows through the apoplastic route due to the transpiration rate of the plants. However, these suberin deposits like Casparian bands force water to flow through the cell-to-cell path, where aquaporins are involved (Steudle 2009). Vandeleur *et al.* (2009) observed that the decrease of *L* by drought conditions could be the result of suberin and lignin depositions. This reduced the apoplastic water movement which was not compensated by the water movement across cell-to-cell pathway. In addition, an increase in suberin contents was also observed in plants after salt treatments which was associated with a decrease of apoplastic pathway and uptake of Na^+ and Cl^- entrance into xylem vessels (Krishnamurthy *et al.* 2011, Ranathunge *et al.* 2011, Schreiber,Franke & Hartmann 2005). In the same way, low temperatures reduced *L* and this reduction was correlated with increased suberin content (Lee *et al.* 2005). Most recently was found that while plants with lower amount of suberin had higher *L* values; plants with higher amounts had the same *L* than control plants (Ranathunge & Schreiber 2011).

On the other hand, the influence of AQPs distribution in the roots in order to regulate *L* should be noted. Hachez *et al.* (2006) studied the localization of PIPs in primary root tips of *Zea mays*. Their results showed that all *ZmPIP* genes were expressed in primary roots, with the exception of *ZmPIP2;7* gene. Likewise, a positive relationship was found between the expression of *ZmPIPs* genes and the developmental stage of the root, where an increase in expression was noted from the elongation to mature zones. Additionally, an increase of the gene expression of *ZmPIP1;5* and *ZmPIP2;5* in the mature zones was observed. As a result of immunocytochemical localization technique, ZmPIP2;1 and ZmPIP2;5 were localized in the exodermis and endodermis. Moreover, a polar localization of ZmPIP2;5 to the external periclinal side of epidermal cells in root apices was observed, suggesting that ZmPIP2;5 could have a important function in the movement and uptake of water.

Drought

Drought is one of the main problems affecting today food production. Drought is known as the condition in which plants cannot get the amount of water needed to carry out its development due to low rainfall and high temperature (Breda *et al.* 1995, Chen *et al.* 2010). In these circumstances, the water potential of soil is similar or even lower than of the root, which hinders the

absorption of water as at the same time the transpiration rate starts to decline (Aroca *et al.* 2001, Breda *et al.* 1995, Duursma *et al.* 2008). Drought is characterized by a decrease in plant transpiration rate to prevent water loss, and by a reduction of *L*, in order to avoid flow of water outside roots (Aroca *et al.* 2006a, Aroca *et al.* 2008b, Gao *et al.* 2010, Trifilo *et al.* 2004).

It is known that under low transpiration conditions, the apoplastic path is reduced, gaining importance the cell-to-cell path (Javot & Maurel 2002, Steudle & Peterson 1998). However, other authors observed that *L* diminution under drought conditions correlates with an increase in the water movement by the apoplastic path (Siemens & Zwiazek 2003, Siemens & Zwiazek 2004).

Several studies have focused on changes in the expression and abundance of aquaporins under drought conditions. And although a clear pattern for each class of aquaporins was not reported, it could be said that in general a reduction of abundance and gene expression of PIP2s occurs (Aroca *et al.* 2008b, Barzana *et al.* 2014, Porcel *et al.* 2006a). Nevertheless, there are some exceptions including the study of Ruiz-Lozano *et al.* (2009), where two out of 4 *ZmPIP2s* genes decreased their expression and the other two remained unchanged.

With regard to PIP1s, the results are unclear. For instance, Vandeleur *et al.* (2009) observed a rise in *VvPIP1;1* gene expression in *Vitis vinifera* and Aroca *et al.* (2007) analysed the expression of three *PIPs* genes with different results between them since one increased, another one decreased and the last one remained unchanged in *Phaseolus vulgaris* plants. In the same way in maize plants, Ruiz-Lozano *et al.* (2009) observed that the expression of one out of three *PIP1* genes was reduced and Barzana *et al.* (2014) analysed five different isoforms of *PIP1* at two different water stress regimes and observed distinct pattern for each gene depending on the kind of drought applied. Under short term drought, the expression of two out of five *PIP1* genes was decreased and that of the another one elevated. Under sustained drought, the expression of all of them remained unchanged.

Other experiments have analyzed the effect of aquaporins overexpression under well-water and drought conditions. This includes the work made by Perrone *et al.* (2012) where *VvPIP2;4* was overexpressed in grapevine roots and it was observed that such overexpression improved stomatal conductance, gas exchange, shoot growth and *L* under well-water conditions, whereas it had a minimum role in *L* under drought. In addition, Aharon *et al.* (2003) observed that PIP1b overexpression had a negative effect during drought stress in tobacco plant because of causing faster wilting. In spite of our studies are focuser in PIP aquaporins, several studies overexpressed TIPs aquaporins. Peng *et al.* (2007) discovered that *PgTIP1* overexpression in *Panax ginseng*

diminished the drought tolerance when plants were grown in 10cm deep whereas when they were grown under 45cm deep, these plants were significantly more tolerant against water stress. Overall, more studies are needed to understand the role of aquaporins under drought conditions.

Hormonal effects on *L* and aquaporin regulation

Abscisic acid (ABA) has been the hormone most studied in relation to *L* and aquaporins. However, ABA effect on *L* is not clear, because in some reports exogenous ABA increased *L* (Aroca *et al.* 2006a, Aroca *et al.* 2008b, Hose,Steudle & Hartung 2000, Ruiz-Lozano *et al.* 2009) but in other reports the opposite was found (Beaudette *et al.* 2007). In plants subjected to drought, an exogenous ABA application rendered higher *L* than plants without exogenous ABA treatment (Ruiz-Lozano *et al.* 2009). At the molecular level, Mahdieh and Mostajeran (2009) found an increase in PIP gene expression and protein abundance in tobacco roots after exogenous ABA application. However, the response of *PIP* gene expression to ABA depends on the dose used (Beaudette *et al.* 2007). Moreover, Ruiz-Lozano *et al.* (2009) found a down-regulation of several *PIP* genes in AM maize plants and an up-regulation in non-AM plants under drought conditions after exogenous ABA application. Most conclusive are the results from plants with different levels of ABA, where a positive correlation was found between ABA contents and expression and abundance of PIP proteins (Parent *et al.* 2009). In addition, Thompson *et al.* (2007) observed an increase of *L* in transgenic tomato plants with higher contents of ABA.

As in the case of ABA, but less studied, contradictory results have been found about the role of ethylene in regulating *L*. Therefore, there are also studies in which ethylene can increase or decrease *L* (Islam,MacDonald & Zwiazek 2003, Li *et al.* 2009).

On the other hand, Boursiac *et al.* (2008a) found that SA application in Arabidopsis plants reduced *L*. It is also known that exogenous IAA application induces a repression of expression of the majority of aquaporins genes in Arabidopsis (Peret *et al.* 2012). In relation with JA only Lee *et al.* (1996b) observed that methyl jasmonate (MeJA) application improved root water free exudation under chilling conditions as well as closing the stomata in rice plants. In brief, little is known about the hormonal effects on *L*.

Role of AM symbiosis in regulating *L*

As it was said above, AM symbiosis is known to improve the water status in plants (Allen *et al.* 1982, Jahromi *et al.* 2008). New investigations have been focused on study of efficiency of

different fungi and their implication on molecular parameters. For instead, Marulanda *et al.* (2003) analysed the capacity of several AMFs to enhance the capacity of the host plant to take up water from the soil. They found that *Rhizophagus irregularis* and *Funneliformis mosseae* among the six AMFs tested had the highest capacity for taking up water. However, the strategies followed by each fungus to protect the host plant against water deficit were different. *F. mosseae* down-regulated *PIP* gene expression, and decreased the abundance of PIP proteins. By contrast, *R. irregularis* did not change the expression of *PIP* genes under drought stress and was most effective in improving plant water uptake (Marulanda *et al.* 2007, Porcel *et al.* 2006a). Therefore, *F. mosseae* seems to enhance a water conservation mechanism. In addition, it has been reported that AM symbiosis improves the apoplastic pathway under both well-watered and drought conditions (Barzana *et al.* 2012). In another work, it was observed that nine out of sixteen aquaporin genes decreased their expression by AM root colonization in maize plants under well-watered conditions, however under drought conditions, six different expression patterns were found in these aquaporins, depending on the degree of drought applied (Barzana *et al.* 2014). Highlighting, ten out of sixteen aquaporins increased their expression in AM roots and six of them did not change under short-term drought conditions. Under sustained drought it was found that the expression of seven aquaporins genes were not changed by AMF, six of them decreased their expressions and three increased their expressions in AM roots (Barzana *et al.* 2014).

Recently, several AM fungal aquaporins have been cloned. Aroca *et al.* (2009) cloned a fungal aquaporin called *GintAQP1* from *R. irregularis*. Its expression varied depending on the host plant species and on environmental conditions. Since it was proven that *GintAQP1* failed to transport water in *Xenopus laevis* oocytes, its function remains unknown (Aroca *et al.* 2009). Li *et al.* (2013b) have cloned two new fungal AQP genes in *R. irregularis*, *GintAQPF1* and *GintAQPF2*, which exhibited capacity for transporting water in heterologous systems, and whose expression increased under drought stress. Thus, the ability of AM plants to take up more water under drought conditions than non-AM plants may be mediated by these fungal AQPs.

Aims of the study

Aims of study

The general aim of the thesis was to investigate the role of jasmonic acid (JA)-related substances, nitric oxide (NO) and arbuscular mycorrhizal (AM) symbiosis in the root hydraulic properties of plants cultivated under well-watered and drought stress conditions.

To achieve this overall objective, the following specific objectives were defined and investigated throughout the chapters presented in this thesis.

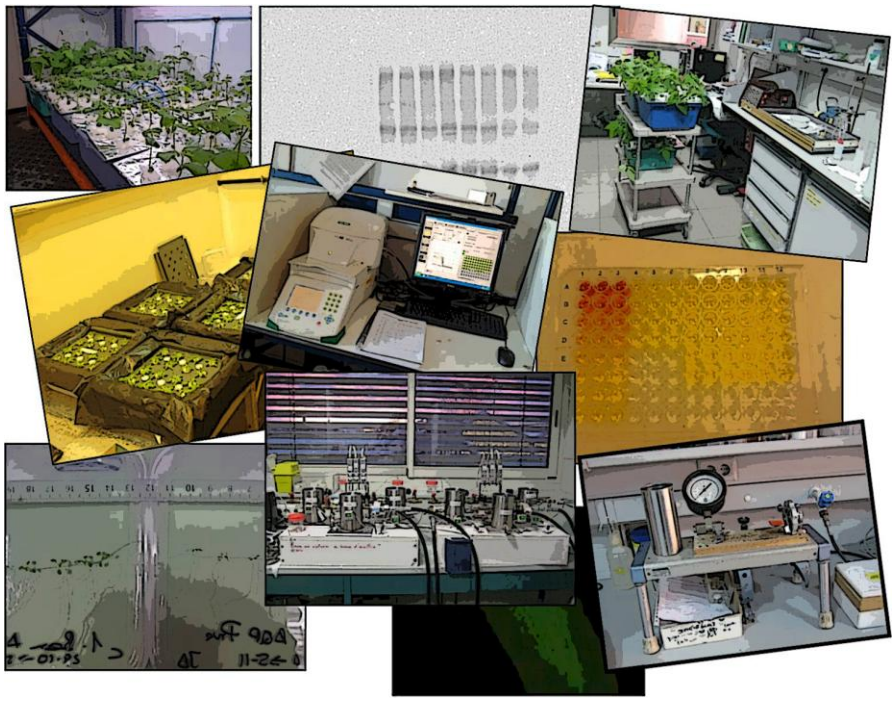
1. To determine the role of jasmonic acid in the regulation of root hydraulic properties, and the involvement of abscisic acid and calcium in such regulation.

2. To study the effect of NO and JA in the establishment and functionality of AM fungi.

3. To analyse changes in the expression and abundance of aquaporins caused by JA, NO and AMF in roots of plants cultivated under well-watered and drought stress conditions.

4. To determine the effects of NO and JA on root hydraulic properties in plants cultivated under to well-watered and drought stress conditions and their possible interactions with other plant hormones.

Material and Methods



Material and Methods

Plant grown under hydroponic conditions and greenhouse experiment

The growth conditions of each experiment will be explained at the beginning of each chapter. This section contains additional information that is not provided in these chapters.

Soil characteristics (Chapters 2, 3 and 4)

Loamy soil was collected from the Granada province (Spain), sieved (5 mm), diluted with quartz sand (0.2 mm) [1:2, soil:sand, v/v in Chapters 2 (bean experiment) and 3 (tomato experiment) and 1:1 in Chapter 4 (lettuce experiment)] and sterilized by steaming (100 ºC for 1 h on three consecutive days). The original soil used in bean and tomato experiments had a pH of 8.2 [measured in water 1:5 (w/v)], 1.5% organic matter and nutrient concentrations (g kg^{-1}) as follows: N, 1.9; P, 1.0; K, 6.9. The characteristics of the soil used in the lettuce experiment were pH of 8.1, 1.8% organic matter and nutrient concentrations (g kg^{-1}) as follows: N, 2.5; P, 6.2; K, 132.

Biological material (Chapters 2, 3 and 4)

Mycorrhizal inoculum was bulked in an open-pot culture of *Trifolium repens* L. mixed with *Sorghum vulgare* Pers. X *Sorghum*×*drummondii* (Steud.) Millsp. & Chase plants and consisted of substrate (vermiculite:sepiolite, 1:1), spores, mycelia, and infected root fragments. Ten grams of inoculum was applied to each pot (1 Kg) at the same time as the seeds. The AMF species was *Rhizophagus irregularis* isolated BEG 121. Uninoculated plants received the same amount of sterilized inoculum and 2 ml aliquot of the filtered AM inocula to provide a general microbial population free of AM propagules.

Physiological parameters

Biomass production

At harvest, shoots and washed roots were separated and weighed independently to obtain fresh weights. Then, the samples were dried in an oven (75ºC) for 2 days to determine the dry weights (DW).

Symbiotic development (Chapters 2, 3 and 4)

The percentage of mycorrhizal root length colonization of several roots was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980).

Leaf relative water content (RWC) (Chapters 2, 3 and 4)

Small leaf pieces from the last fully developed leaves of several different plants from each treatment were weighed (fresh weight (FW)) immediately after harvesting, then placed in a water saturated vial at 4ºC for 48 h and weighed (turgid weight (TW)). The samples were then dried in an oven at 75ºC for a period of 48h and their dry weights (DW) were obtained. Then RWC was calculated using the following equation: ((FW – DW)/ (TW-DW) x 100).

Stomatal conductance (gs) (Chapters 2, 3 and 4)

Stomatal conductance was measured with a porometer system (Porometer AP4; Delta-T Devices Ltd, Cambridge, UK) following the manufacturer's instructions. Stomatal conductance measurements in tomato and bean plants were taken in the apical petiole of the second and third leaf of the last fully developed leaf from several different plants from each treatment. By contrast, gs measurements in lettuce were taken in the first green leaf fully developed in several different plants from each treatment. The measurements were taken 2 h after sunrise.

Daily water consumption (Chapters 2, 3 and 4)

During one week, daily water consumption of each plant was controlled. The amount of water to be supplied to each pot per day to maintain constant the percentage of soil moisture was recorded. The methods used were gravimetrically method and ML2 ThetaProbe (AT Delta-T devices Ltd., Cambridge, UK) method.

Gravimetric method. This method was only used in Chapter 3. Previously at this analysis, the soil field capacity was calculated which is the difference between the water content that has a soil that was saturated and then drained (150 ml).

Considering soil field capacity previously measured, moisture percentages were adjusted by weighing each pot and reaching the required amount of water that corresponded to that percentage (1150g well-watered plants and 1050g droughted plants).

ML2 ThetaProbe method (AT Delta-T devices Ltd., Cambridge, UK). This method was used in Chapter 2 and Chapter 4. ThetaProbe measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil. The volumetric soil moisture is the ratio between the volume of water present and the total volume of the sample. This is a dimensionless parameter and it is expressed as a percentage (%vol). The moisture percentage to well-watered plants was 20% and to drought conditions 11% and 14% in bean plants and lettuce plants, respectively.

Chlorophyll content (Chapter 3)

The samples (100 mg aprox.) were frozen with liquid nitrogen and then crushed in a mortar. To each sample was added 1.5 ml of 100% methanol. The resulting mixture was transferred into a 2 ml Eppendorf tube and agitated for 30 minutes. At the end of agitation period samples were centrifuged at 17,700 *g* for 5 min at 4 °C and the supernatant was recovered. Thereafter, 1:25 diluted samples were measured in a spectrophotometer at 652.4 and 665.2 nm. Two replicates per sample were performed. Total chlorophyll concentration was calculated as: $C\ a+b = 1.44 \times A_{665.2} + 24.93 \times A_{652.4}$ according to Lichtenthaler (1987).

Percentage of yellow leaves (Chapter 3)

Leaves were separated into yellow and green and the dry weight was determined separately. So the percentage of yellow leaves was calculated using the following formula: YLDW/ (YLDW + GLDW) where YLDW is the dry weight of yellow leaves and GLDW is the dry weight of green leaves.

Root hydraulic conductivity (*L*)

L was measured by three methods:

1. Free exudation method (Chapters 1, 2 and 4)

L of bean, tomato and lettuce plants were measured with free exudation method as described previously (Aroca 2006). Under these conditions, water circulates through roots following the osmotic gradient between the root bathing solution and the root xylem. Therefore, according to Steudle's model (Steudle & Peterson 1998), water mostly flows through the cell-to-cell pathway. Bean plants were excised with a razor blade just below the cotyledons, in the case of tomato and lettuce plants, their stems were cut just below the first branches. In plants grown in pots, they were immersed in aerated nutrient solution, resembling hydroponic conditions. A pipette connected to a silicon tube was attached to the stem. The liquid exuded from the root in the first 15 min was discarded to avoid phloem contaminations. Plants were maintained under exuding conditions for 90 minutes, and the exudates were collected and weighed. Also, the root dry weight of each plant was determined after incubation during 2 days at 75ºC. The osmolarity of the exuded sap was determined using a cryoscopic osmometer (Osmomat 030, Gonotec Gmbh, Berlin, Germany). *L* was calculated as: $L = Jv/\Delta\Psi s$, where Jv is the exuded sap flow rate expressed in a root dry weight basis and $\Delta\Psi s$ is the osmotic potential gradient between the exuded sap and the solution. According to Fiscus (1986) we assume that reflection coefficient of bean plants was very close to 1 (0.98 ± 0.01), so we did not include it in the calculation of *L*. We assumed the same value for lettuce plants.

2. Pressure chamber method (Chapter 1)

L of bean and tomato roots was measured by pressurizing the roots in a pressure chamber (SF-Press-Root, SolFranc, Tarragona, Spain). The stems of tomato and bean plants were excised

with a blade, three centimetres above the root. Plastic tubes were connected to the stems to collect the exudates. The root systems were placed inside pressure chamber with the nutrient solution and chemical of the different treatments. Finally, the roots were subjected to different pressures (0.2, 0.3, 0.4, 0.5 MPa) for 1 min at each pressure. The exuded sap was collected and weighed. For Arabidopsis, pressure chamber measurements were performed as described by Boursiac *et al.* (2005) and Javot *et al.* (2003). In all cases, sap flow was expressed in mg g (root dry weight)$^{-1}$ h $^{-1}$ and plotted against pressure (MPa), with the slope being the *L* value in mg g (root dry weight)$^{-1}$ h^{-1} MPa $^{-1}$.

3. High pressure flow meter method (HPFM) (Chapter 3)

Root hydraulic conductivity (*L*) was calculated using a high pressure flow meter (Lt, kg cm^{-3} s^{-1} MPa^{-1}) (HPFM, Dynamax, Inc., Houston). Detached roots were connected to the HPFM and water was pressurized into the root from 0 to 0.5 MPa in the transient mode to calculate root hydraulic conductance (Kr). *L* was determined by dividing Kr by the root dry weight (Calvo-Polanco *et al.* 2014a).

Molecular measurements

Quantitative real-time RT-PCR

Expression analyses of known PIP genes were carried out by quantitative real-time RT-PCR, using an iCycler (Bio-Rad, Hercules, CA, USA) (Benabdellah,Ruiz-Lozano & Aroca 2009). Total RNA was isolated from roots by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay *et al.* 1987). DNase treatment of total RNA and reverse transcription were done following Qiagen´s protocol (Quantitect Reverse Transcription KIT Cat#205311, Qiagen, CA). The PCR mix contained 1 µl of cDNA (100 ng µl^{-1}), 10.5 µl of Master Mix (Bio-Rad Laboratories S.A, Madrid), 8.6 µl of deionized water, and 0.45 µl of each primer pair (1µM). The PCR program was different depending on the plant species and gene analysed.

The relative abundances of all transcripts were calculated by using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen 2001). Expression analyses were carried out in three independent RNA samples coming from a pool of three different root samples, and were repeated two times for each RNA sample.

1. Bean gene expressions (Chapters 1 and 2)

The bean PIP genes analysed were: *PvPIP1;1* (Acc. No. U97023), *PvPIP1;2* (Acc. No. AY995196), *PvPIP1;3* (Acc. No. DQ855475), *PvPIP2;1* (Acc. No. AY995195), *PvPIP2;2* (Acc. No. EF624001), and *PvPIP2;3* (Acc. No. EF624002) (to see sequence primers Table M1). Specific primers are described in Benabdellah *et al.* (2009). The PCR program consisted of a 3 min incubation at 95 ºC to activate the hot-start recombinant Taq DNA polymerase, followed by 32 cycles of 30 s at 94 ºC, 30 s at annealing temperature and 30 s at 72 ºC. Annealing temperatures were 58 ºC for *PvPIP1;1, PvPIP1;3, PvPIP2;1* and *PvPIP2;3*, and 60 ºC (50s) for *PvPIP1;2* and *PvPIP2;2*. Standardization was carried out based on the expression of the *P. vulgaris* ubiquitin gene in each sample, as measured by using *P. vulgaris* ubiquitin-specific primers (Acc. No. KF378810）.

Table M1. Quantitative real-time PCR primers sequence of aquaporin genes from *P.vulgaris*.

P.vulgaris	**Gene**	**Forward:**	**Reverse:**
Constitutive	Ubiquitin	CCAAGGAACTTCAGATTGCTG	GTCATCACCATCATCCATTCC.
Aquaporin gene	*PvPIP1;1*	ATTAGGTGGTGCATGTGTCG	CAGCCGTACATCACACATAGAG
	PvPIP1;3	GTGGGTAAAAGAACCAGACG	CACATCGCTCATGGTCATC
	PvPIP2;1	CTCCAACAAGGCTATGGTGT	CCCACAACAACTGTAGCTGA
	PvPIP2;2	GAAGGAAAAAAACATGGAGGGGA AGGAGC	AGGGGACCTGAGGACACCCATG
	PvPIP2;3	CTTCCTCCCACATTCATTCTACCAC CAATG	ACTCGGTGTTACCGGAATACTGGT

Table M2. Quantitative real-time PCR primers sequence of aquaporin genes from *S. lycopersicum*.

S. lycopersicum	**Gene**	**Forward:**	**Reverse:**
Constitutive	Ubiquitin	ATGGCCGCACCCTTGCAGAC	CAAGTCACCGCCACGGACCC
Aquaporins gene	*SlPIP1;1*	GAAGAGTTGAAGAGACCTTTT	GGAGAAAGAAAACTTTAGATAC
	SlPIP1;2	GAGGAACACCTTGGCCTATTG	GTGTATGTTATTTGCAGATAAC
	SlPIP1;5	GTTATCTGTCTTTAAACAATGGG	CAAAGATAAAAATAAAGAACCTTC
	SlPIP1;7	GGATCTGAATTCAATCATTTTCC	GGAAAAGGCAAGGTACTTTTG
	SlPIP2;1	GTACAATTATCATGAAGAATTGAA	GGACAATAGCCTTGCTATTTTC
	SlPIP2;4	GATTTCTAATTTCATCAATCCAATG	CAACAATGTGAGTTGAAACTATTG
	SlPIP2;6	GATAAAAGATGGAATAATTTGAG	CCCAACATATACACTATTATTAT
	SlPIP2;8	CAATAACTAAAGCATTCAATTGATC	GAAAAATATGACCAATACTAATAAG
	SlPIP2;9	CAAGTTATCAACTTTCATTCAC	CGAAAGAAGAATAGACCACC
	MC	GAGAATTTCAAGGAAGTTCAA	GGCTTTATTTCACACAGAGATA
	AOS2	AGATTTTCTTCCCGAATATGCTGAA	ATACTACTGATTTCATCAACGGCAT

2. Tomato gene expressions (Chapters 1 and 3)

Root aquaporins expression were determined for eight tomato PIP genes, *SlPIP1;1* [Tigr. No.:TC175784], *SlPIP1;5* [Tigr. No.:TC178447], *SlPIP1;7* [Tigr. No.:TC170092], *SlPIP2;1* [Tigr. No.;TC170322], *SlPIP2;4* [Tigr. No.:TC181456], *SlPIP2;6* [Tigr. No.: TC175989], *SlPIP2;8* [Tigr. No.:TC180270] and *SlPIP2;9* [Tigr. No.:TC173223] as described in Sade *et al.* (2009) (to see sequence primers table M2). The PCR program consisted of a 3 min incubation at 95 ºC to activate the hot-start recombinant Taq DNA polymerase, followed by 31 cycles of 30 s at 94 ºC, 30 s at annealing temperature and 30 s at 72 ºC. Annealing temperatures were 58 ºC for *SlPIP1;1*, *SlPIP1;5*, *SlPIP 1;*7, *SlPIP 2;1*, *SlPIP 2;4*, and *SlPIP2;6*, 55 ºC for *SlPIP2;9* and 57 ºC for *SlPIP 2;8.*

To determine the JA deficiency of *def-1* tomato plants, the expression analyses of JA biosynthetic enzyme allene oxide synthase 2 (Acc. No. AF230371) (AOS 2) and multicystatin (Acc. No. AF083253) (MC) genes, both genes being responsiveness to MeJA (Lopez-Raez *et al.* 2010), were analysed in WT and *def-1* roots. The primers used and the PCR protocol were the same as described by López-Ráez *et al.* (2010).

All standardization was carried out based on the expression of the *S. lycopersicum* ubiquitin gene in each sample, as measured by using *S. lycopersicum* ubiquitin-specific primers (Acc. No. XM_004249837).

3. Fungal aquaporins expression (Chapters 2 and 3)

For fungal AQPs, the analysed genes were: *GintAQP1* (Acc.No. FJ861239) (to see sequence primers table M3) described by Aroca *et al.* (2009) and *GintAQPF1* (Acc. No. JQ412059) and *GintAQPF2* (Acc. No. JQ412060) described by Li *et al.* (2013b). The primers used to amplify fungal genes were described previously (Aroca *et al.* 2009, Li *et al.* 2013b). The PCR program consisted of a 3 min incubation at 95 ºC to activate the hot-start recombinant Taq DNA polymerase, followed by 40 cycles of 30 s at 94 ºC, followed by annealing temperature and 30 s at 72 ºC. Annealing temperatures were 60 ºC (30 s) for *GintAQP1*, 55 ºC (50 s) for *GintAQPF1*, and 58ºC (30 s) for *GintAQPF2*. Standardization was carried out based on the expression of the ribosomal 18S gene (Gint18S, Acc. No. HE817884) in each sample (Aroca *et al.* 2009)

Table M3. Quantitative real time RT-PCR primers sequence of aquaporins genes of *R. irregularis.*

Rhizophagus irregularis	**Gene**	**Forward:**	**Reverse:**
Constitutive	*GINT18S*	TGTTAATAAAAATCGGTGCGTTGC	AAAACGCAAATGATCAACCGGAC
Aquaporins gene	*GintF1*	TCAAAATGCTAGATGCAGAAC	TTAGTTTTGGGATTCTATGTC
	GintF2	ATGGCGGATGAACGTGGACCG	CTAGGCTACTGCTCTATGCTC
	GintAQP1	AGGACTCGGAGGTAGTGATGC	GCCGGATATATCACTCCAAAGC

Northern blot (Chapter 4)

Total RNA was extracted from lettuce roots as described above. Total RNA samples (15 mg each) were fractionated by electrophoresis on a MOPS–formaldehyde–formamide 1.5 % agarose gel, and transferred by capillary action overnight to Hybond-N membranes (Amersham Biosciences, Piscataway, NJ, USA) using 20X SSC (3 M NaCl and 0.3 M sodium citrate).

The RNA was fixed by baking at 80 ºC for 30 min. Pre-hybridization was performed at 65 ºC for 2 hours in a solution of NaH_2PO_4 (pH 7.2), 1mM EDTA and 7% SDS (w/v). 32P-labelled cDNAs of the 30'-UTR of *LsPIP1* gene (Porcel *et al.* 2006a) was added to the solution. Hybridization was performed overnight, followed by five consecutive 5 min washings, the first two were made with 2X SSC and 0.1% SDS (w/v) at room temperature, and the next two were made with 0.5X SSC and 0.1% SDS (w/v) at 65ºC, and the last one was made with 0.5X SSC and 0.1% SDS (w/v) at 60ºC. Detection of the labeled probe was performed by phosphorimaging. Quantification of gene expression was performed by dividing the intensity value of each band by the intensity of the corresponding rRNA stained with ethidium bromide (Martınez and Chrispeels, 2003). Blots were repeated three times with two different sets of plants.

Microsome isolation (Chapters 1, 2, 3 and 4)

Microsomal membranes were isolated from roots as described by Hachez *et al.* (2006). Briefly, roots were homogenized in a grinding buffer (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 250 mM sorbitol, 0.2 mM aminoethylbenzenesulfonylfluoride, 2 µg ml^{-1} aprotinin, 1 µg ml^{-1} leupeptin, 1 µg ml^{-1} chymostatin, 1 µg ml^{-1} pepstatin). A supernatant was collected after centrifugation at 15,000 *g* for 10 min. It was filtered through a double layer of cheesecloth and centrifuged again at

100,000 *g* for 2 h. The pellet was resuspended in 40 µl of 5mM KH_2PO_4, 330 mM sucrose and 3 mM KCl, pH 7.8. Finally the samples were sonicated for 15 seconds.

Enzyme-linked Immunosorbent Assay (ELISA)(Chapters 1, 2 and 3)

Plates containing 1 µg of microsomes in the case of beans roots and 2 µg of microsomes for tomato roots in each well, were incubated overnight at 4ºC with coating buffer (0.05 M carbonate/bicarbonate, pH 9.6). The plates were then washed three times for 15 minutes with TTBS (Tris-buffered-saline with 0.05% Tween 20) and blocked for 1 h at room temperature with 1% BSA in TTBS, then washed again three times for 10 minutes with TTBS. After that, the plates were incubated with dilutions of antibodies (different depending on the kind of plant) raised against 26 first aa of N-terminal peptide of PvPIP1;3 (Acc. No. DQ855475), and the last 12 aa of C-terminal peptide of PvPIP2;1 (Acc. No. AY995195),(Marulanda *et al.* 2010). Also, an antibody against phosphorylated PIP2 at the C-terminal end of PvPIP2;1 (CAIKALG{pSER}FRSNA) was used. These antibodies recognize several PIPs from different plant species including tomato (Calvo-Polanco *et al.* 2014a). To check each PIP2 antibody specificity, we made an ELISA test where we cross reacted each one of the target peptides with each antibody (Table M4). The results showed that each antibody was very specific to its target protein. These antibodies were developed in rats to PIP1 and in rabbits to PIP2 (no phosphorylated and phosporylated isoforms) by Abyntek Company (Bilbao, Spain). The quantification of aquaporin amounts was done by means of standard curves, using specific peptides as standards. Total protein amount was quantified by the Bradford method using BSA as a standard (Bradford & Williams 1976). Goat anti-rat Ig coupled to horseradish peroxidase (Sigma) to PIP1 and goat anti-rabbit Ig coupled to horseradish peroxidase (Sigma) to different PIP2s isoform, were used as secondary antibody at a specific dilution (different depending on the kind of plant). The signal was developed using a 3, 3´, 5, 5´ tetramethylbenzidine (TMB) substrate (SIGMA), which detects horseradish peroxidase (HRP) activity, yielding a blue color (Amax = 370nm and 652nm) that turns to yellow (Amax = 450nm) upon addition of a 2 M sulphuric acid solution.

The dilutions used in each experiment were for Chapters 1 and 2, the primary antibody dilution was 1:2,000 and secondary antibody dilution was 1:10,000. In the Chapter 3, tomato aquaporin abundance was measured using a primary antibody dilution of 1:200 and secondary antibody dilution of 1:10,000.

Peptide/ Antibody	PIP2	PIP2PH
Pept PIP2	**1.21**	0.11
Pept PIP2PH	0.09	**1.02**
BSA	0.08	0.07

Table M4. Specificity of antibodies to recognize their corresponding peptides. The table shows the specificity of each antibody to link at different aquaporin proteins and BSA (bovine serum albumin). We have antibodies against the C-terminal peptide PIP2 and one antibody against phosphorylated PIP2 at the C-terminal, like PIP2PH (Ser-280). These results were obtained using ELISA technique.

Western blot analysis (Chapter 4)

Fifteen micrograms of extract microsomal membranes were solubilised for 15 minutes at 60ºC in denaturing buffer (27 mM Tris / HCl, 0.7% SDS, 3.3% glycerol, 0.0016% bromophenol blue, and 1% DTT). Proteins were separated by SDS-PAGE gel of 12% acrylamide. and were transferred to a membrane of polyvinylidene fluoride (PVDF, Millipore) for 1 hour at 110 mA, the membranes were preincubated for 1 minute in pure methanol and 5 minutes in the buffer.

Then, the membranes were blocked for 2 hours at room temperature with 5% (w / v) milk powder in TTBS. After that, the membranes were incubated overnight at 4 ° C with a 1: 500 dilutions of antibodies raised against amino-terminal peptide of PvPIP1;3 (previously mentioned). This region is highly conserved in different PIP1 aquaporins of different plant species. Next, three washes were performed each fifteen minutes with TTBS, starting the incubation with secondary antibody at a dilution of 1: 5000. The signal was detected using a chemiluminescent reagent (West-Pico, Super Signal, Pierce, Rockford, IL, USA). Microsomes were isolated from two different root samples. The equal loading of the proteins in the different lines was confirmed by staining the gel with coomassie brilliant blue. To quantify the immunoblot signal, the intensity of each band was measured using Adobe PhotoShop 8.0.1 (Adobe Systems, Mountain View, CA), corrected for the background and normalized against the intensity of the corresponding coomassie brilliant blue band (Aroca *et al.* 2005).

Calcium exudation rate by the xylem (Chapter 1)

A pressure chamber was used to collect the sap exudates of four plants of each treatment (control plants and plants treated for 24 h with 1 mM EGTA). For this, bean plants were grown under hydroponic conditions as described in Chapter 1, placed inside a pressure chamber at 0.25 MPa until 1 ml of sap was obtained. Calcium content was determined by the Ionomic Service of Estación Experimental del Zaidín, CSIC, Granada, Spain by means of ICP-OES technique (ICP-OES 720-ES Agilent Technologies, Santa Clara, CA, USA). The calcium exudation rate was expressed as μmol ml^{-1} h^{-1} g^{-1} root dry weight.

Cytosolic Ca^{2+} accumulation (Chapter 1)

Small root pieces were obtained from control and MeJA (100 μM)-treated bean plants. Measurements were taken 1 and 24 h after application of MeJA. Pieces taken 2 cm from the root apex and also root apieces were analysed. The pieces of root were incubated in a solution containing 20 μM Fluo-4/AM dye, 50 mM sorbitol and 5 mM HEPES (pH 7.0) and 0.03% (v/v) Pluronic, at room temperature for 90 min in the dark, followed by washing with 50 mM sorbitol and 5 mM HEPES. Before measurements, samples were kept in the dark at room temperature for 25 minutes. Fluorescence was examined with a Nikon Eclipse inverted microscope equipped with epifluorescence and appropriate filter sets (peak excitation, 590 nm; peak emission, 617 nm). Photographs were taken with a DS-Fi1 Nikon camera. Root samples were mounted in a chamber with a clean cover slip attached to the bottom. The roots were observed with green fluorescence (520 nm). The recovery of fluorescence was quantified by means of ImageJ software (Rasband W.S., NIH, http://imagej.nih.gov/ij/) which allows the measurement of the mean grey value within a region of interest.

Cell image analysis of Arabidopsis GFP seedlings (Chapter 1)

Detection of aquaporins labelled with Green Fluorescent Protein (GFP). We used three kinds of transgenic Arabidopsis seeds expressing a specific aquaporin fused to GFP (p35S:: PIP1.1-GFP, p35S::GFP-PIP1.2 and p35S::GFP-PIP2.2) and a control membrane protein (p35S::GFP-LTi6a), all under the control of the 35S CaMV promoter (p35S) (Boursiac *et al.* 2005, Cutler *et al.* 2000).

Images were captured at 1 cm from root tip with an inverted confocal laser-scanning microscope (LSM 510 AX70, Zeiss, Göttingen, Germany) with a 40 × water immersion or a 63 × oil immersion objective, essentially as described by Boursiac *et al.* (2005). Excitation wave length (as provided by an argon laser) was 488 nm and the fluorescence emitted by the GFP constructs was detected with a filter set for fluorescein isothiocyanate (BP 500 to 530). Fluorescence was quantified by means of ImageJ software as described (Boursiac *et al.* 2008a).

Hormone root concentration

Root ABA (Chapters 1, 2 and 3) and IAA (Chapters 2 and 3) accumulation

IAA and ABA root concentrations in tomato and bean plants were determined as explained by Bacaicoa *et al.* (2011). The concentration of IAA and ABA were analysed in root extracts using high performance liquid chromatography-electrospray-mass spectrometry (HPLC-ESI-MS/MS). The extraction and purification of these hormones were carried out using the method described by Dobrev and Kamínek *et al.* (2002), with some variations. Frozen plant tissue (0.5 g), previously ground to a powder in a mortar with liquid N, was homogenized with 5 ml of pre-cooled (-20 ºC) methanol:water (80:20, v/v) and 2.5 mM Na diethyldithiocarbamate (DDTC). The deuterium labeled internal standards ([$^2H_5$] indol-3-acetic acid, (D-IAA); [$^2H_6$] (+)-cis, trans-abscisic acid, (D-ABA) were added (100 µl of a stock solution of 400 ng ml^{-1} of each standard in methanol) to the extraction medium. After overnight extraction at -20 ºC, solids were separated by centrifugation at 12,000 *g* for 10 min at 4 ºC using a Centrikon T-124 centrifuge with an A8.24 rotor (Kontron Instruments, Cumbernauld, United Kingdom) and re-extracted for 1 h with an additional 4 ml of extraction mixture. Supernatants were passed through a Strata C18-E cartridge (3 cm^3, 200 mg) (Phenomenex, Torrance, CA; Ref. 8B-S001-FBJ), preconditioned with 4 ml of methanol followed by 2 ml of extraction medium. After evaporation at 40 ºC using a Labconco Vortex Evaporator (Labconco Co., Kansas City, MO), 0.5 ml of 1 M formic acid was added. Then, hormones were extracted successively with two portions of 5 and 4 ml of diethyl ether, and the organic phase was evaporated to dryness. The residue was re-dissolved in 250 µl of methanol:0.5% acetic acid (40:60, v/v). Before the injection in the HPLC-ESI-MS/MS system, the solution was centrifuged at 8,000 *g* for 5 min.

Hormones were quantified by HPLC-ESI-MS/MS using a high-pressure liquid chromatograph (2795 Alliance HT; Waters Co., Milford, MA) coupled to a 3200 Q TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an

electrospray interface. A reverse-phase column (Synergi 4 µm Hydro-RP 80A, 150x2 mm; Phenomenex, Torrance, CA) was used. A linear gradient of methanol and 0.5% acetic acid in water was used: 35% A for 1 min, 35% to 95% A in 9 min, 95% A for 4 min and 95% to 35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 ml min^{-1}, the injection volume was 40 µl and column and sample temperatures were 30 and 20 ºC, respectively.

Table M5. Optimal parameters for the detection of IAA, ABA, MeJA, MeSA, SA and JA used in the HPLC-ESI-MS/MS analysis. Deuterium labeled internal standars (D-IAA, D-ABA, D-MeJA, D-MeSA, D-SA and D-JA). Abbreviation : DP, declustering potential; EP, entrance energy; CEP, collision cell entrance potential, CE, collision energy, CXP, collision cell exit potential.

Compound	Transition		Ionization and Collision parameters				
	Q1 (uma)	Q3 (uma)	Dwell (ms)	DP (V)	EP (V)	CEP (V)	CE (V)
IAA	174	130	40	-30	-3	-12	-14
	174	128	40	-30	-3	-12	-22
D-IAA	179	135	40	-20	-7	-12	-14
	179	133	40	-20	-7	-12	-26
ABA	263	153	40	-25	-3.5	-20	-16
	263	219	40	-25	-3.5	-20	-16
D-ABA	269	159	40	-25	-12	-14	-14
	269	225	40	-25	-12	-14	-18
MeJA	225	151	80	-26	-8	-15	-18
	225	133	80	-26	-8	-15	-21
D-MeJA	227	151	80	-26	-8	-15	-19
	227	133	80	-26	-8	-15	-21
MeSA	153	121	60	-36	-4	-12	-19
	153	65	60	-36	-4	-12	-41
D-MeSA	156	121	60	-26	-4.5	-12	-23
	156	65	60	-26	-4.5	-12	-39
SA	137	93	40	-25	-6	-10	-20
	137	65	40	-25	-6	-10	-38
D-SA	141	97	40	-30	-5.5	-10	-20
	141	69	40	-30	-5.5	-10	-36
JA	209	59	40	-35	-7	-12	-22
	209	165	40	-35	-7	-12	-20
DHJA	211	59	40	-34	-8	-13	-21
	211	167	40	-34	-8	-13	-20

The detection and quantification of both hormones was carried out using multiple reaction monitoring (MRM) in the negative-ion mode, employing multilevel calibration curves with deuterated hormones as internal standards.

The source parameters are: curtain gas: 172.37 kPa, GS1: 310.26 kPa, GS2: 413.69 kPa, ion spray voltage: -4000 V, and temperature: 600 ºC. Data samples were processed using Analyst 1.4.2 Software from Applied Biosystems/MDS Sciex (Ontario, Canada).

Root JA (Chapter 2), MeJA (Chapters 1 and 2), MeSA (Chapter 3) and SA (Chapters 2 and 3) concentrations

Extraction protocol for this group of hormones is as follows: 0.2 g of frozen plant tissue (previously ground to a powder in a mortar with liquid N) was homogenized with 2 ml of MeOH/H_2O/HCOOH (90/9/1, v/v/v). The deuterium-labelled internal standard for each hormone (D-MeJA, D-MeSA, D-SA and D-JA) was added (50 µl of a stock solution of 1.000 ng ml^{-1} in methanol) to the extraction medium. After one hour of mixing the samples at 2000 rpm using the Multi Reax shaker (Heidolph, Schwabach, Germany), the solids were separated by centrifugation at 12,000 *g* for 15 min. 0.5 ml of supernatants was separated and 0.3 ml of 0.2% acetic acid was added. Before its injection in the HPLC-ESI-MS/MS system the samples were centrifuged at 12,000 *g* for 10 min and the supernatants were introduced into chromatographic vials. The hormones were quantified by HPLC (2795 Alliance HT; Waters, Milford, MA, USA) linked to a 3.200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using a reverse-phase column (Synergi 4 µ Hidro-RP 80A, 150x2 mm, Phenomenex, Torrance, CA, USA). A linear gradient of methanol and 0.2% acetic acid in water was used: 60% A for 3 min, 60% A to 85% A in 9 min, 85% A for 1 min and 85% to 60% A in 1 min, followed by a stabilization time of 4 min. The flow rate was 0.2 ml min^{-1}, the injection volume was 40 µl, and the column and sample temperatures were 30º C and 20º C respectively.

The detection and quantification of MeJA and MeSA were performed by multiple reaction monitoring (MRM) in the positive-ion mode, employing a multilevel calibration graph with deuterated hormones as internal standard. Compounds dependents parameters are listed in Table M5.

The MeJA and MeSA source parameters were: curtain gas: 20 psi, GS1: 45 psi, GS2: 50 psi, ion spray voltage: 5.000 V and temperature: 500º C.

Whereas, JA and SA detection and quantification were performed MRM in the negative-ion mode, employing a multilevel calibration graph with deuterated hormones as internal standards. The monitoring fragmentation processes are listed in the Additional Table M5.

The JA source parameters were: curtain gas: 22 psi, GS1: 45 psi, GS2: 55 psi, ion spray voltage: -4.000 V and temperature: 550 ◦C.

The SA source parameters were: curtain gas: 20 psi, GS1: 45 psi, GS2: 50 psi, ion spray voltage: -4.000 V and temperature: 500 ◦C.

Data samples were processed using Analyst 1.4.2. (Applied Biosystems/MDS Sciex).

Statistical analysis

Hydroponic experimental data were subjected to analysis of variance (ANOVA) with treatments as sources of variation (Chapter 1). However, greenhouse experimental data (chapters 2, 3 and 4) were subjected to multifactorial ANOVA analysis with three factors (biological, chemical and abiotic factors). Post-hoc comparisons with the least-significant difference (LSD) tests were used to investigate differences between groups. In the case of dye roots, aquaporin expression and abundance and xylem calcium concentration, Student's *t*-student was used to check differences between the two groups (treated or not treated, Chapter 1). Also, we made linear regression of all parameters measured checking all possible combinations in order to find out new relations among factors (these were only made in greenhouse experiments).

Chapter 1: Enhancement of root hydraulic conductivity by methyl jasmonate and the role of calcium and abscisic acid in this process

Chapter 1: Enhancement of root hydraulic conductivity by methyl jasmonate and the role of calcium and abscisic acid in this process

Objective

The aim of this study was to investigate the effects of MeJA on *L*, and to elucidate if these effects were mediated by calcium and ABA. The plant species chosen for this purpose was common bean (*Phaseolus vulgaris*) because it has been used extensively to study root hydraulic properties (Aroca 2006, Aroca *et al.* 2006a, Aroca *et al.* 2006b, Benabdellah *et al.* 2009). Consequently, *P. vulgaris* plants were treated with MeJA together with $LaCl_3$, heparin or EGTA or with the ABA biosynthesis inhibitor fluridone (Chae *et al.* 2004, Hossain *et al.* 2011). However, we also took advantage of *sitiens*, a tomato mutant defective in ABA synthesis (Taylor *et al.* 1988) and *def-1*, a mutant defective in JA (Howe & Ryan 1999) to analyse the involvement of endogenous levels of these two plant hormones in the response to MeJA. Moreover, different Arabidopsis lines expressing several PIP proteins linked to the green fluorescence protein (Boursiac *et al.* 2005) were used to visualize *in vivo* the accumulation of PIP proteins in the roots in response to MeJA treatment.

Experimental design and growth conditions

In this study, several experiments were carried out and three different plant species were used.

Experiments with *Phaseolus vulgaris* (bean)

Seeds of bean (cv Borlotto) were germinated in moist perlite for one week under dark conditions. After that, seedlings (7 days old) were transferred to aerated 8 l containers filled with 80% nutrient solution (Aroca *et al.* 2006a). Plants were grown in a controlled-environment growth chamber at 23:20ºC (day:night), in a photoperiod of 16:8 h (day:night), with a photosynthetic photon flux density of 300 µmol m^{-2} s^{-1}. After an additional 1week of growth, the roots were exposed to different chemical treatments:

1. Dose-dependent effects of MeJA. We applied different concentrations of MeJA (0, 1, 10, 100, 500 and 1000 µM MeJA) and *L* measurements were taken 1 or 24 h after MeJA addition in both treated and non-treated plants. Root samples were immersed in liquid nitrogen and stored at -80ºC until further use. One and 24 h corresponded to 6 and 5 h after the lights were turned on, respectively.

2. Effects of calcium channel blockers and chelators of calcium. The plants were supplied with MeJA (100 µM), two calcium channels blockers (1 mM heparin, 1 mM $LaCl_3$), an extracellular calcium chelator (1 mM EGTA) or a mixture of MeJA with each one of the above compounds. Heparin, $LaCl_3$ and EGTA were applied 24 h before MeJA addition. Root samples and *L* measurements were taken 1 and 24 h after MeJA addition in both treated and non treated plants.

3. Effects of fluridone, an inhibitor of ABA biosynthesis (Chae *et al*. 2004). Fluridone (10 µM) was applied 24 h prior to MeJA addition (100 µM). We analysed ABA concentration and measured *L* at 1 and 24 h after MeJA application.

Experiments with *Solanum lycopersicum* (tomato)

Tomato seeds were sown in sterile vermiculite for one week and then seedlings were transplanted to aerated 8 l containers filled with a 80% nutrient solution (Aroca 2006). The nutrient solution was replaced every seven days. The plants were grown in the same conditions as bean plants but for two months. Measurements of *L* were done 24 h after MeJA (100µM) application. We did two different experiments with two different tomato mutant lines:

1. Tomato mutant plants that do not accumulate JA. In this experiment the mutant *def-1*, which is deficient in JA accumulation (Howe & Ryan 1999) and the corresponding wild type *Castle mart* were used.

2. Tomato mutant plants that do not accumulate ABA. The tomato mutant plant *sitiens* is deficient in the enzyme activity required for the final step of ABA biosynthesis (Taylor *et al*. 1988). Thus, *sitiens* plants and the corresponding wild type *Rheinlands Ruhm* were used.

Experiments with *Arabidopsis thaliana* (Arabidopsis)

The Arabidopsis seeds were sterilized for 7 min in a solution containing 3.4 g l^{-1} Bayrochlore (Bayrol, Mundolsheim, France) and 86% ethanol, followed by 3 rinses with absolute

ethanol. The seeds were then dried under sterile conditions and kept at 4°C for 24 hours in darkness before sowing.

Two different experiments were undertaken:

1. Effects of MeJA (100 µM) on root water transport. Sterilized seeds of Arabidopsis were germinated and plants grown in half-strength Murashige and Skoog (1/2 MS) medium supplemented with sucrose (10 g l^{-1}) and agar (7 g l^{-1}; 1/2 MS agar) for 10 days.

Plants were then transferred to hydroponic culture for two weeks in the following nutrient solution [1 mM KNO_3, 0.5 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.5 mM NaH_2PO_4, 50 mM FeEDTA, 50 mM H_3BO_3, 12 mM $MnSO_4$, 0.7 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.24 mM MoO_4Na_2, 100 mM Na_2SiO_3, 0,5 mM $CaCl_2$]. Finally, *L* was measured with a pressure chamber as described by Boursiac *et al.* (2005) and Javot *et al.* (2003).

2. Detection of aquaporins labelled with Green Fluorescent Protein (GFP). Transgenic Arabidopsis seeds expressing a specific aquaporin fused to GFP were used (Boursiac *et al.* 2005, Cutler *et al.* 2000). We checked lines expressing three PIPs (p35S:: PIP1.1-GFP, p35S::GFP-PIP1.2, p35S::GFP-PIP2.2) and a control membrane protein (p35S::GFP-LTi6a), all under the control of the 35S CaMV promoter (p35S). These plants were grown in 1/2 MS medium for six days. They were then moved to fresh plates one day prior to 100 µM MeJA application to the medium. Since leaves never touched the medium, MeJA was only applied to the roots.

Results

MeJA dose-response experiment

In a preliminary experiment, treatments of bean roots with different concentrations of MeJA (0 µM, 1 µM, 10µM, 100µM MeJA) during 1 or 24 h were performed to determine which MeJA dose was optimal to produce an effect on root hydraulic conductivity (*L*; Fig. 1.1). Increases in exogenous MeJA concentration induced a progressive rise of *L*. The most significant effect was observed after application of 100µM MeJA. Therefore, we chose exposure to 100 µM MeJA for our experiments. Plants treated with 100 µM MeJA had about 100 times higher MeJA concentration in their roots than untreated roots (Fig. 1.2). The MeJA effects on *L* could not be caused by an increase in the osmotic potential of the nutrient solution since it was 63 ± 3 KPa and 70 ± 5 KPa for 100 µM

MeJA treated and untreated solutions, with no significant differences after a t-test ($p>0.05$). In other studies it has also been found that MeJA effects saturate above 100-200 µM, and that doses higher than 1 mM may cause toxicity (Gould *et al.* 2009, Kim *et al.* 2007, Yu *et al.* 2011).

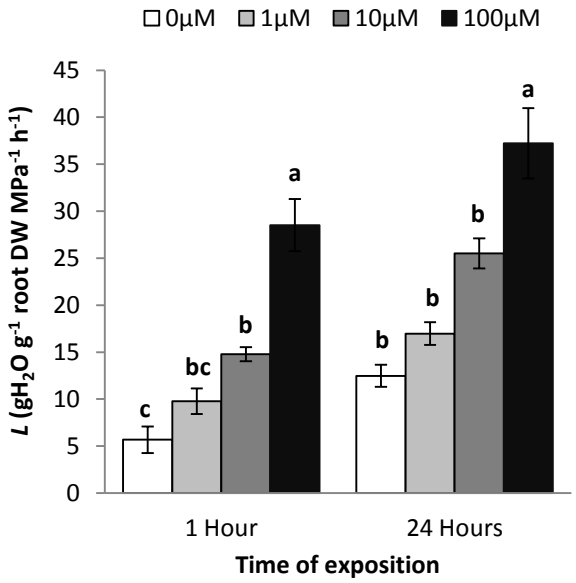


Figure 1.1. Root hydraulic conductivity (*L*) of *P. vulgaris*. plants were subjected to 0, 1, 10 or 100 µM MeJA during 1 and 24 h. *L* was measured by the free exudation method. Bars represent mean ± SE. Within each time period different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=5).

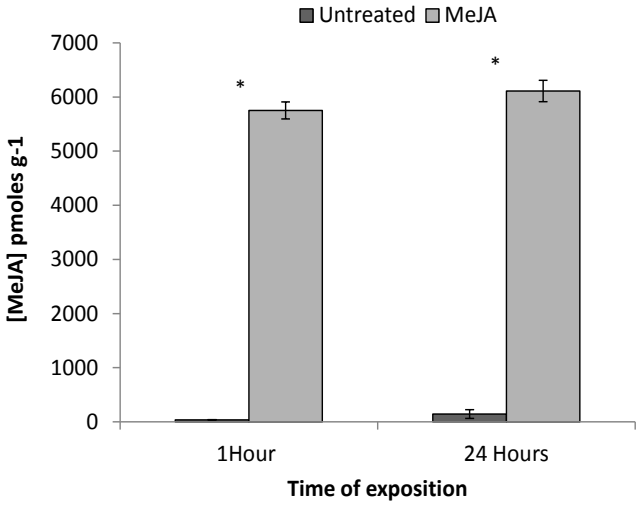


Figure 1.2. MeJA concentration (pmol g^{-1} root FW) of roots of *P. vulgaris* plants after 1 or 24 h of exposure to 100 µM MeJA. Bars represent mean ± SE (n = 4). Asterisks mean significant differences ($p < 0.05$) after t-student test.

So, we further checked the effects of 0.5 and 1 mM MeJA addition on *L*. When we added 500 μM MeJA, we observed the same effect on *L* as with 100 μM JA (Fig. 1.3). However, addition of 1 mM JA caused an inhibition of *L* after 24 h of exposure (Fig.1.3). Therefore, MeJA concentrations higher than 100 μM were not applied in further experiments. The enhancement of *L* by 100 μM MeJA determined by the free exudation method was confirmed using the pressure chamber technique (Fig.1.4). It should be mentioned that using the free exudation method water only circulates through the cell-to-cell pathway (Steudle & Peterson 1998), but when the pressure chamber was used, water circulates through both pathways, cell-to-cell and apoplastic ones. So, MeJA increased the water flow through both pathways.

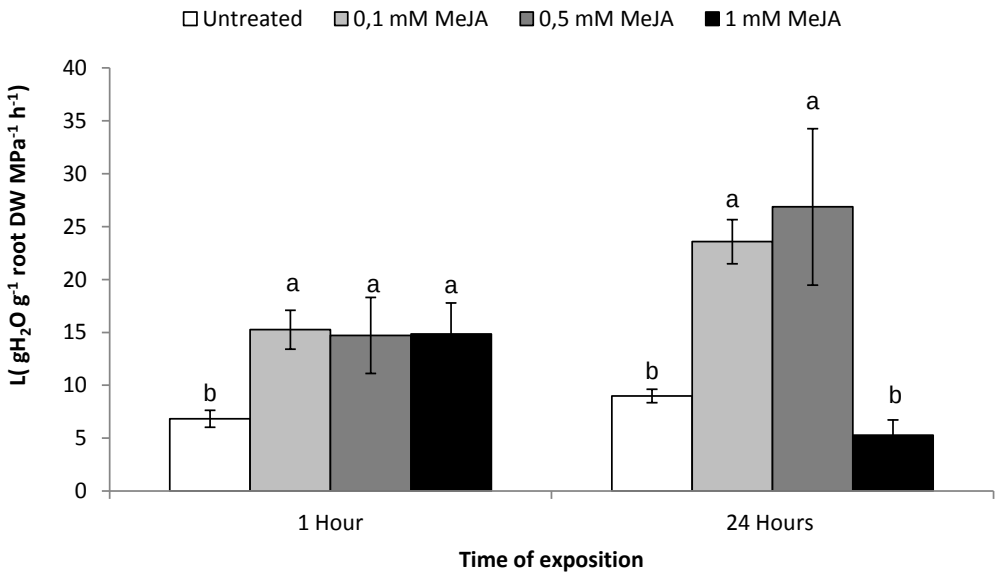


Figure 1.3. Root hydraulic conductivity (*L*) of *P. vulgaris* plants subjected to 0 (untreated), 0.1, 0.5 and 1 mM MeJA during 1 and 24 h. *L* was measured by the free exudation method. Bars represent mean ± SE. Within each time period different letters mean significant differences ($p<0.05$) after ANOVA and LSD test (n =8).

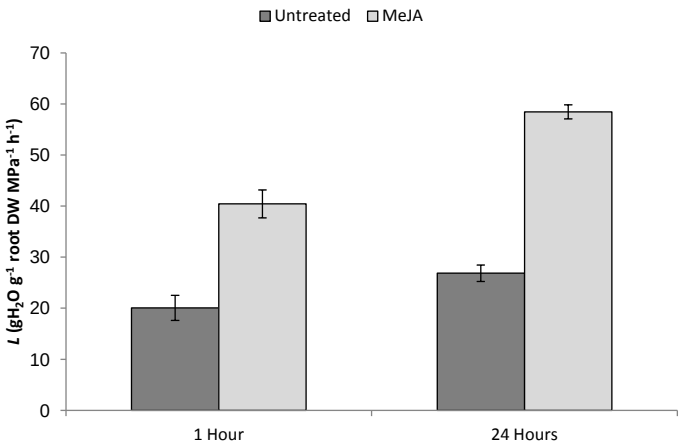


Figure 1.4. Root hydraulic conductivity (*L*) measured with a pressure chamber in roots of *P. vulgaris* plants after 1 or 24 h of exposure to 100 µM MeJA. Bars represent mean ± SE (n = 8). Asterisks mean significant differences ($p < 0.05$)

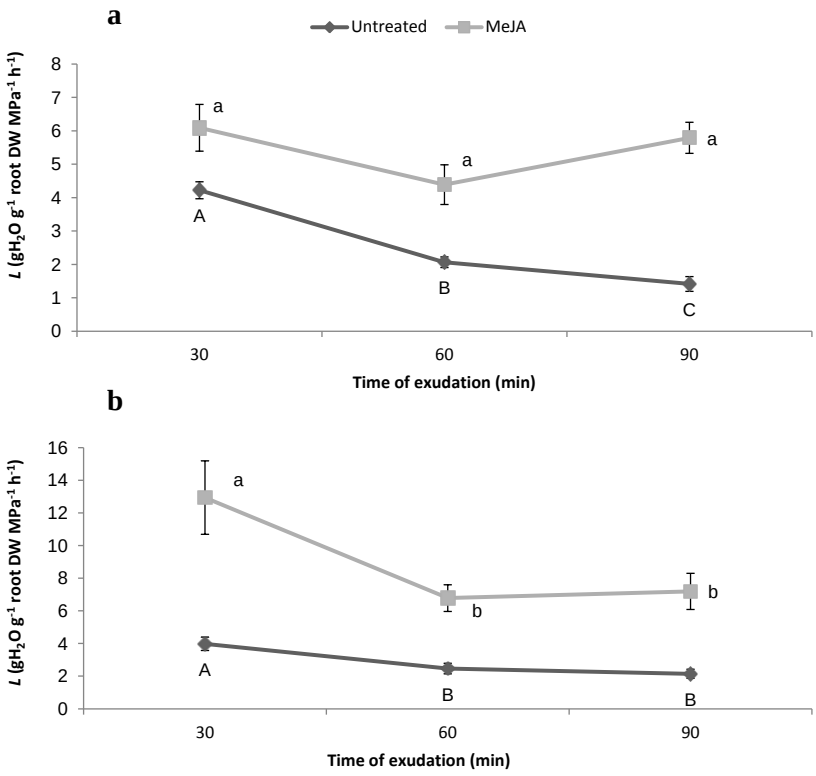


Figure 1.5. Root hydraulic conductivity (*L*) of *P. vulgaris* plants, untreated and treated with 100 µM MeJA during 1 **(a)** or 24 h **(b)**. *L* was measured each 30 min during 90 min by the free exudation method. Bars represent mean ± SE. Within each treatment different letters mean significant differences of *L* along the time ($p<0.05$) after ANOVA and LSD test (n=8).

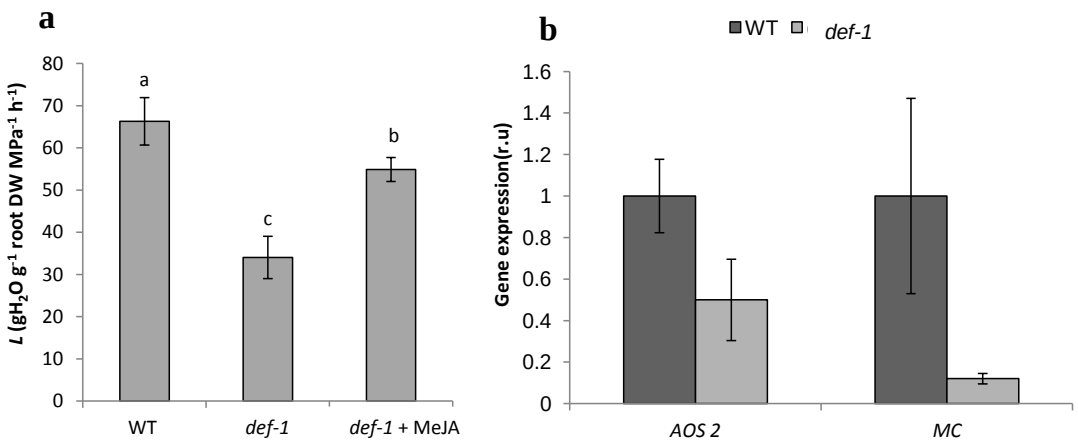


Figure 1.6. (a) *L* values of wild type (WT) of *S. lycopersicum* and *def-1* mutant line deficient in JA biosynthesis, either untreated (*def-1*) or after 24 h of 100 µM MeJA application (*def-1*+MeJA). *L* was measured with a pressure chamber. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=8). **(b)** Relative expression of *SlAOS2* and *SlMC* genes of *S. lycopersicum* in WT and *def-1* roots. Bars represent mean ± SE (n = 6). Asterisks mean significant differences ($p < 0.05$) after t-student test.

Therefore, for the next experiment with bean plants, only the free exudation method was used, keeping in mind that this method only quantifies the cell-to-cell water flow. Furthermore, we calculated *L* of 100 µM MeJA and untreated bean roots at 30 min intervals (Fig.1.5), and at all intervals MeJA treated roots had higher *L* than untreated roots. Additionally, we tested if the low levels of JA in the tomato mutant *def-1* resulted in low levels of *L* (Fig. 1.6a). *L* was indeed lower in *def-1* than in WT plants, and application of 100 µM MeJA during 24 h partially restored *L* values in the mutant. In these particular tomato plants the MeJA levels were below the detection limit (4 pmol g^{-1} FW) in both WT and *def-1* plants.

However, the impairment of accumulating JA by the *def-1* plants is well supported by the literature (Howe *et al.* 1996, Howe & Ryan 1999, O'Donnell *et al.* 2003). Also, we checked the gene expression levels of two genes regulated by MeJA (AOS2 and MC, Lopez-Raez *et al.* 2010), and both genes were down regulated in *def-1* roots (Fig.1.6b). The overall results establish that exogenous MeJA increased *L* in both bean and tomato plants, and that lower endogenous levels of JA result in lower values of *L*.

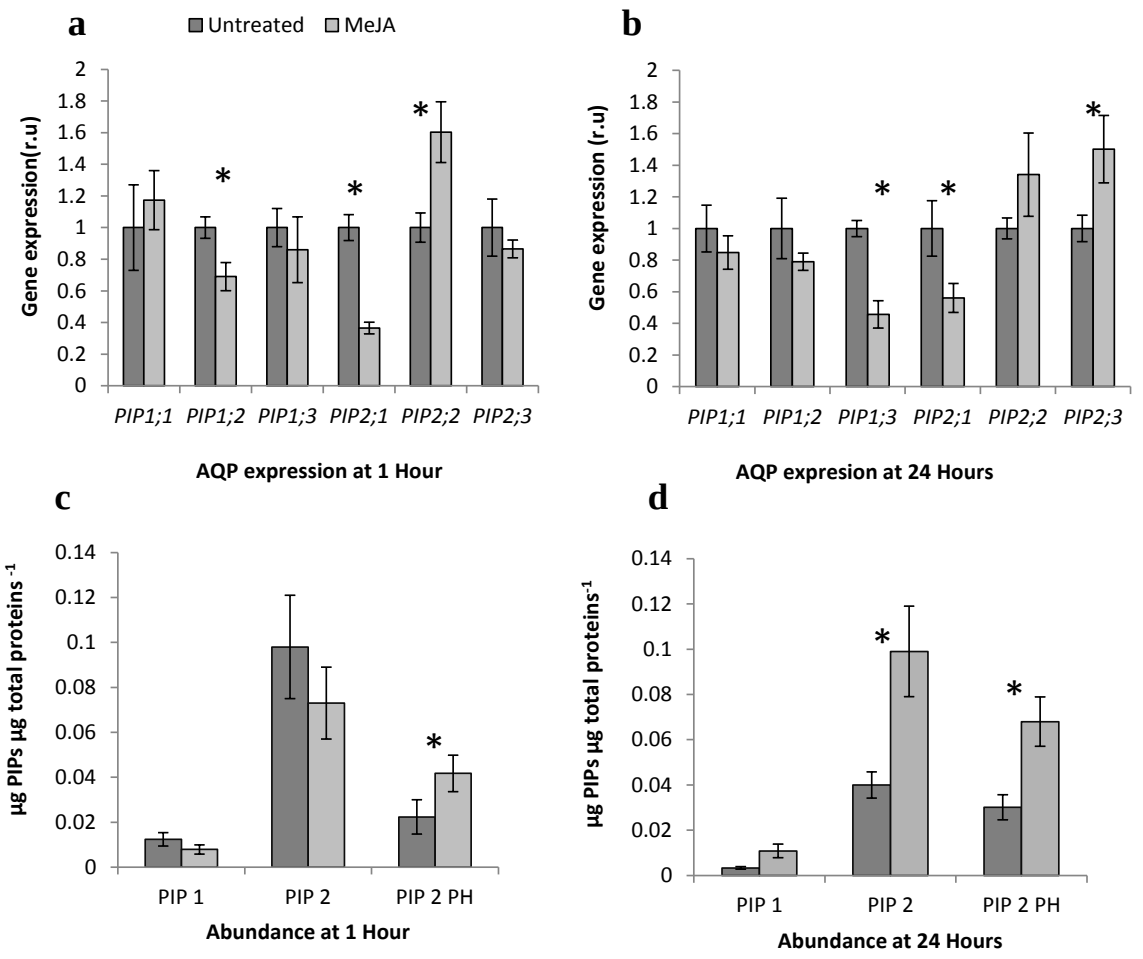


Figure 1.7. Relative expression of *PvPIP1;1*, *PvPIP1;2*, *PvPIP1;3*, *PvPIP2;1*, *PvPIP2;2*, *PvPIP2;3* at 1 h **(a)** or 24 h **(b)** (n=5),and abundance of PIP1, PIP2, phosphorylated at Ser-280 PIP2 (PIP2PH) proteins in untreated roots or roots treated with 100 µM MeJA after 1 h **(c)** or 24 h **(d)** (n=6). Bars represent mean ± SE. Asterisks mean significant differences ($p<0.05$) between untreated and treated roots after Student's *t*-test.

MeJA effects on *PIP* expression and PIP abundance in *P. vulgaris* roots

Gene expression of the six different PIP aquaporins of *P. vulgaris* known so far (*PvPIP1;1*, *PvPIP1;2*, *PvPIP1;3*, *PvPIP2;1*, *PvPIP2.2*, *PvPIP2;3*) was analysed (Fig. 1.7). One h after the addition of 100 µM MeJA (Fig. 7a), gene expression of *PvPIP1;2* and *PvPIP2;1* aquaporins was found to be decreased, whereas gene expression of *PvPIP2;2* was enhanced. Expression of the other three genes did not change. After 24 h of application of MeJA (Fig. 1.7b) gene expression of *PvPIP1;3* and *PvPIP2;1* was down-regulated, while that of *PvPIP2;3* was up-regulated. Expression of the other three aquaporin genes did not change. The abundance of PIP1 and PIP2 proteins and of

the PIP2 proteins phosphorylated at the Ser-280 was analysed in control roots and roots treated with 100 µM MeJA for 1 or 24h (Fig. 1.7 c-d). We used ELISA assays with specific antibodies: anti-PIP1, anti-PIP2 (Marulanda *et al.* 2010), and anti-PIP2 phosphorylated at Ser-280 (anti-PIP2PH). The number of the Ser residue refers to the PvPIP2;1 protein, but these residues are conserved among all PIP2 proteins (Prak *et al.* 2008). Combinations of anti-PIP2 and anti-PIP2PH antibodies and their antigen peptides established that each antibody only recognizes its corresponding peptide (Fig. M4). Figures 1.7 c-d shows that there was a ~ 2-fold increase in the phosphorylation of Ser-280 of PIP2 proteins (PIP2PH) after 1 h and 24 h of exposure to MeJA. After 24 h the total amount of PIP2 proteins without phosphorylation in their C-terminal region was also increased by 2.5-fold (Fig. 1.7d). In contrast, PIP1 protein levels did not increase in response to MeJA treatment.

Effects of MeJA on PIP abundance in intact roots of *A. thaliana*

To visualize how PIP aquaporins responded *in vivo* to MeJA we used transgenic *A. thaliana* plants that constitutively express fusions of GFP with three of the most highly expressed PIP isoforms, *At*PIP1;1 *At*PIP1;2 and *At*PIP2;2. Similar to bean and tomato, *L* of *A. thaliana* plants is responsive to MeJA and was increased by ~50% after a treatment with 100 µM MeJA for 24 h (Fig. 1.8a).

Laser scanning confocal microscopy of root cells revealed that plants treated with MeJA in the same conditions had a higher fluorescence of GFP-PIP1;2 (+109%) and GFP-PIP2;2 (+30%) than plants grown under standard conditions (Fig. 1.8b). However, the opposite effect was observed in plants expressing a PIP1;1-GFP fusion protein, with a 34% decrease in abundance after MeJA treatment. As a control, we also analysed another membrane-associated protein called LTi6 (Boursiac *et al.* 2005). MeJA had no effect on the abundance of a GFP-LTI6a reporter protein.

Calcium accumulation inside the cells

Since calcium is involved in MeJA signaling, we analysed several root sections stained with the calcium dye FLuo 4 AM, to track possible variations in $[Ca^{2+}]_{cyt}$ owing to MeJA treatment. We took image samples at the root tip and 2 cm above the tip. The recordings showed that at both root sites, MeJA-treated roots had a more intense fluorescent signal than control roots after 24 h of exposure, but not after 1 h (Fig. 1.9). These results indicate that $[Ca^{2+}]_{cyt}$ increased after 24 h of addition of MeJA.

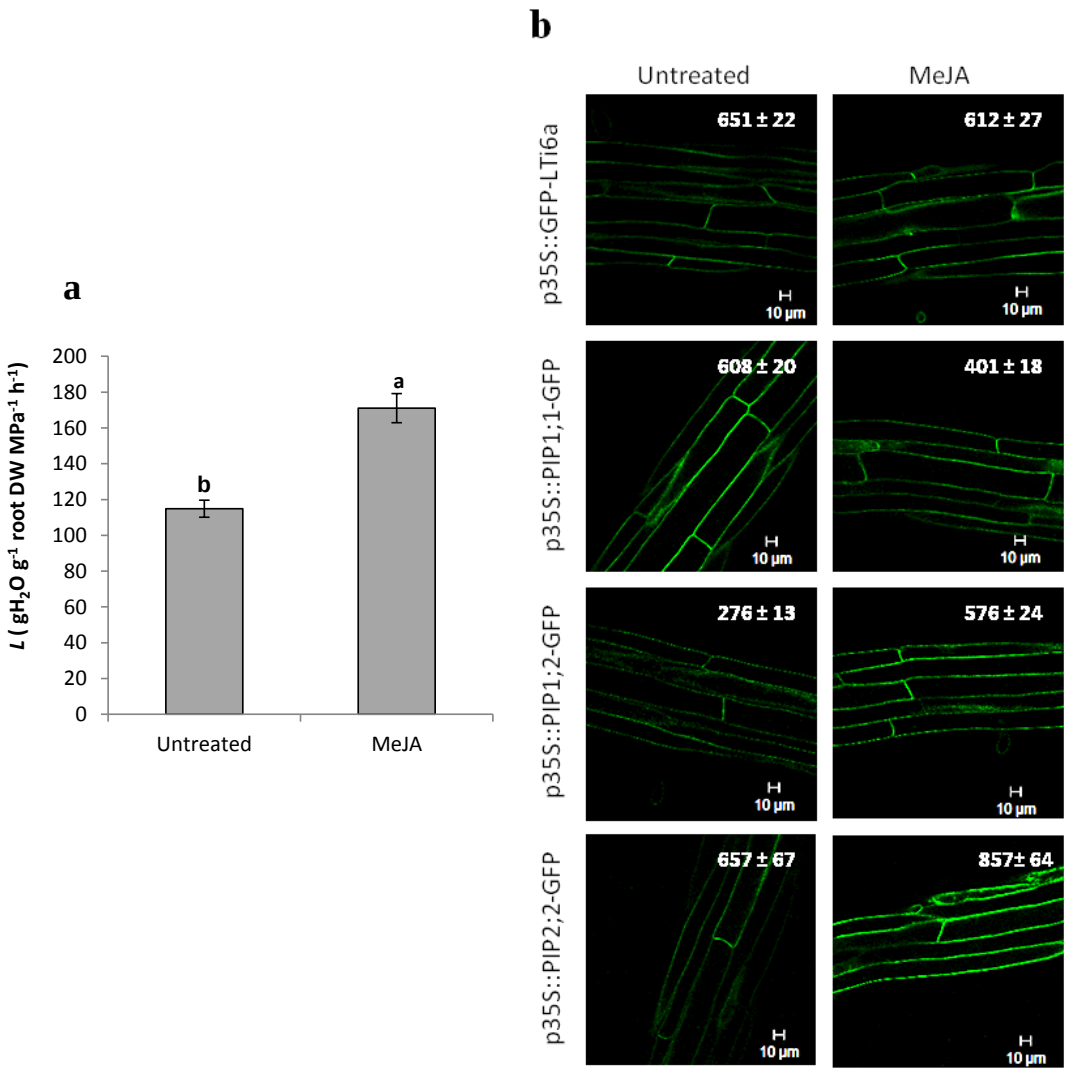


Figure 1.8. (a) Root hydraulic conductivity (*L*) of wild type Arabidopsis plants either untreated or exposed to 100 µM MeJA for 24 h. *L* was measured by a pressure chamber technique, as described in the Materials and Methods. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) between untreated and treated roots after t-student test (n=55). **(b)** Representative confocal images of Arabidopsis root cells expressing the indicated fusions with GFP, all under the control of a p35S promoter. The p35S::GFP-LTi6a line was used as a control. Plants were either untreated or treated with 100 µM MeJA for 24h. Numbers inside the figures represent the mean ± SE green intensity of at least 30 different roots.

Effects of calcium channel blockers and a calcium chelator on MeJA-enhanced root hydraulic conductivity (*L*)

Since we observed that intracellular calcium ions increased in root cells only after 24 h of exposure to MeJA, the following experiments were done only after 24 h of exposure to MeJA. We used chemicals that either reduce the external concentration of calcium (EGTA), block several calcium channels located in cell membranes ($LaCl_3$) or block IP_3-dependent calcium channels (heparin) (Amelot *et al.* 2012, Klaas *et al.* 2011, Poutrain *et al.* 2009, White 2000, Yamamoto,Kanaide & Nakamura 1990, Yang *et al.* 2013).

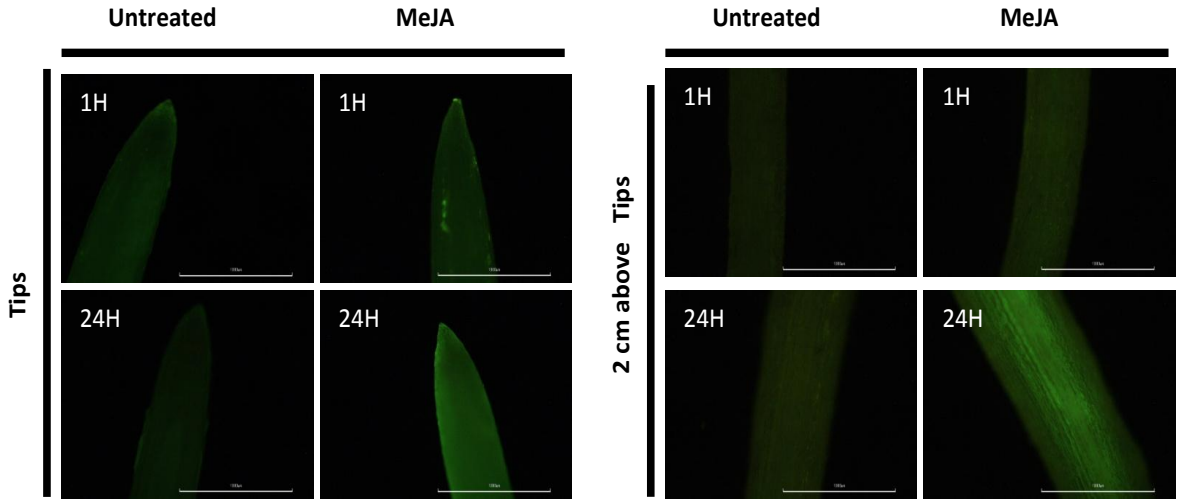


Figure 1.9. Staining of *P. vulgaris* roots with Fluo-4/AM dye to determine $[Ca^{2+}]_{cyt}$. Images were taken at root tips and at sections 2 cm above tips, in untreated roots and roots treated with 100 µM MeJA for 1 or 24 hours. Representative images are shown.

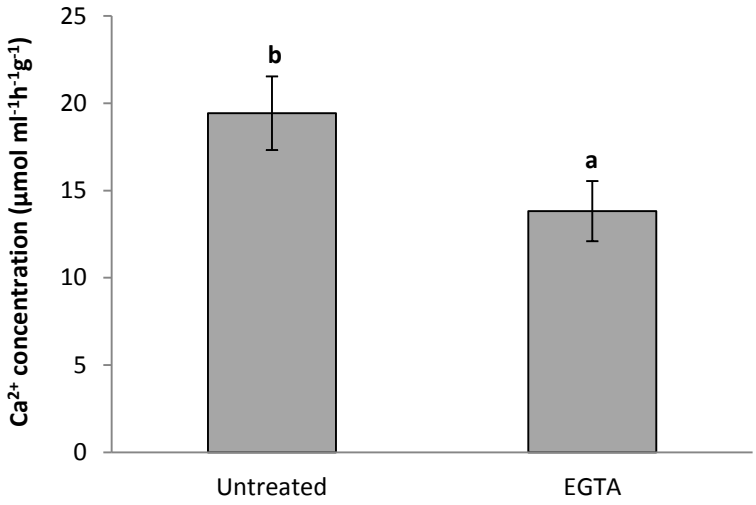


Figure 1.10. Apoplastic calcium concentration of untreated plants and plants treated with (1mM) EGTA (apoplastic calcium chelator) during 24 h. Scholander chamber at 0.25 MPa was used to get the exudates (n=4)(t-test, $P \leq 0.05$).

EGTA experiment

We first checked the capacity of EGTA to eliminate calcium ions from the medium (Fig 1.10). For this, calcium exudation was measured in untreated and EGTA-treated plants. A reduction of calcium exudation rate in plants treated with EGTA was observed, indicating that EGTA is an effective chelator of apoplastic calcium. Its effects on MeJA-dependent regulation were then investigated. After treatments for 24 hours with MeJA or EGTA+MeJA, plants had similar *L* values, both higher than in untreated control plants (Fig. 1.11a). We also checked that EGTA alone had no significant effect on *L*.

Lanthanum chloride and heparin experiments

Secondly, we used lanthanum chloride ($LaCl_3$) and heparin. These compounds block the calcium channels of plant membranes. MeJA+$LaCl_3$ treatment partially inhibited the increase of *L* caused by MeJA (Fig. 1.11b). So, when calcium movement inside the root cells was impeded, the increase of *L* caused by MeJA was reduced. A heparin treatment had even more pronounced effects (Fig. 1.11c), and completely abolished the enhancement of *L* caused by MeJA treatment.

Involvement of ABA on the MeJA action on *L*

MeJA can induce the synthesis of ABA (Adie *et al.* 2007), which in turn can increase *L* (Aroca 2006). To investigate this relationship, we designed experiments using beans and fluridone, an inhibitor of ABA biosynthesis.

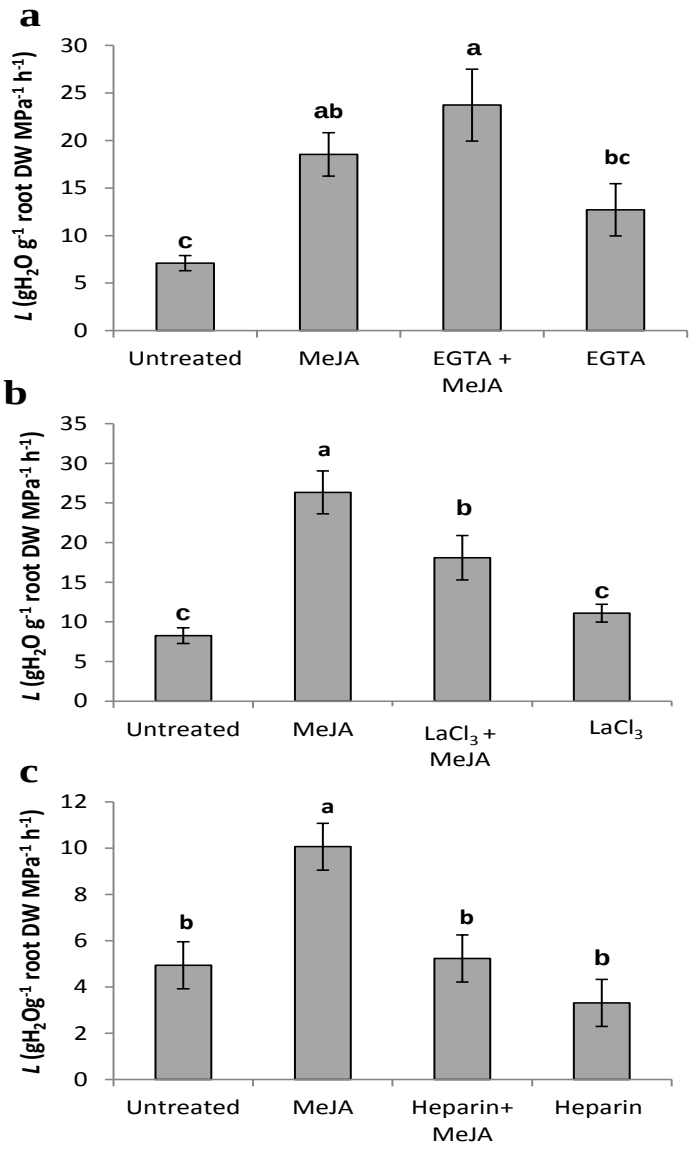


Figure 1.11. Root hydraulic conductivity (*L*) measured by the free exudation method in *P. vulgaris* roots treated with 100 µM MeJA during 24 h plus 1 mM EGTA **(a)**, 1 mM $LaCl_3$ **(b)** or 1 mM heparin **(c)**. Chemicals were added 24 h before MeJA addition. Bars represent mean ± SE (n=15). Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test.

We first checked whether MeJA can indeed induce an ABA accumulation and whether such increase can be abolished by a fluridone treatment. The data showed that MeJA application induced an increase of ABA concentration which after 1 h of fluridone treatment was completely inhibited, but after 24 h of exposure to MeJA inhibition was only partial (Fig. 1.12b).

Water transport was then investigated in control bean plants and plants treated with fluridone, MeJA or fluridone+MeJA. Similar to its effect on ABA levels, fluridone inhibited partially or completely the increase in *L* caused by treatments with MeJA for 1 h or 24 h, respectively (Fig. 1.12a). Fluridone alone did not cause any effect on *L* at any time point (Fig. 1.12a).

We also investigated the regulation of *L* by MeJA in *sitiens* plants, a tomato mutant plant that is deficient in ABA synthesis. After 24 h application of MeJA, *sitiens* plants showed an increase of *L*, similar to wild type plants (Fig. 1.13a). However, since MeJA did not increase ABA contents in *sitiens* roots (Fig. 1.13b), it is clear that MeJA does not need to enhance ABA concentration in roots to get an increase of *L* in this particular mutant.

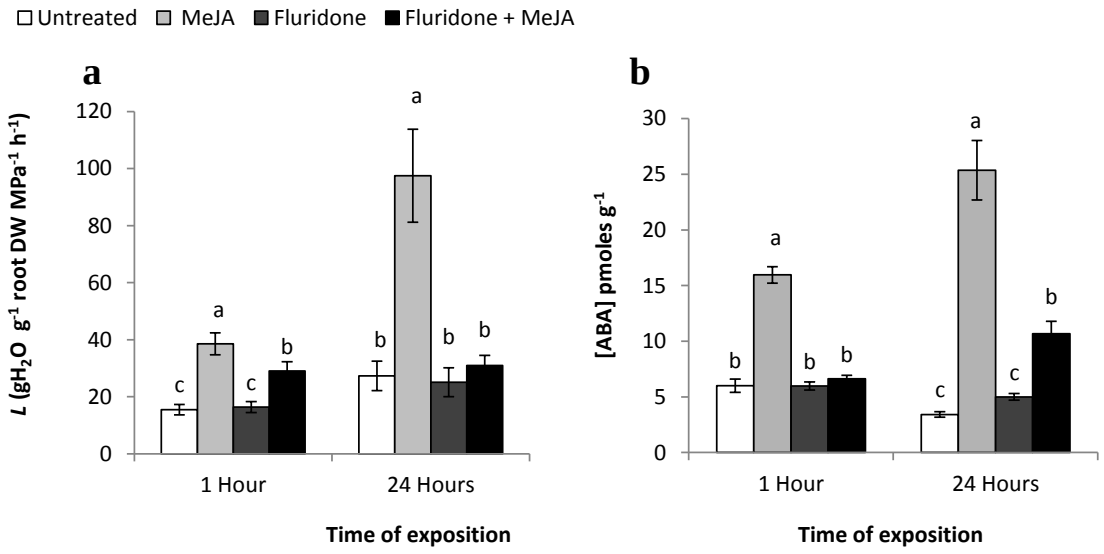


Figure 1.12. Root hydraulic conductivity (*L*) measured by the free exudation method **(a)** and ABA concentrations **(b)** in roots of *P. vulgaris* plants after 1 or 24 h of exposure to 100 µM MeJA plus 10 µM of fluridone. Fluridone was applied 24 h before MeJA addition. Bars represent mean ± SE (n = 8). Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test.

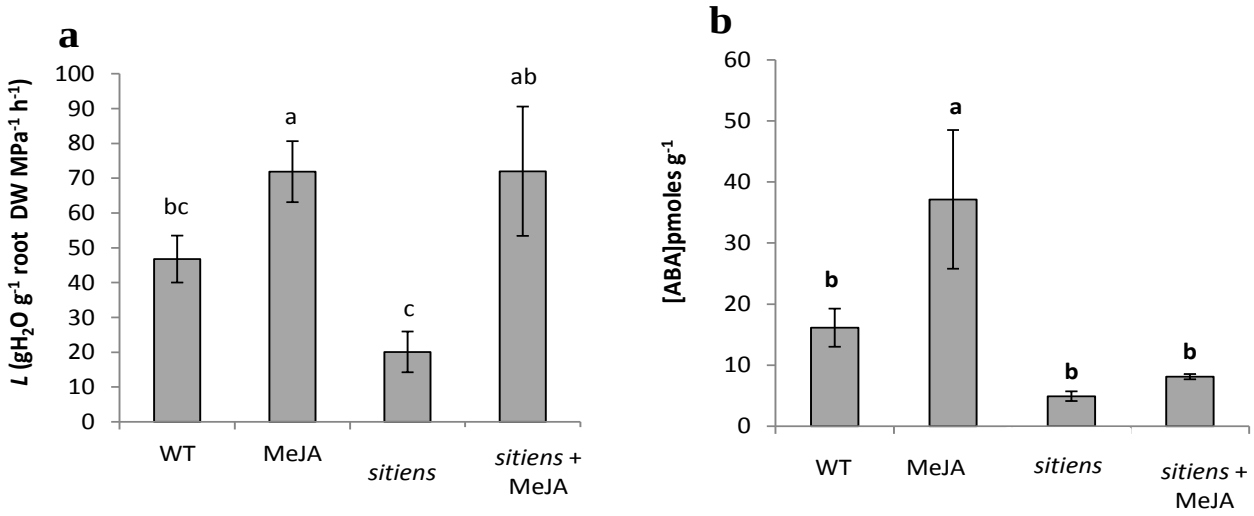


Figure 1.13. Root hydraulic conductivity (*L*) measured with a pressure chamber **(a)** and ABA concentrations **(b)** of roots of wild type (WT) tomato and ABA-deficient (*sitiens*) plants after 24 h of exposure to 100 µM MeJA. Bars represent mean ± SE (n = 5). Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test.

Discussion

Effects of MeJA on *L* and PIP aquaporins

There is already much information about the role of MeJA under stress conditions and in regulating stomatal opening (Gehring *et al.* 1997, Hossain *et al.* 2011, Munemasa *et al.* 2007, Raghavendra & Reddy 1987, Suhita *et al.* 2004). However, few investigations have been made to elucidate the role of MeJA on the regulation of root water transport (Lee *et al.* 1996b).

Our study has established that *L* increased when MeJA was applied to plant roots. This effect was observed in three different plant species, *P.vulgaris*, *A.thaliana* and *S. lycopersicum*. So, exogenous MeJA increases *L*, similar to ABA (Aroca *et al.* 2008b, Kudoyarova *et al.* 2011, Mahdieh & Mostajeran 2009, Ruiz-Lozano *et al.* 2009). Moreover, we confirmed that the free exudation method is valid to measure *L* in bean plants. Using MeJA deficient tomato plants, we corroborated that plants with lower amounts of MeJA (*def-1* plants; Howe *et al.* 1996, Howe & Ryan 1999, Li *et al.* 2002, O'Donnell *et al.* 2003) have lower *L* than wild type plants, with *L* being partially restored by exogenous MeJA. Thus, endogenous levels of MeJA also contribute to regulation of *L*. Similar results were found in tomato and maize plants with lower endogenous

levels of ABA, which showed lower values of *L* than wild type or untransformed plants (Nagel,Konings & Lambers 1994, Parent *et al.* 2009).

Since PIP aquaporins are known to significantly contribute to *L* (Javot *et al.* 2003, Perrone *et al.* 2012, Postaire *et al.* 2010), we studied how MeJA regulates PIP expression, abundance and phosphorylation state. Changes in *PIP* gene expression may not explain the increase in *L* observed after MeJA treatment in *P. vulgaris* plants. In fact, the expression of three *PIPs* was down-regulated (*PvPIP1;2*, *PvPIP1;3* and *PvPIP2;1*), and only the expression of *PvPIP2;2* and *PvPIP2;3* was up-regulated after 1 and 24 h of exposure to MeJA. This apparent lack of relationship between *PIP* expression and *L* could be because other *P. vulgaris PIP* genes that are not yet identified contribute significantly to *L* enhancement. The PvPIP2;3 protein has a high capacity for transporting water compared to PvPIP2;2 protein (Zhou *et al.* 2007), so its enhanced expression by MeJA after 24 h could be related to the enhancement of *L*. Also, a collaborative interaction between PvPIP2;2 and PvPIP2;3 proteins could take place as recently reported Li *et al.* (2013a) for PIP2 proteins of cotton.

Obviously, a reverse genetic approach is needed to corroborate these possibilities. At the same time, we cannot discard the possibility that an interaction between PIP1 and PIP2 monomers could take place to enhance *L* in MeJA treated roots (Fetter *et al.* 2004, Zelazny *et al.* 2007).

Our next step was to analyse PIP protein abundance and phosphorylation state. Here we found a relationship between increase in *L* by MeJA and the increase in the amount of PIP2 proteins phosphorylated at Ser 280. The phosphorylation of this Ser residue is responsible for the water transport activity of several PIPs proteins (Azad *et al.* 2008, Johansson *et al.* 1998, Törnroth-Horsefield *et al.* 2006). Also, after 24 h of MeJA exposure an increase in PIP2 protein amount took place, which may be related to the increase in *PvPIP2;3* gene expression.

Altered subcellular localization of PIPs can also lead to changes in *L* values (Boursiac *et al.* 2008a, Boursiac *et al.* 2005). Thus, taking advantage of *A. thaliana* lines that constitutively express PIP proteins fused to GFP (Boursiac *et al.* 2008a), we found that MeJA treatment increased the amounts in the plasma membrane of two of the three major PIP proteins. These effects may be responsible for the increase of *L*. However, it remains to be determined if this phenomenon is due to enhanced trafficking of PIPs from intracellular membranes to the cell surface or to enhanced stability of the proteins at the plasma membrane.

Role of calcium in the regulation of *L* by MeJA

Based on the finding that MeJA regulates *L*, we were interested in elucidating how calcium may be involved in this process. Calcium is a highly mobile element, whose apoplastic and cytosolic concentrations can change in response to external stimuli. Previous work showed that MeJA can move calcium ions into the cytosol from apoplastic spaces or from intracellular stores such as the vacuole or the endoplasmic reticulum (Islam *et al.* 2010, Sun *et al.* 2006a, Sun *et al.* 2006b).

Using a fluorescent dye, we first confirmed that MeJA treatment induced an increase in $[Ca^{2+}]_{cyt}$ in *P. vulgaris* roots after 24 h of application, but not after 1 h. Hence, in contrast to short-term (1 h) effects, the long-term increase of *L* observed after 24 h of exposure to MeJA could be linked to changes in $[Ca^{2+}]_{cyt}$. This idea was further explored using calcium chelators (EGTA) and calcium channel blockers ($LaCl_3$ and heparin). The lack of effects of EGTA on MeJA-induced *L* after 24h showed that apoplastic calcium may not be involved in this process. However, it is possible that minimal entrance of calcium ions could cause the response to MeJA. When we applied some calcium channels blockers ($LaCl_3$ and heparin) the *L* value was lower than with MeJA alone, especially after heparin application. Hence, the enhancement of *L* caused by MeJA could be mediated by mobilization of internal calcium stores, particularly from those dependent on IP_3 calcium channels, which are localized in endoplasmic reticulum and tonoplast and are inhibited by heparin (Poutrain *et al.* 2009, Sun *et al.* 2009b, White 2000). The involvement of calcium in the induction of stomatal closure by MeJA has been also documented (Gehring *et al.* 1997, Herde *et al.* 1997, Munemasa *et al.* 2011a).

Involvement of ABA on MeJA-enhanced *L*

Many studies have demonstrated a role for ABA and MeJA in transpiration and defence against pathogens (Adie *et al.* 2007, Herde *et al.* 1997, Hossain *et al.* 2011, Suhita *et al.* 2004).The relationship between the two signaling molecules is still unclear. For instance, (Lorenzo & Solano 2005) found that ABA activates the expression of genes by the MeJA signaling pathway, and others found, that it is MeJA that activates the synthesis of ABA (Adie *et al.* 2007, Hossain *et al.* 2011). It is also known that exogenous ABA application increased *L* (Adie *et al.* 2007, Kudoyarova *et al.* 2011, Mahdieh & Mostajeran 2009, Ruiz-Lozano *et al.* 2009).

Here we found that the initial *L* response to MeJA is partially dependent on ABA, since fluridone treatment did not abolish completely the effect of MeJA. However after 24 h of exposure to MeJA the increase of *L* was totally dependent on the rise of ABA induced by MeJA. In fact, ABA contents also increased in fluridone-treated roots after 24 h of exposure to MeJA, but *L* did not increase. So, we could distinguish two ways of action of MeJA on *L*, a short-term one, partially independent of ABA and calcium (1 h of exposure) and a long-term one, dependent on calcium and ABA (24 h of exposure). In fact, the increase of cytosolic calcium in guard cells induced by MeJA is mediated by ABA (Munemasa *et al.* 2011a). Similar ABA-dependent and ABA-independent pathways for MeJA signaling have been previously found for other MeJA effects (Munemasa *et al.* 2007, Suhita *et al.* 2004). To confirm the MeJA ABA-independent signaling pathway, we analysed the response of *L* to MeJA addition in the ABA-deficient tomato mutant *sitiens* (Taylor *et al.* 1988). This mutant is more susceptible to water deficit-induced embolism, most probably because overall low hydraulic properties (Secchi *et al.* 2013). Results confirmed the existence of an ABA-independent MeJA signaling pathway in the regulation of *L* by MeJA. The same occurs for stomatal regulation (Islam *et al.* 2010, Suhita *et al.* 2004).

In summary, we report here for the first time, and in three different plant species, that exogenous MeJA causes an increase of *L*. We postulate both calcium-dependent and independent, as well as, ABA-dependent and independent pathways for this effect of MeJA. The regulation of *L* by MeJA could be important under abiotic stress conditions such as drought or salinity, where soil water availability is restricted (Aroca,Porcel & Ruiz-Lozano 2012), since it is known that MeJA improves water status of plants subjected to these kind of stresses (Anjum *et al.* 2011, Wu *et al.* 2012a). In future research the involvement of MeJA in controlling *L* under water deficit and salt stress conditions will be studied.

Chapter 2: Arbuscular mycorrhizal symbiosis and methyl jasmonate prevent the inhibition of root hydraulic conductivity caused by drought

Chapter 2: Arbuscular mycorrhizal symbiosis and methyl jasmonate prevent the inhibition of root hydraulic conductivity caused by drought

Objective

The aim of this work was to study the effects of MeJA on *L* and on the expression and abundance of AQPs in AM plants grown under greenhouse conditions and subjected to drought. We also analysed how changes in the endogenous levels of other hormones, such as, IAA, ABA and SA affect *L* after MeJA application.

Experimental design

Plants of *Phaseolus vulgaris* were used in the experiment following a combined factorial design with three factors: (1) biological factor, with plants inoculated or not with the AM fungus (AMF) *Rhizophagus irregularis*, (2) chemical factor formed by untreated plants and plants treated with 200 µm MeJA, and (3) abiotic factor, with plants grown under well-watered or drought stress conditions. We had a total of 64 plants and 8 different treatments. Four replicates of each treatment were used for the measurement of *L*, and the other 4 replicates were frozen in liquid nitrogen immediately after harvest for later use in molecular and biochemical determinations described in the materials and methods section. For all treatments, physiological measurements and the collection of plant samples were carried out 3 h after sunrise, to avoid effects of diurnal fluctuations on plant processes.

Growth conditions

The experiment lasted 4 weeks from seed germination and was conducted under greenhouse conditions with temperatures between 19 to 25°C , 16/8 light/dark, a relative humidity of 50-60% and a photosynthetic photon flux density of 800 µE m^{-2} s^{-1}, as measured with a light meter (LICOR; Lincoln, NE, USA model LI-188B). After two weeks from the beginning of the experiment, all plants received 10 ml of 80% Hewitt`s nutrient solution (Hewitt 1952). Besides, MeJA-treated plants received three doses of 10 ml of 200 µM MeJA which were applied during the last week of

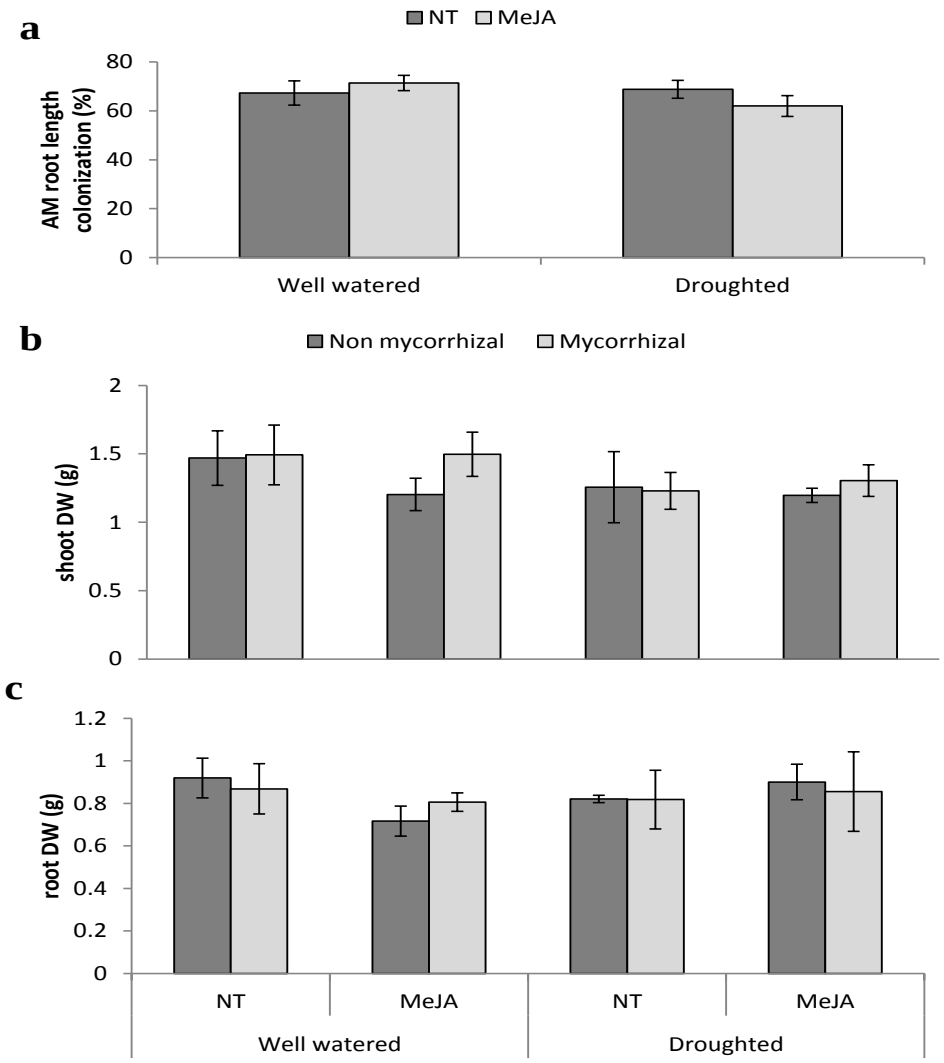


Figure 2.1. (a) Effect of water regime and MeJA treatment on the percentage of mycorrhizal root length colonization in bean roots by *Rhizophagus irregularis*. Seven plants of each treatment were analysed. Untreated plants (NT, dark bars) and plants treated with 200µl MeJA (grey bars) under well-watered or drought conditions. None of the treatments had significant differences after ANOVA and LSD tests. Bars represent mean ± SE (n=7). Dry weights of shoots **(b)** and roots **(c)** of *P.vulgaris* plants untreated (NT) or treated with 200µM MeJA and no inoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars), under well-watered and drought conditions. None of the treatments had significant differences after ANOVA and LSD tests. Bars represent mean ± SE (n=4).

experiment. The doses were supplied throughout the week, the first one coincided with the beginning of drought treatment; the second application was applied after two days of drought and the last dose being applied the day before harvest. Drought treatment was also applied the last week before harvest. Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd.,

Cambridge, UK). Water was supplied daily to maintain soil at field capacity during the first 3 weeks after germination. Then, half of the plants were subjected to drought (55% of field capacity) while the other half was maintained at field capacity. Plants were maintained under such conditions for 7 days before harvest.

Results

Mycorrhizal colonization and plant growth

After four weeks of growth, uninoculated bean roots did not show mycorrhizal colonization. The percentage of AM root length colonization was over 60 % for all AM plants, without significant differences among treatments (Fig. 2.1a). Also, no differences in shoot or root dry weights were observed among treatments (Fig. 2.1b and c).

Root hydraulic conductivity (*L*)

When plants were grown under well-watered conditions, *L* was unaffected by the application of exogenous MeJA but it was decreased by AM symbiosis (Fig. 2.2a). Drought treatment diminished *L* in non-AM plants, and both, MeJA application or AM symbiosis counteracted this inhibition (Fig. 2.2a). Overall, MeJA treatment had a positive effect on *L* as reported by the ANOVA analysis (Table 2.1).

Relative water content (RWC) and stomatal conductance(gs)

AM plants raised RWC regardless of water regime, except if MeJA was added under drought conditions (Table 2.1; Fig. 2.2b). Overall, drought treatment decreased RWC as indicated by ANOVA analysis (Table 2.1). In contrast, well-watered AM plants treated with MeJA reached the maximum values of RWC (Fig. 2.2b).

MeJA-treated AM plants had lower stomatal conductance than non-AM counterparts under well-watered conditions (Fig. 2.2c). In general, a decrease of stomatal conductance was observed in plants subjected to drought (Table 2.1), being significant only for non-AM plants treated with MeJA (Fig. 2.2c).

Plant PIP gene expression

The expression of the six known plant *PIP* genes in *P.vulgaris* was analysed by q-RT-PCR in root tissues. The expression of *PvPIP1;1* and *PvPIP2;3* was not altered by any treatment (Table 2.1; Fig. 2.3). *PvPIP1;2* gene expression decreased in AM plants under well-watered conditions (Fig. 2.3). The combination of AM symbiosis and MeJA increased the expression of *PvPIP2;1* and *PvPIP2;2* genes under drought conditions (Fig. 2.3), while MeJA treatment also increased *PvPIP1;2* expression in non-AM plants under well-watered conditions. Under drought conditions, MeJA treatment increased *PvPIP1;3* and *PvPIP2;1* expression in non-AM and AM plants, respectively, and decreased *PvPIP2;2* expression in non-AM plants (Fig. 2.3).

Table 2.1. Significance of sources of variation after three-way ANOVA analyses for following parameters, such as root hydraulic conductivity (*L*), relative water content(RWC), stomatal conductance (gs), several PIPs gene expressions and abundances, and the content of methyl jasmonate (MeJA), jasmonic acid (JA), indole-3-acetic acid (IAA),abscisic acid (ABA) and salicylic acid (SA). The sources of variance were AM symbiosis (AM), water regime (WR) and MeJA addition (MeJA), as well as their interactions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant effect.

	AM	WR	MeJA	AMxWR	AMxMeJA	WRxMeJA	AMxWRxMeJA
L	ns	ns	*	**	ns	ns	ns
RWC	***	*	ns	*	ns	ns	*
gs	ns	**	ns	ns	ns	ns	*
PvPIP1;1	ns	ns	ns	ns	*	ns	ns
PvPIP1;2	***	ns	ns	**	ns	ns	ns
PvPIP1;3	***	ns	ns	ns	ns	**	*
PvPIP2;1	ns	**	ns	**	*	ns	ns
PvPIP2;2	**	*	ns	*	*	ns	ns
PvPIP2;3	ns	ns	ns	ns	*	ns	ns
PIP1	ns	ns	ns	ns	ns	ns	ns
PIP2	ns	ns	ns	ns	ns	ns	ns
PIP2Ph	**	ns	ns	**	ns	ns	**
MeJA	***	ns	***	ns	***	ns	*
JA	***	***	ns	***	ns	ns	ns
IAA	***	***	ns	**	**	***	***
ABA	ns	***	ns	ns	ns	ns	ns
SA	ns	ns	ns	**	*	*	**

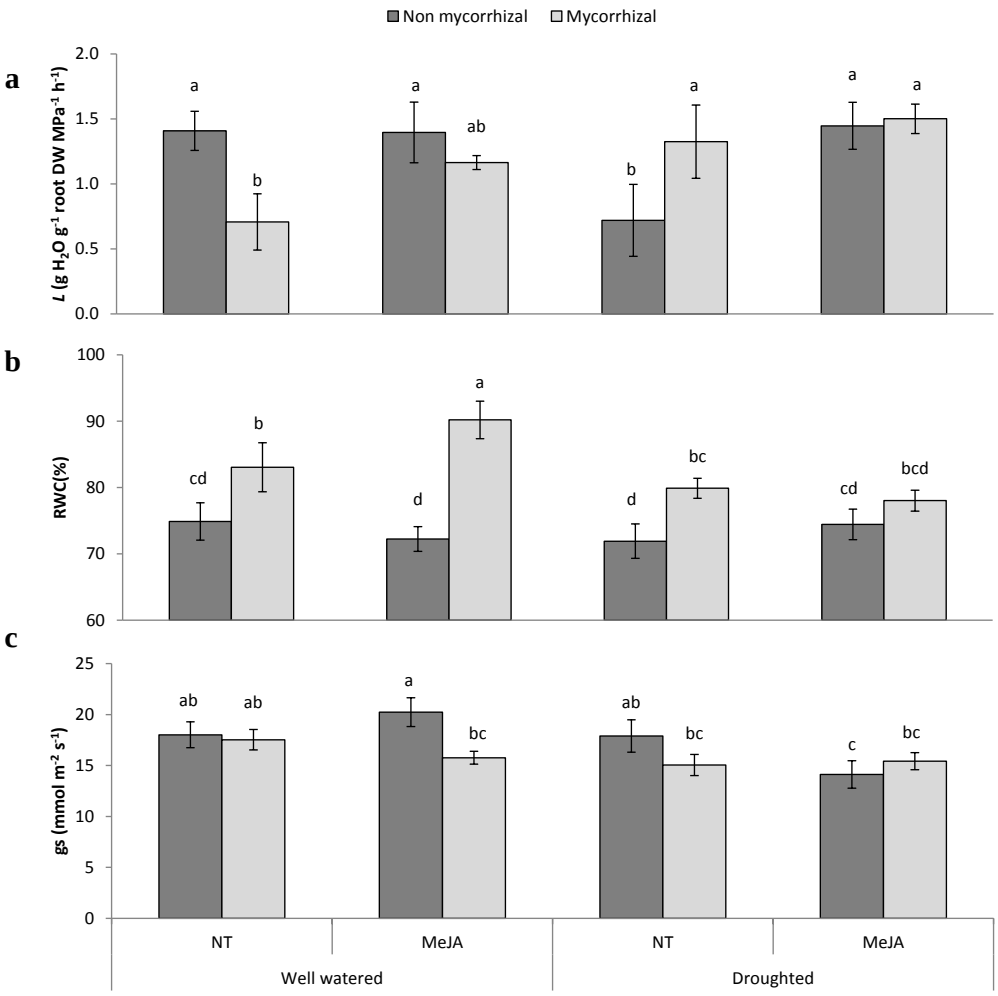


Figure 2.2. (a) Root hydraulic conductivity (*L*), **(b)** relative water content (RWC), **(c)** and stomatal conductance (gs) of *P. vulgaris* plants. Plants were either uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars). Plants were treated with 200 µM MeJA or did not receive MeJA applications (NT). Plants were either cultivated under well-watered conditions or under drought conditions for seven days. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test. *L* was measured in four different plants and RWC and stomatal conductance were measured in seven different plants.

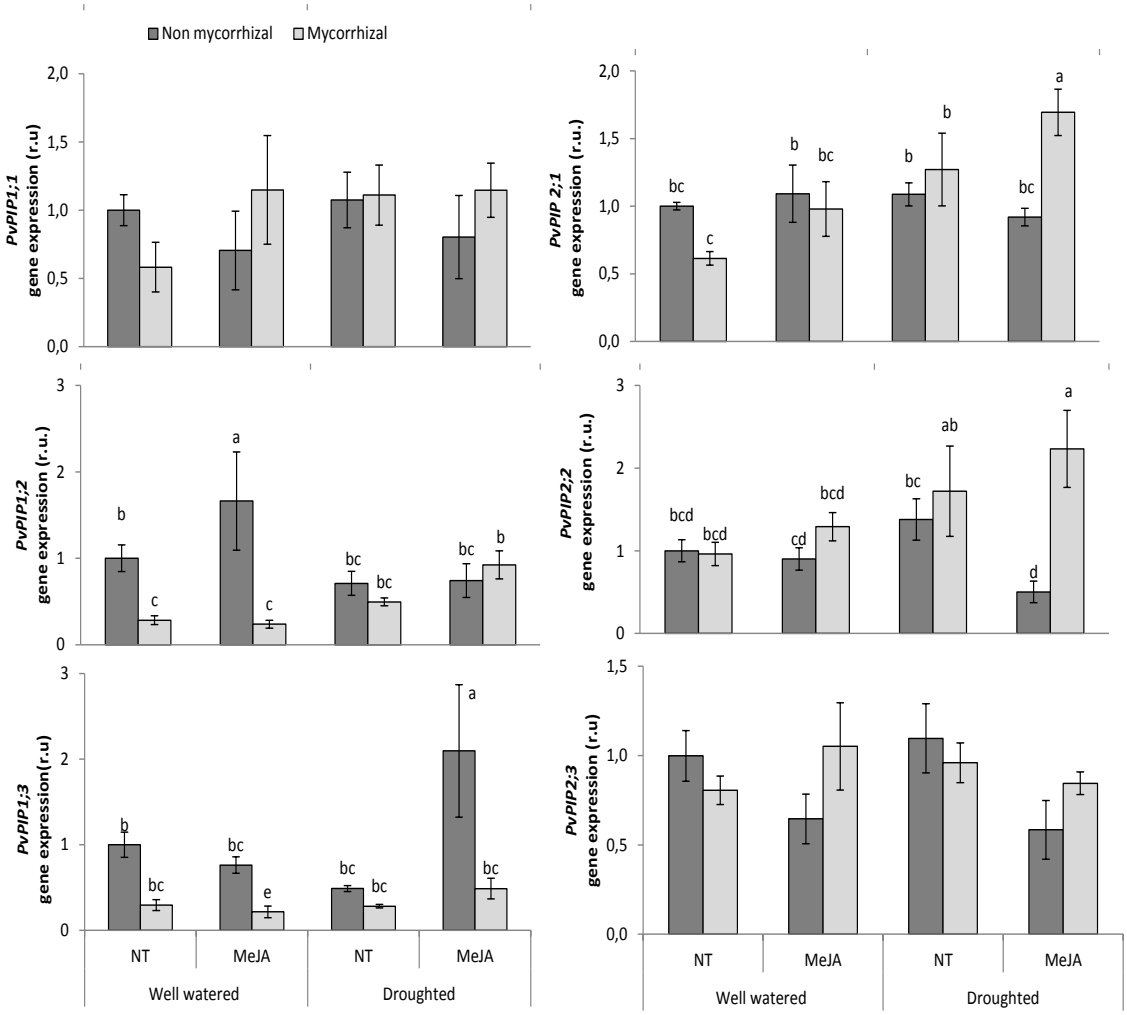


Figure 2.3. Relative expression of *PvPIP1;1*, *PvPIP1;2*, *PvPIP1;3*, *PvPIP2;1*, *PvPIP2;2* and *PvPIP2;3* genes determined by q-RT-PCR in *P. vulgaris* roots uninoculated (dark bars) and inoculated with *Rhizophagus irregularis* (grey bars). Plants were treated with 200 µM MeJA or did not receive MeJA applications (NT). Plants were either cultivated under well-watered conditions or under drought conditions for seven days. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=6).

Plant PIP protein abundance and phosphorylation state

Abundance of PIP1, PIP2 and PIP2 phosphorylated proteins (PIP2PH) at Ser-280 were analysed by ELISA, finding significant changes only for PIP2Ph (Fig.2.4a; Table 2.1). The presence of AMF or the application of MeJA decreased PIP2PH abundance under well-watered conditions. Drought treatment reduced PIP2PH amount in non-AM plants untreated with MeJA, and the

exogenous MeJA application counteracted this effect (Fig. 2.4a). On the contrary, drought treatment caused an increase in the amount of PIP2PH proteins in AM plants in the absence of MeJA (Fig. 2.4a). A significantly ($p < 0.05$) positive correlation was found between *L* values and PIP2PH protein amount (Fig. 2.4b).

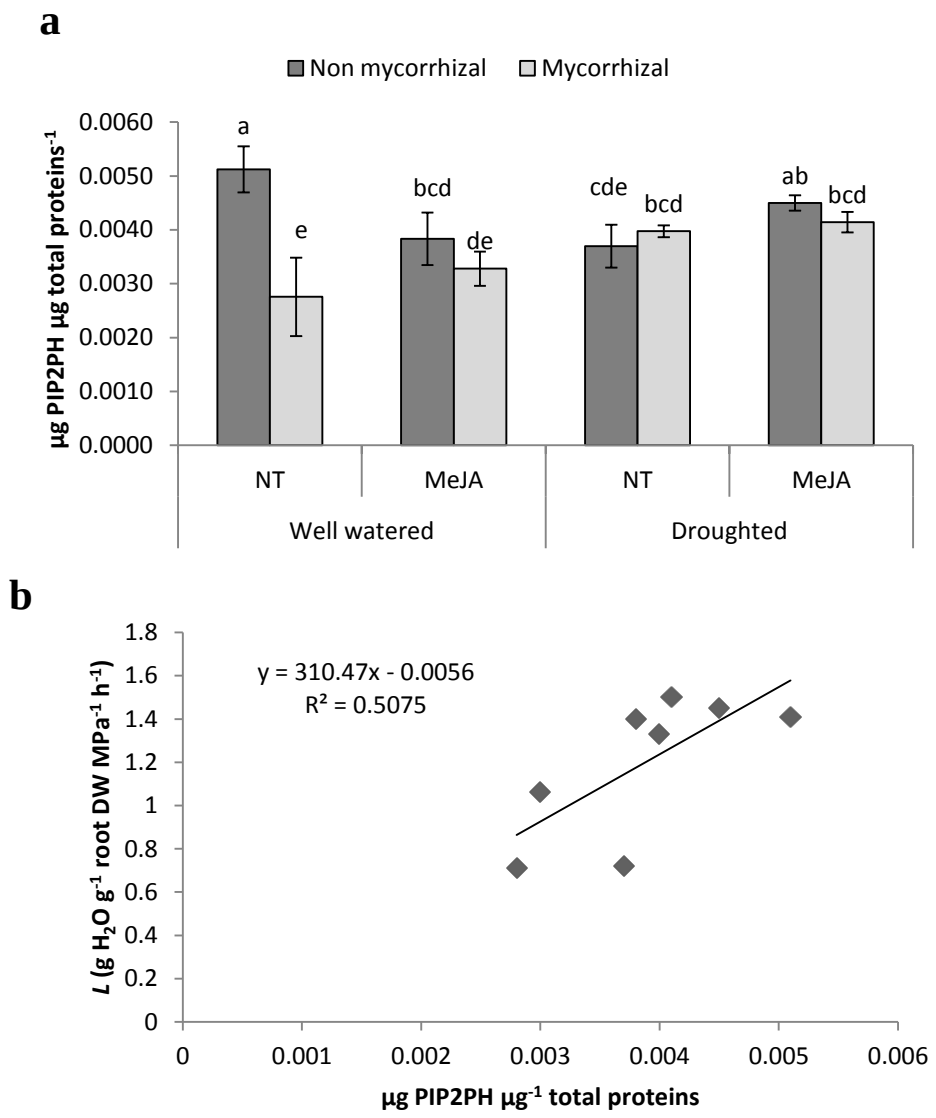


Figure 2.4. (a) Abundance of phosphorylated PIP2s at Ser-280 (PIP2PH) proteins in roots of *P. vulgaris* untreated (NT) or treated with 200 µM MeJA, in non-AM plants (dark bars) and AM plants with *Rhizophagus irregularis* (grey bars) under well-watered or drought conditions. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=5). **(b)** Representation of lineal model established between *L* and PIP2PH.

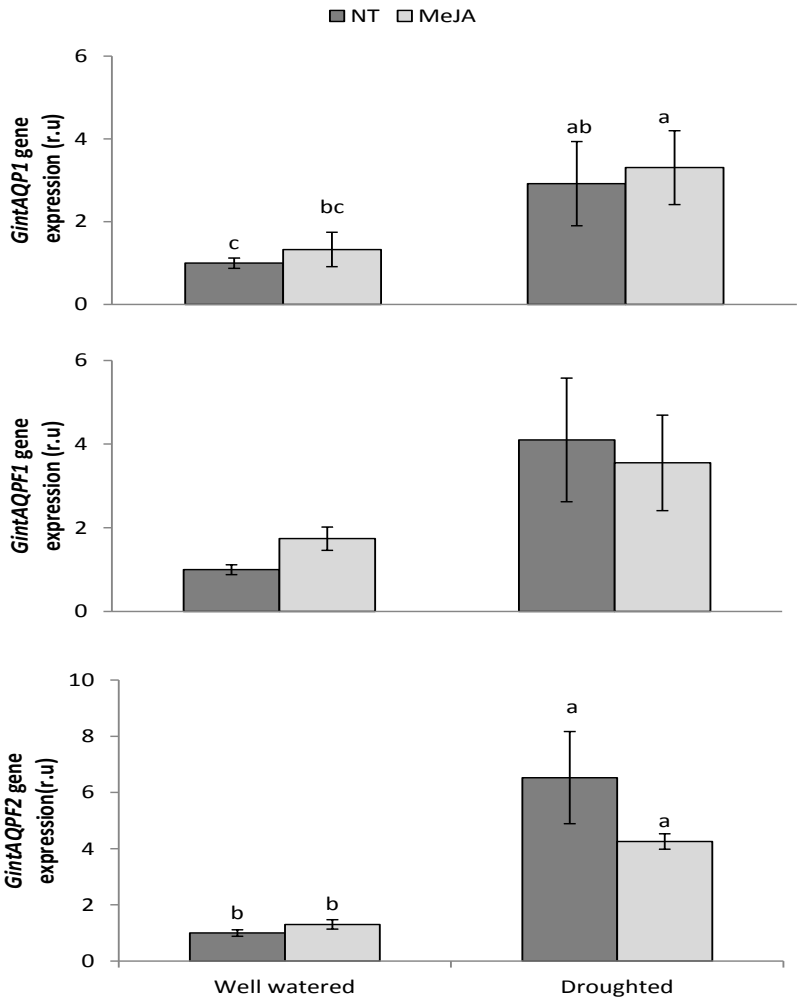


Figure 2.5. Expressions of *GintAQP1*, *GintAQPF1* and *GintAQPF2* genes of AM roots. Untreated roots (dark bars) and roots treated with 200µM MeJA (grey bars) under well-watered or drought conditions. Bars represent mean±SE (n=6). Different letters indicate significant differences among treatments ($p<0.05$) after ANOVA and LSD tests.

Expression of fungal AQPs

The gene expression of the three known AQPs in *R. irregularis* was analysed (Fig. 2.5). Expression of *GintAQP1* and *GintAQPF2* genes was lower under well-watered conditions than under drought conditions. MeJA application had no effect on these genes. The expression of *GintAQPF1* gene did not change significantly ($p>0.05$) under any treatment.

Concentrations of hormones

Effects of *R. irregularis* colonization and exogenous application of MeJA on the concentration of several hormones, i.e. JA, ABA, SA and IAA, in root tissues were analysed.

The application of MeJA increased MeJA concentration in roots except in AM plants under well-watered conditions (Table 2.1; Fig. 2.6a). Under drought conditions, the increase of MeJA content in roots by MeJA addition was lower in AM plants than in non-AM plants (Fig. 2.6a). AM symbiosis or drought separately had no effect on endogenous MeJA.

Plants untreated with MeJA had the same JA content as plants treated with MeJA (Table 2.1; Fig. 2.6B). However, AM plants showed lower JA contents than non-AM plants under well-watered conditions (Fig. 2.6b). Also, drought treatment diminished JA contents in non-AM plants to the values of AM plants (Fig. 2.6b).

AM plants had higher IAA concentrations than non-AM plants regardless of the treatment. However, MeJA application diminished IAA content in AM plants under well-watered conditions while the opposite was observed under drought conditions (Fig. 2.6c). Non-AM plants did not change their IAA contents by any treatment (Fig. 2.6c). Additionally, drought stress enhanced ABA content in all roots, but AM symbiosis or MeJA had no further effects (Table 2.1; Fig. 2.6d). Drought treatment increased SA content only in non-AM plants without MeJA treatment (Fig. 2.6e), and the root content of SA correlated negatively ($p < 0.05$) with *L* values (Fig. 2.6f).

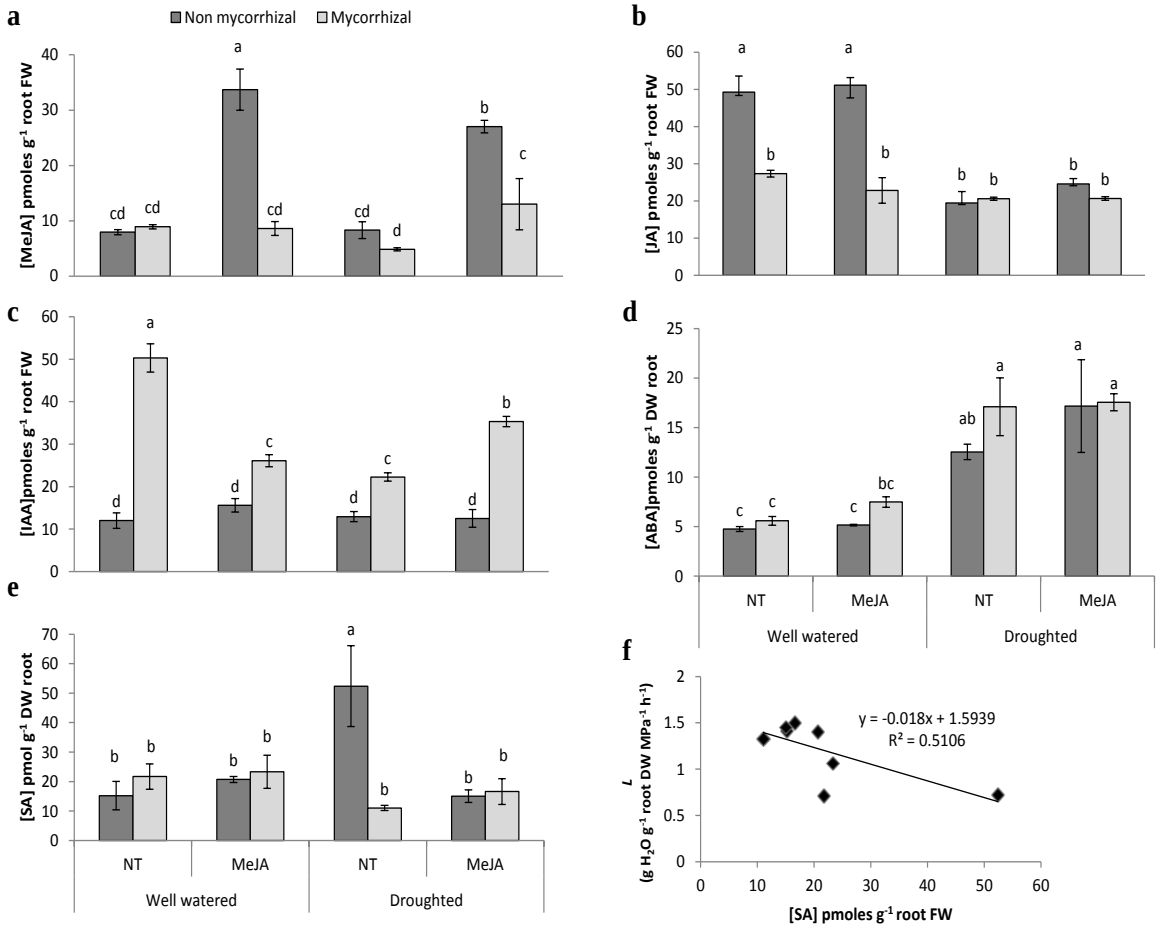


Figure 2.6. (a) Methyl jasmonate (MeJA), **(b)** jasmonic acid (JA), **(c)** indole-3-acetic acid (IAA) **(d)** abscisic acid (ABA) and **(e)** salicylic acid (SA) root concentration of beans roots untreated (NT) or treated with 200µM MeJA and uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars), under well-watered and drought conditions. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=4). **(f)** Representation of lineal model established between *L* and SA.

Discussion

It is known that a fine regulation of root hydraulic properties is crucial for plants to tolerate drought stress (Salih *et al.* 1999) and that root hydraulic properties could be affected by AM symbiosis (El-Mesbahi *et al.* 2012) and MeJA (Chapter 1: Sanchez-Romera *et al.* 2014). The aim of the present study was to elucidate how exogenous MeJA affects root hydraulic properties and levels of endogenous hormones depending on the AM fungal presence and water regime.

All plant responses to cope with environmental stresses are regulated by a crosstalk between different hormones and signal molecules. For instance, it is known that ABA controls *L* (Aroca *et al.* 2008b, Ruiz-Lozano *et al.* 2009). However, *L* is also co-regulated by other hormones such as JA, ethylene, SA or IAA (de Ollas *et al.* 2013, Hossain *et al.* 2011, Sanchez-Romera *et al.* 2014).

Little information is available regarding the role of JA on root hydraulic properties (chapter 1: Sanchez-Romera *et al.* 2014). The most studied function of JA regards the protection of plants against biotic stresses and its role in regulating stomatal conductance (Hossain *et al.* 2011, Wasternack 2007). This last effect is not so clear under well-watered conditions, since there are reports in which JA induced stomatal closure (Akter *et al.* 2013, Akter *et al.* 2012, Gehring *et al.* 1997, Herde *et al.* 1997) while others reported that JA had no effect on stomatal aperture (Horton 1991). Furthermore, it is known that stomatal closure is due to a combined action of ABA and JA (Hossain *et al.* 2011). Under drought conditions, de Ollas *et al.* (2013) observed an increase in the endogenous content of these hormones and an induction of stomatal closure. Here, we did not find any data supporting the combined action of ABA and JA under drought conditions. First, we could see an increase of ABA content in plants subjected to drought, regardless of AMF and MeJA applications. However, we observed the opposite effect in endogenous JA contents and any effect in endogenous MeJA content. Second, the water stress did not cause any modification in stomatal conductance. A decrease of stomatal conductance was only found after MeJA application in non-AM plants and no interaction between ABA and MeJA was observed. Although Anjum *et al.* (2011) found that exogenous application of MeJA improved leaf water content under both water regimes, well-watered and drought, in our study we could not see such an effect (Fig. 2.1b; Table 2.1).

On the other hand, Sanchez-Romera *et al.* (2014) found that plants treated with MeJA increased their *L* under hydroponic conditions. In our experiment, with plants growing on a solid substrate in pots, the plants cultivated under well-watered conditions did not show this effect, regardless of the AMF presence. However, the MeJA-treated plants increased *L* under drought conditions (Fig. 2.1a; Table 2.1), supporting previous results (Chapter1: Sanchez-Romera *et al.* 2014).

Non-AM plants which had the lowest *L* values under drought conditions, presented higher SA content in their roots, thus supporting findings by Boursiac *et al.* (2008a) in Arabidopsis. Proietti *et al.* (2013) found negative crosstalk for the combined action of JA and SA. Therefore, SA production could play an important role in the reduction of *L* under drought conditions, this effect being counteracted by AM symbiosis or MeJA addition. Since there was a negative correlation between SA contents and *L*, we propose that the reduction of SA levels could be a mechanism by which AM symbiosis usually avoid *L* diminutions under drought conditions (Barzana *et al.* 2012), since SA contents are usually high under drought conditions (Bandurska & Cieslak 2013).

Addition of MeJA increased *L* and decreased stomatal conductance as an anti-stress strategy for non-AM plants. *PvPIP1;2* gene expression was increased in non-AM plants as a result of exogenous MeJA application in well-watered plants. On the other hand, MeJA application increased the expression of *PvPIP1;3* gene and decreased the expression of *PvPIP2;2* gene in non-AM plants under drought conditions. Regardless of their expression, the abundance of Ser-280 phosphorylated PIP2 proteins was increased in non-AM plants treated with MeJA under water deficit conditions. This phosphorylation at the C-terminal tail of the AQP, activates it and thus the transport of water by the cell-to-cell path is increased (Azad *et al.* 2008, Prado *et al.* 2013), which is the water path measured by our *L* determination method (Steudle & Peterson 1998). Moreover, a positive correlation was found between PIP2PH amount and *L*, reinforcing the regulatory role of phosphorylation on Ser-280.

By establishing symbiotic relationships with AMF and plants have advantages to survive under adverse conditions. AMFs are able to get water from the soil that is inaccessible to plants due to better soil exploration through their hyphae (Marulanda,Azcon & Ruiz-Lozano 2003). AM symbiosis has been widely studied in a great variety of plants and under various abiotic stress conditions. It is known that *L* could be increased by AM symbiosis under drought conditions (Barzana *et al.* 2012).

The expression of plant AQPs under drought conditions could be modified by AM symbiosis. Thus, *SlPIP2;1* expression was increased in tomato AM roots after 10 days of drought (Aroca *et al.* 2008a). Also, *ZmPIP1;2, ZmPIP1;5, ZmPIP2;5* and Zm*PIP2;6* expressions and the abundance of ZmPIP1;2, ZmPIP2;1 and ZmPIP2;5 proteins were raised in maize AM roots after 4 days of withholding water (Ruiz-Lozano *et al.* 2009). However, Porcel *et al.* (2006a) observed a decrease of PIP2 amount in lettuce plants by *Funneliformis mosseae* symbiosis. Nevertheless, we did not see any effect in plant AQPs expression which could explain the high values of *L* found under drought conditions in AM plants. In fact, AQP in AM plants showed low or similar expression than in non-AM plants, regardless of water regime. This could be due to a high IAA root concentration in AM plants, since repression of AQP gene expression in Arabidopsis has been reported after exogenous IAA application (Peret *et al.* 2012).

Taking into account that AM fungi have their own AQPs, these could facilitate the transport of water from the soil to the host roots (Aroca *et al.* 2009, Li *et al.* 2013b). Fungal AQPs are known to increase their expression under drought conditions (Aroca *et al.* 2009, Li *et al.* 2013b), which could also contribute to the increase of *L* caused by the AM symbiosis.

It is known that ABA and JA are necessary for the establishment of the symbiosis as well as for the root colonization process (Herrera-Medina *et al.* 2007, Leon-Morcillo *et al.* 2012a, Martin-Rodriguez *et al.* 2011). We focused on MeJA and AM symbiosis, showing that exogenous application of MeJA in AM plants did not modify the effect caused by the fungus regardless of the water regime. Only the expression levels of *PvPIP2;1* and *PvPIP2;2* got cumulative effects under drought conditions.

In conclusion, both AM symbiosis and MeJA could be used by plants to overcome adverse conditions. We found that AMF improved plant water status under both well-watered and drought conditions. The effects of MeJA addition were only observed under drought conditions. Reduction of *L* caused by drought could be due to an accumulation of SA in root tissues, and this accumulation was prevented by both MeJA addition and AM symbiosis. On the other hand, we found data supporting that PIP activity can be partly governed by phosphorylation at Ser-280.

Chapter 3: Involvement of *def*-1 mutation in the regulation of root hydraulic conductivity by arbuscular mycorrhizal fungi

Chapter 3: Involvement of *def-1* mutation in the regulation of root hydraulic conductivity by arbuscular mycorrhizal fungi

Objective

The aim of the present study was to investigate the involvement of internal JA contents in the regulatory activity of AMF on *L* under both well-watered and drought stress conditions. For that, tomato plants deficient in JA synthesis (*def-1*; Howe *et al.* 1996) and wild type (WT) plants were grown, without AM inoculum or inoculated with the AM fungus *Rhizophagus irregularis* under two water regimes (well-watered or drought). Plant growth, AMF colonization, water status, *L*, aquaporins expression and abundance, and hormonal contents were determined.

Experimental design

Plants of *Solanum lycopersicum* were used in an experiment following a combined factorial design with three factors: (1) biological factor, with plants inoculated or not with the AMF *Rhizophagus irregularis*, (2) genotype factor formed by WT plants and mutant plants which are deficient in JA (*def-1*), and (3) abiotic factor, in which plants were grown under well-watered or drought conditions. Finally, we had a total of 10 replicates in 8 different treatments. Six replicates of each treatment were used for the measurement of *L*, and the remaining 4 replicates were frozen in liquid nitrogen immediately after harvest for later use in other molecular and biochemical determinations described in the Materials and Methods section. For all the treatments, physiological measurements and the collection of plant samples were carried out 3 h after sunrise, in order to avoid diurnal fluctuations in plant processes.

Growth conditions

The experiment lasted 11 weeks from seed germination and was conducted under greenhouse conditions with temperatures from 19 to 25°C, 16/8 h light/dark, a relative humidity of 50-60% and a photosynthetic photon flux density of 800 µE m^{-2} s^{-1}, as measured with a light meter (LICOR; Lincoln, NE, USA model LI-188B). Before the beginning of drought treatment, all plants received 10 ml of 80% Hewitt`s nutrient solution (Hewitt 1952). Soil moisture was controlled gravimetrically and water was supplied daily to maintain soil at field capacity before starting the

drought treatment. Ten days before harvest, half of the plants were subjected to drought (60% of field capacity) while the other half was maintained at field capacity until harvest.

Table 3.1. Significance of sources of variation after three-way ANOVA analyses for following parameters, shoot dry weigh (SDW), root hydraulic conductivity (*L*), relative water content (RWC), stomatal conductance (gs), *SlPIP* isoforms gene expressions, PIPs abundance, methyl salicylic acid content (MeSA), indole-3-acetic acid content (IAA), abscisic acid content (ABA) and salicylic acid content (SA). The sources of variance were AM symbiosis (AM), water regime (WR) and genotype, as well as their interactions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant effect.

	AM	WR	Genotype	AMxWR	AMx Genotype	WRx Genotype	AMxWRx Genotype
SWD	**	ns	ns	ns	ns	ns	ns
L	**	ns	*	ns	ns	ns	ns
RWC	ns	ns	*	*	ns	ns	ns
gs	ns	***	ns	ns	**	ns	*
SlPIP1;1	ns	**	ns	**	ns	ns	ns
SlPIP1;5	ns	ns	ns	ns	ns	ns	ns
SlPIP1;7	*	***	***	*	*	***	ns
SlPIP2;1	ns	**	ns	ns	ns	ns	**
SlPIP2;4	ns	***	ns	ns	ns	ns	ns
SlPIP2;6	ns	***	ns	**	ns	ns	ns
SlPIP2;8	ns	ns	ns	**	ns	*	ns
SlPIP2;9	ns	ns	ns	***	ns	**	ns
PIP1	ns	ns	**	ns	ns	ns	ns
PIP2	***	*	ns	ns	ns	ns	ns
PIP2Ph	**	***	*	ns	ns	ns	ns
MeSA	***	ns	***	ns	ns	ns	*
IAA	***	ns	ns	*	**	ns	ns
ABA	**	ns	***	ns	ns	*	ns
SA	**	***	ns	*	ns	**	*

Results

Mycorrhizal colonization and plant growth

After 11 weeks of plant growth and having subjected the plants to a period of 10 days with a controlled water regime, root AM colonization was determined. Uninoculated plants did not exhibit any mycorrhizal colonization. Inoculated plants displayed a percentage of root length colonization

around 60%, with no significant differences regardless of water regime or genotype (Fig. 3.1a). No significant differences ($p>0.05$) in root dry weight were observed among treatments (Fig. 3.1c). Instead, WT AM plants showed lower shoot dry weight than non-AM plants only under drought conditions (Fig. 3.1b). Overall, AM plants were smaller than non-AM plants but no effects of drought treatment or genotype were observed on shoot dry weight (Table 3.1).

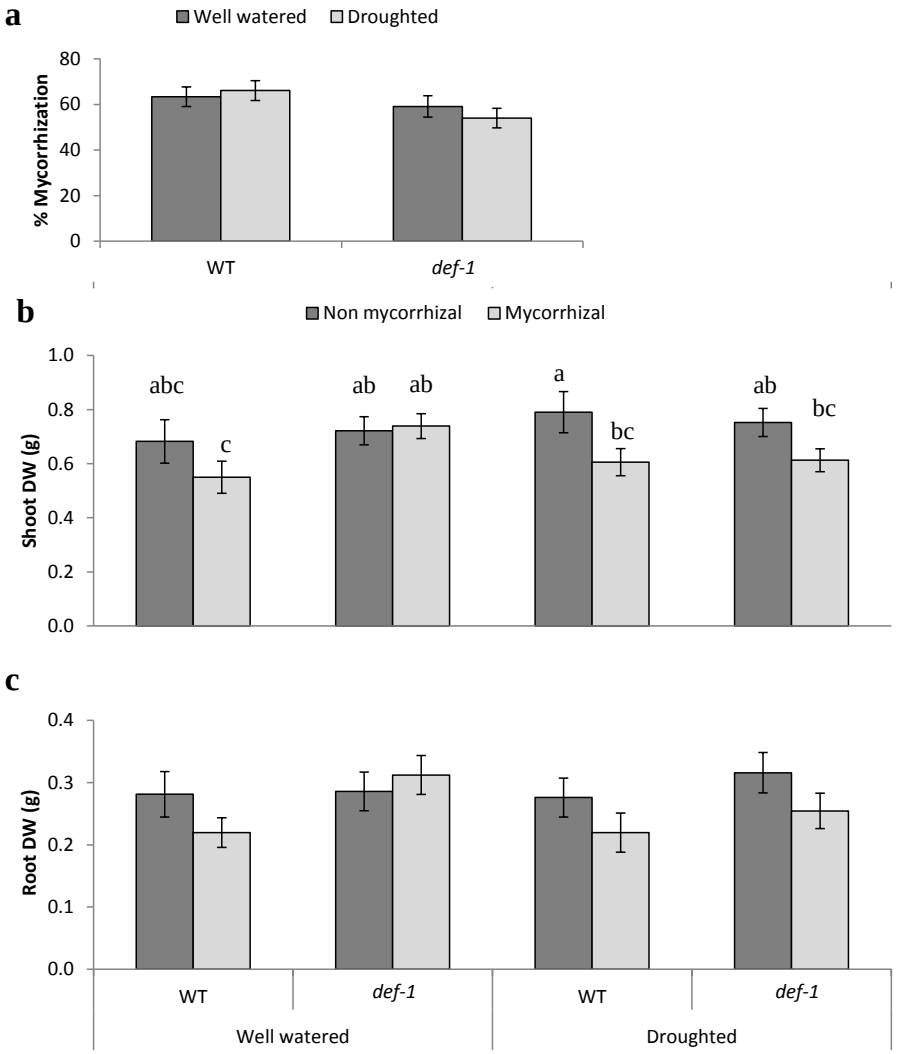


Figure 3.1 (a) Percentage of mycorrhizal root length colonization in tomato roots by *Rhizophagus irregularis*. Nine plants of each treatment were analysed. WT plants (dark bars) and *def-1* plants (grey bars) grown under well-watered or drought conditions. (b) Dry weights of shoots and (c) roots of WT or *def-1* tomato plants, uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars), were grown under well-watered and drought conditions. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=6).

Root hydraulic conductivity (*L*)

L was determined by the HPFM method (see Material and Methods section). *Def-1* plants, which are deficient in JA, showed different behaviour of *L* depending on the water regime (Fig. 3.2a). Under well-watered conditions *def-1* plants had higher *L* than WT plants (without AMF presence), whereas under drought conditions, both kinds of plants showed similar *L*. On the other hand, *L* remained constant in AM plants, not being affected by water regime or plant genotype (Fig. 3.2a).

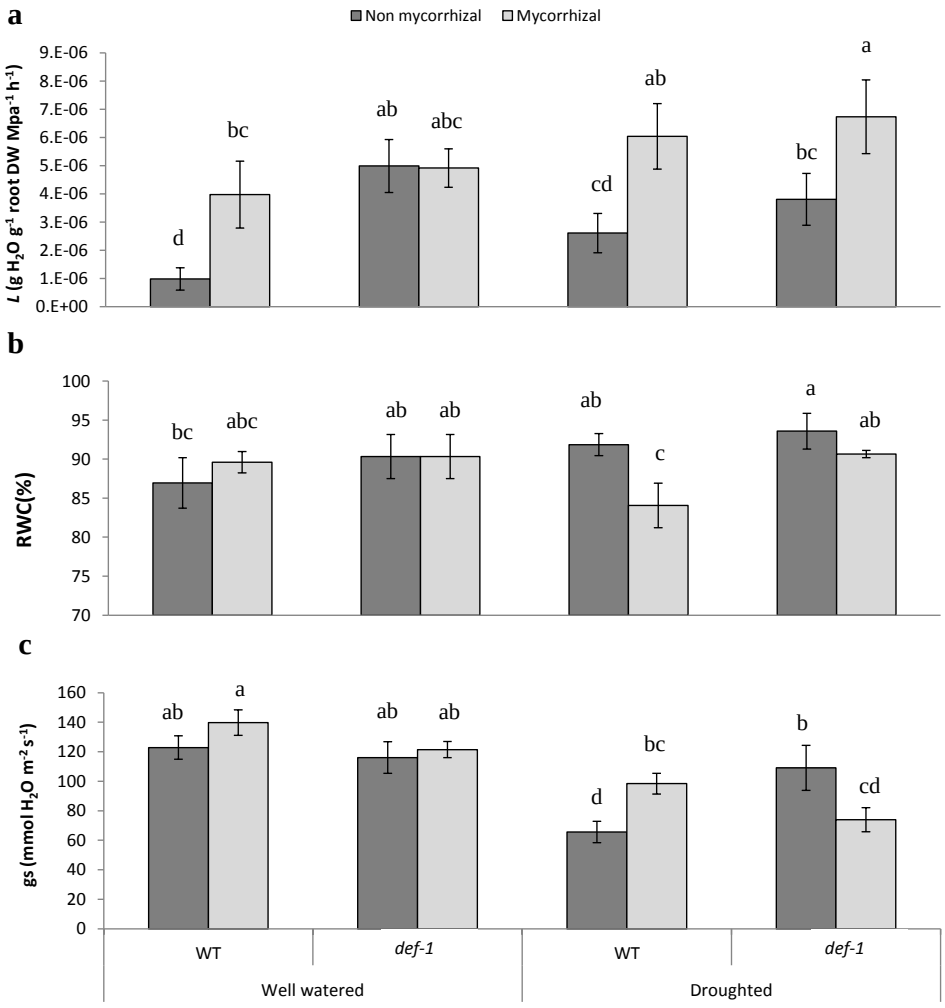


Figure 3.2. (a) Root hydraulic conductivity (*L*) measured by the HPFM method (n=6), **(b)** relative water content (RWC) (n=6), **(c)** stomatal conductance (gs) (n=20) of WT and *def-1* tomato plants. Plants were either uninoculated plants (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars) and were cultivated under well-watered conditions or subjected to drought conditions during ten days. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test.

Thus, *L* of AM plants was always higher than *L* of non-AM plants, except for *def-1* plants under well-watered conditions (Fig. 3.2a). ANOVA analysis showed that *L* was significantly up-regulated by AM ($p < 0,01$) and *def-1* genotype ($p < 0,05$; Table 3.1). Drought treatment had no effect on *L* (Table 3.1).

Relative water content (RWC) and stomatal conductance (gs)

Neither AMF nor plant genotype caused changes in RWC under well-watered conditions (Fig. 3.2b). In contrast, under drought conditions AMF decreased RWC in WT plants but not in *def-1* ones (Table 3.1; Fig. 3.2b). Overall *def-1* plants had higher RWC than WT plants (Table 3.1; Fig. 3.2b).

Stomatal conductance (gs) was mainly reduced by drought (Table 3.1; Fig. 3.2c). Only under drought conditions differences in gs among treatments were observed. Hence, AM symbiosis increased gs in WT plants, but decreased it in *def-1* plants. Also, *def-1* plants showed higher gs than WT ones when not inoculated (Fig. 3.2c). The only plants that did not decrease their gs by drought treatment were *def-1* ones without inoculation (Fig. 3.2c).

Plant *PIP* and fungal *AQP* gene expressions

The expression of eight plant *PIP* genes was analysed in roots of *S. lycorpersicum* by q-RT-PCR. More precisely, three *SlPIP1s* and five *SlPIP2s* genes were analysed. We found significant differences in the expression of two out of three *PIP1s* under drought conditions. Drought treatment diminished *SlPIP1;1* expression in all treatments except in WT plants inoculated with the AMF (Table 3.1; Fig. 3.3). Also, *def-1* plants had higher expression of *SlPIP1;7* gene than WT plants, this effect being decreased by AM symbiosis. The *SlPIP1;7* expression was significantly regulated by all factors (Table 3.1; Fig. 3.3). The opposite happened for *SlPIP1;5* expression, which was not regulated by any factor (Table 3.1; Fig. 3.3).

On the contrary, *PIP2s* genes showed more differences in their expression under well-watered conditions than under drought conditions (Fig. 3.4). For instance, the expression of *SlPIP2;1*, *SlPIP2;8* and *SlPIP2;9* genes in *def-1* plants was higher than in WT plants under well-watered conditions and absence of AMF (Fig. 3.4). However, *SlPIP2;4* and *SlPIP2;6* expressions were similar in both kinds of plants. Moreover, we observed that AMF decreased the expression of several genes such as *SlPIP2;4*, and *SlPIP2;6* in WT plants and *SlPIP2;1*, *SlPIP2;6*, *SlPIP2;8* and *SlPIP2;9* in *def-1* plants under well-watered conditions. Under drought conditions the only

differences observed were that *SlPIP2;6* and *SlPIP2;9* expressions were higher in WT AM plants than in WT non-AM plants (Fig. 3.4).

The expressions of three fungal AQP genes from *R. irregularis* were analysed by q-RT-PCR (Fig. 3.5). No significant differences in the expression of two of them (*GintAQPF2* and *GintAQP1*) were observed. Only the expression of *GintAQPF1* was up-regulated in *def-1* plants under well-watered conditions (Fig. 3.5).

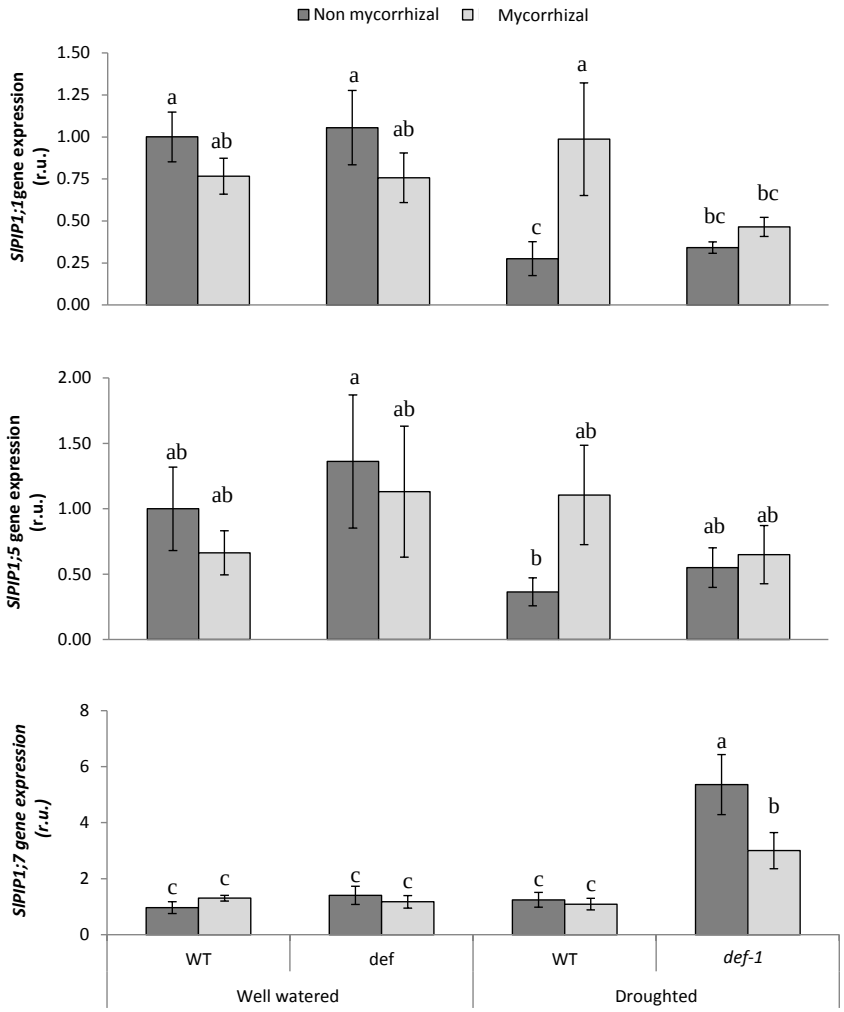


Figure 3.3. Relative expression of *SlPIP1;1*, *SlPIP1;5*, and *SlPIP1;7* genes determined by quantitative real-time RT-PCR in WT and *def-1* tomato roots. Plants uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars) were grown under well-watered conditions or were subjected to drought conditions for ten days. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=6).

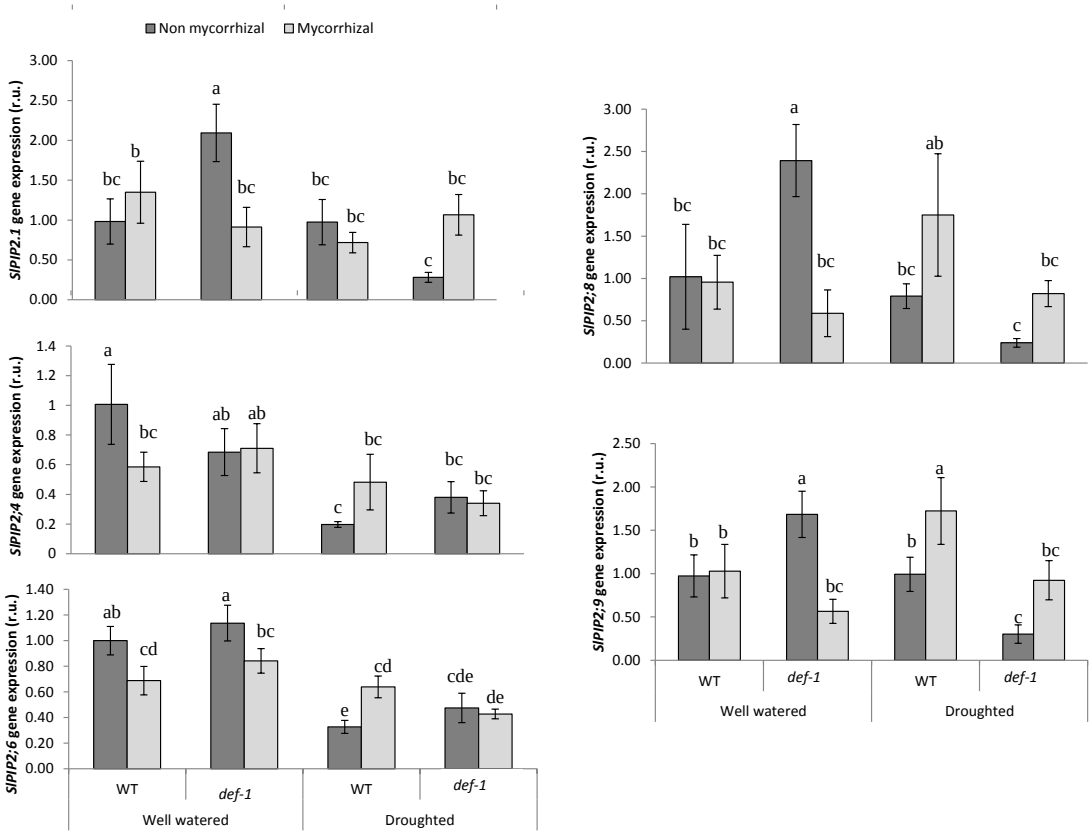


Figure 3.4. *SlPIP2;1*, *SlPIP2;4*, *SlPIP2;6*, *SlPIP2;8* and *SlPIP2;9* genes determined by quantitative real-time RT-PCR in WT and *def-1* tomato roots. Plants uninoculated (dark bars) or not inoculated with *Rhizophagus irregularis* (grey bars) were grown under well-watered conditions or were exposed to drought conditions. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=6).

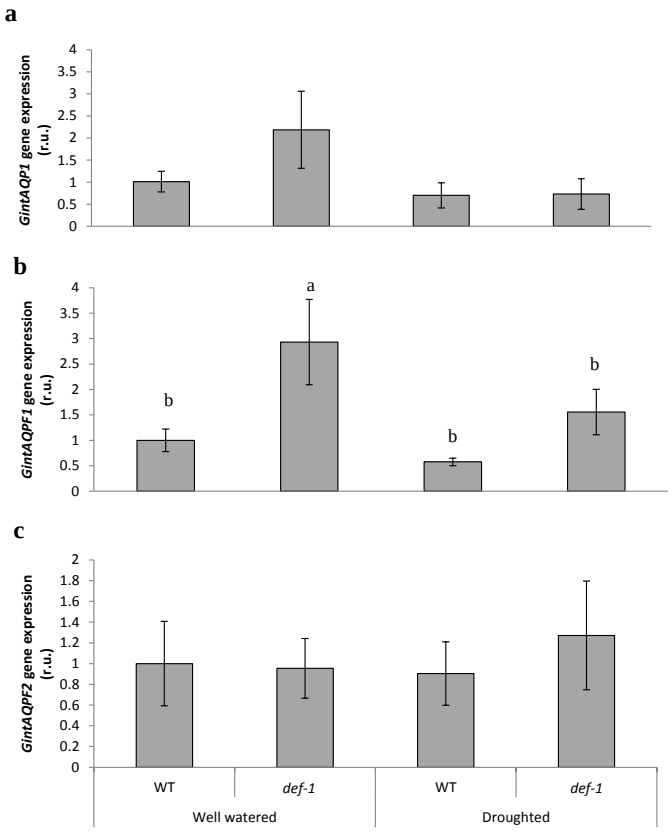


Figure 3.5. (a) *GintAQP1*, **(b)** *GintAQPF1* and **(c)** *GintAQPF2* gene expression of WT root tomato plants (dark bars) and *def-1* root tomato plants (grey bars) under well-watered or drought conditions. Bars represent mean ± SE (n=6). Different letters indicate significant differences among treatments ($p<0.05$) after ANOVA and LSD tests.

Plant PIP proteins abundance

We analysed the abundance of PIP1, PIP2 and PIP2 phosphorylated proteins (PIP2PH) at Ser-280 by ELISA technique (Barzana *et al.* 2014, Boursiac *et al.* 2005). PIP1 abundance was not significantly affected by either AMF or water regimen. However, ANOVA analysis showed that the genotype factor had an effect on the abundance of PIP1. *Def-1* roots had lower PIP1 abundance than WT roots (Table 3.1; Fig. 3.6a).

Under well-watered conditions, uninoculated WT roots had more PIP2PH abundance than *def-1* roots and WT AM roots (Fig. 3.6c). However, the abundance of PIP2 was the same in WT and *def-1* roots, being reduced by AMF presence (Table 3.1; Fig. 3.6b). On the other hand, the drought treatment reduced PIP2PH abundance in all roots, except in non-AM *def-1* roots. Nevertheless, in general all AM plant had lower PIP2PH concentration than non-AM plants (Table 3.1; Fig. 3.6c).

Additionally, after studying statistical regression between *L* and the amount of each of these AQPs, a negative linear regression was observed between *L* and the abundance of PIP1 and PIP2PH (Fig. 3.7 a. and Fig. 3.7b).

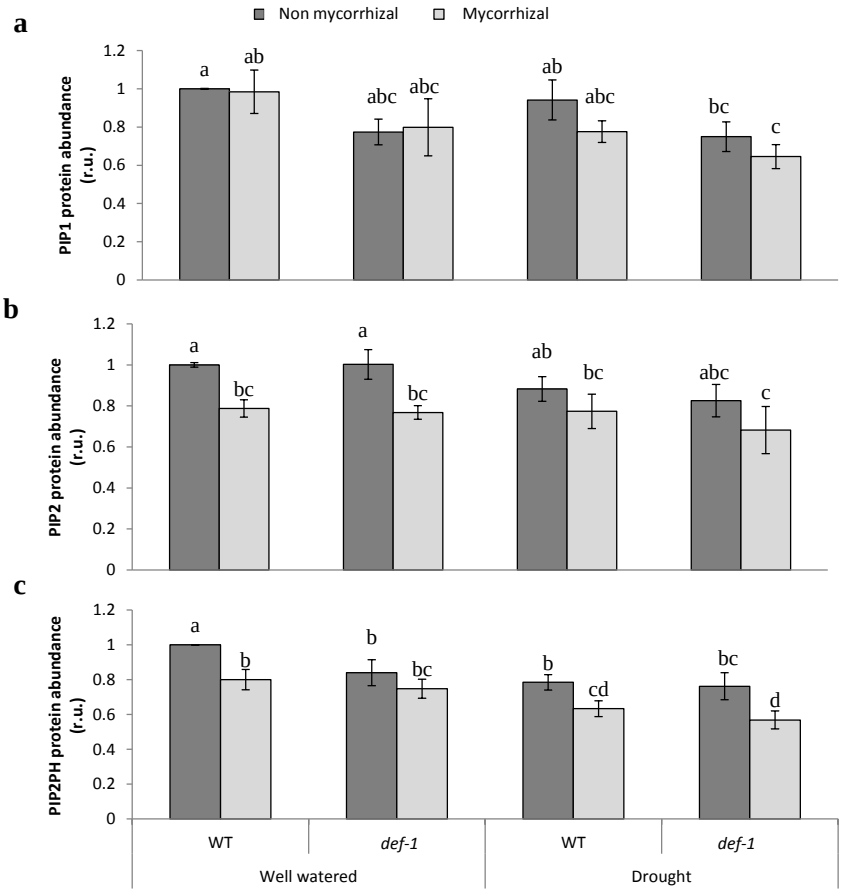


Figure 3.6. Abundance of **(a)** PIP1s, **(b)** PIP2s and **(c)** PIP2s phosphorylated at Ser-280 (PIP2PH) proteins in WT and *def-1* tomato roots. Plants were uninoculated (dark bars) and inoculated by *Rhizophagus irregularis* (grey bars) and were grown under well-watered or drought conditions. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=6).

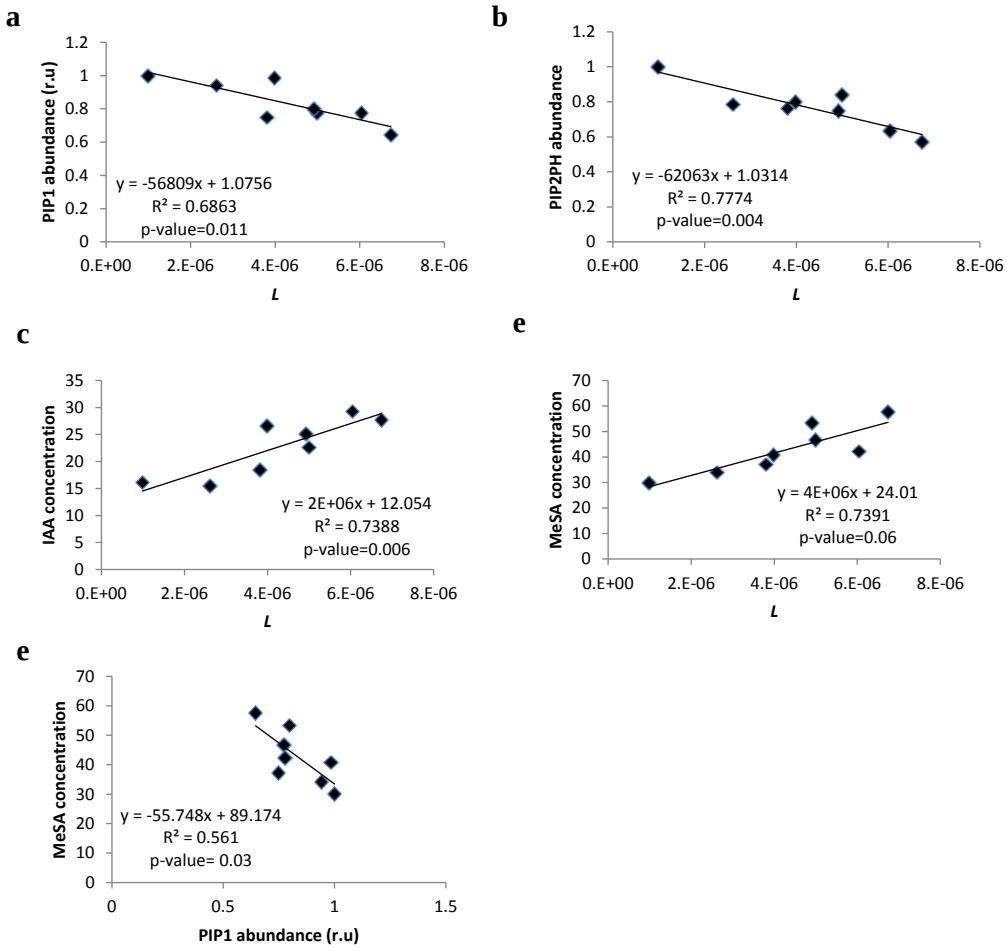


Figure 3.7. Representation of lineal regression model established between different parameters. **(a)** PIP1 abundance- , **(b)** PIP2PH abundance-*L*, **(c)** IAA concentration -*L*, **(d)** MeSA concentration-*L* and **(d)** MeSA concentration- PIP1 abundance.

Hormone concentrations

The root contents of four hormones (ABA, IAA, SA and MeSA) were measured. Firstly, *def-1* plants had higher content of IAA, SA and MeSA than WT plants under well-watered conditions in absence of AMF (Fig. 3.8). However, these concentrations changed when the plants were subjected to drought, because *def-1* plants had lower SA content and higher ABA content than WT plants, always in absence of AMF (Fig. 3.8).

Secondly, AMF presence increased the concentration of IAA and MeSA in all plants except in *def-1* ones under well-watered conditions (Fig. 3.8c and Fig. 3.8d). ABA content increased by

AM symbiosis only in *def-1* plants under well-watered conditions (Fig. 3.8a). On the other hand, SA contents were increased by AM symbiosis in both kinds of plants, but only under drought conditions (Table 3.1; Fig. 3.8b). The linear regression analysis showed that there is a positive linear regression between IAA and *L* and between MeSA and *L*. Instead, the PIP1 abundance exhibited a negative linear regression against MeSA (Fig. 3.7).

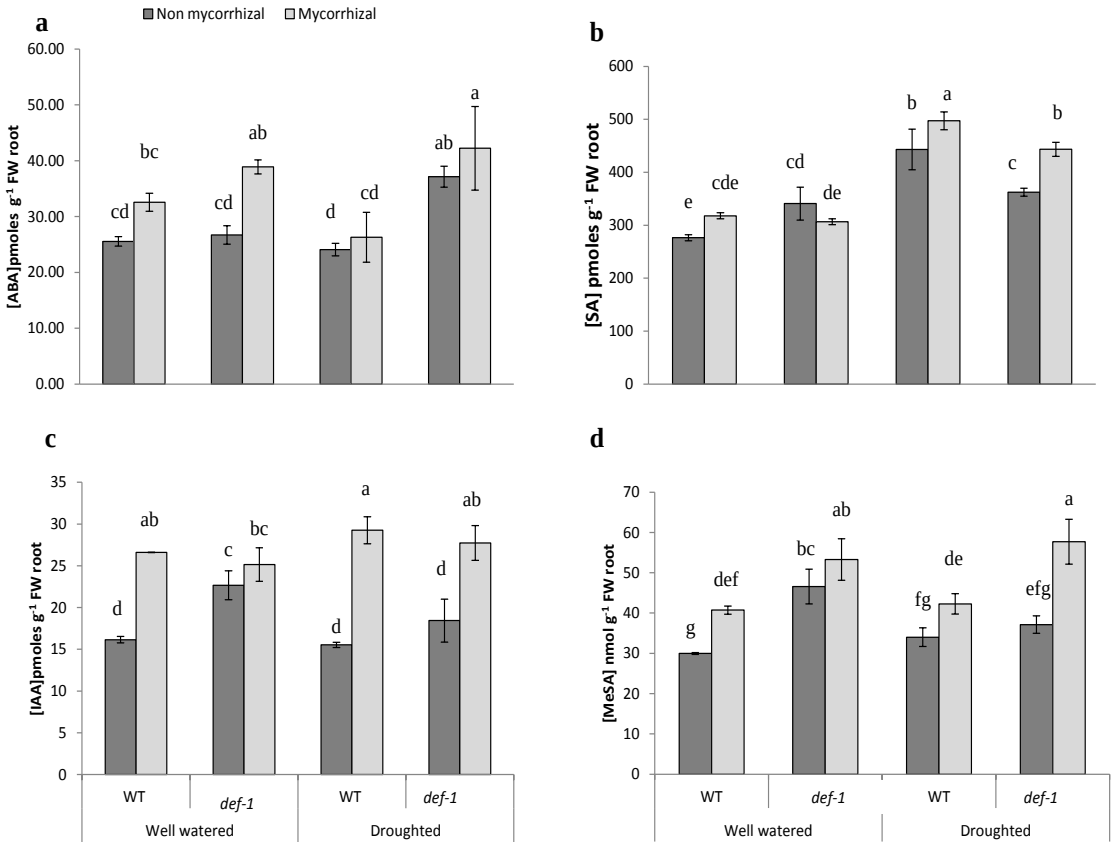


Figure 3.8. (a) Abscisic acid (ABA), **(b)** indole-3-acetic acid (IAA), **(c)** salicylic acid (SA), **(d)** methyl salicylic acid (MeSA) root concentration of WT and *def-1* plants which were uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars), and grown under well-watered or drought conditions. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test (n=4).

Discussion

The mechanisms by which plants regulate their ability to absorb and transport water are still poorly understood. It is known that the interaction between different plant hormones and the presence of AMF affect with the ability of plants to take up water under optimal and stressed conditions (Aroca *et al.* 2008a, Barzana *et al.* 2014). The aim of this work was to get new insights into the effects of the interaction between JA and AMF on root hydraulic conductivity (ability to take up water), in plants growing under well-watered or drought stressed conditions. For this purpose we used the tomato mutant plants (*def-1*), which are defective in JA-biosynthesis (Howe *et al.* 1996, Howe & Ryan 1999, O'Donnell *et al.* 2003).

Effect of *def-1* mutation on root hydraulic properties

There is little information about the possible role of JA in the regulation of root hydraulic properties, although several studies found that exogenous JA improved plant stress tolerance (Anjum *et al.* 2011, Lee *et al.* 1996a, Walia *et al.* 2007).

Lee *et al.* (1996b) observed that plants treated with MeJA increased their RWC and root bleeding rate under cold conditions. More recently, Anjum *et al.* (2011) noted that MeJA-treated plants were more resistant against drought, and that they achieved better RWC. In general, it was observed that MeJA application provided benefits to plants under well-watered and drought conditions (Anjum *et al.* 2011). In support of this hypothesis, a previous study found that *L* increased in different plant species (tomato, bean, arabidopsis) under hydroponic conditions after exogenous applications of MeJA (chapter 1: Sanchez-Romera *et al.* 2014).

Here we used mutant plants whose JA response is affected by impairment of the JA biosynthesis pathway, called *def-1* (Howe *et al.* 1996, Howe & Ryan 1999, O'Donnell *et al.* 2003), and we found that these plants had higher *L* than WT plants under well-watered conditions. Therefore, these results contrast those obtained previously (Chapter 1: Sanchez-Romera *et al.* 2014), where *def-1* plants had lower *L* values than WT plants and this effect was counteracted by MeJA application. These differences in *L* behaviour between the two studies could be caused by the conditions in which the plants were grown, because in the present work the plants were grown in soil and in previous studies, plants were grown under hydroponic conditions. Similarly, Matsuo *et al.* (2009) analysed three different genotypes of rice grown under hydroponic and soil conditions, the plants with the highest *L* value were grown under hydroponics, however the lowest *L* value was

found in plants grown under soil conditions. This was due because the maturation of the exodermal root cells varied depending on the kind of medium in which the root had been cultivated (Matsuo,Ozawa & Mochizuki 2009, Meyer,Seago Jr & Peterson 2009), affecting the water absorption properties of these roots (Hachez *et al.* 2012). Hence, the results obtained under hydroponic conditions cannot be extrapolated to plants growing on soil.

At the molecular level, we observed that WT plants had greater abundance of PIP2PH with respect to *def-1* plants under well-watered conditions. Sanchez-Romera *et al.* (2014: Chapter 1) also observed an increase in the abundance of PIP2PH proteins in MeJA-treated bean plants. In any case, in the present study such protein increase was not accompanied by higher *L*. By contrast, *L* and the abundance of aquaporins were not affected by the plant genotype under drought conditions. Therefore, it is possible that JA is involved in the regulation of aquaporins synthesis and their post-translational modifications, specifically by regulating the concentration of aquaporins in its active state under well-watered conditions. However, we found a negative correlation between *L* and PIP2Ph (P-value<0.005) and PIP1 (P-value<0.02) abundances. Therefore, protein abundance did not explain the increase of *L* caused by *def-1* mutation. Similarly, Sutka *et al.* (2011) found a negative correlation between *L* and transcripts abundance of some *PIP2* genes. So, different PIP subcellular localization could cause also this negative correlation (Boursiac *et al.* 2005, Boursiac *et al.* 2008b). Thus, it is possible that the presence of higher amounts of PIP1 proteins caused retention of these proteins in internal membranes.

Our results showed an increase in gene expressions of *SlPIP2;1, SlPIP2;8* and *SlPIP2;9* in *def-1* plants under well-watered conditions. These are all PIP2 aquaporins, which are known to have a greater capacity to transport water (Fetter *et al.* 2004). These data help to understand the increase of *L* in *def-1* plants under well-watered conditions. However, the expression of remaining *PIP* aquaporins did not change in *def-1* plants. In the same way, Sanchez-Romera *et al.* (2014: Chapter 1) noted that not all aquaporins responded in the same way to the exogenous application of MeJA. Although most aquaporins decreased their expression, others increased it or did not change. Therefore, we believe that JA negatively regulates the expression of some aquaporins and positively regulates the abundance of aquaporins. In addition, previous studies show that *PIP* gene expression is not always followed by protein amount (Aroca *et al.* 2005, Marulanda *et al.* 2010, Muries *et al.* 2011).

By contrast, under drought conditions, both plant genotypes maintained similar levels of expression for most aquaporins, except for an increase of *SlPIP1;7* in *def-1* plants, and a decrease of

SlPIP2;9 in WT plants. Hence, we hypothesized that under drought stress conditions, plants regulate the abundance and expression of aquaporins by a route different to JA-signaling pathway.

Effects of *def-1* mutation on the response to AM symbiosis

There are several studies dealing with the involvement of JA in the establishment and development of AM symbiosis. However, the results obtained to date are unclear. From one side, there are studies where it has been observed that AM plants have higher level of MeJA than non-AM plants (Hause *et al.* 2002). Instead, some JA deficient mutant plants had more AM colonization (Herrera-Medina *et al.* 2008, Leon-Morcillo *et al.* 2012a) while in another case, these JA mutant plants showed less mycorrhizal colonization (Isayenkov *et al.* 2005). Consequently, according with our outcomes, we do not support any of these findings because the fungal infection rate did not show differences between the two types of plants, nor was affected by water regimes used. Such results could be caused because we checked the AM colonization after eleven weeks of growth, and JA could be more involved in the initial phases of colonization (Hause *et al.* 2002).

It is also known that AM symbiosis can regulate the plant growth. In some cases, the growth is not affected by AMF presence (Aroca *et al.* 2008b, El-Mesbahi *et al.* 2012, Ruiz-Lozano *et al.* 2009), however in other cases, the AM plants are bigger (Aroca *et al.* 2008a, Jahromi *et al.* 2008) or smaller (Ouziad *et al.* 2006) than non-AM plants. Our results showed that AM plants mainly decreased their shoot dry weight, except in *def-1* plants grown under well-watered conditions. Therefore, JA may interfere in the dialogue between the fungus and the plant, and probably also in the nutrient exchange (see review by Leon-Morcillo,Ocampo & Garcia-Garrido 2012b) under well-watered conditions, and in our study caused a descent of growth.

More outstanding was the presence of AMF during the drought period, since both AM plants (WT and *def-1*) exhibited opposite behaviours to regulate RWC and gs, and to cope with drought conditions. Specifically, *def-1* AM plants had higher RWC and lower gs than AM WT plants. Therefore the AMF role was affected by *def-1* mutation, suggesting that JA is involved in the regulation of RWC by AMF, probably because JA, like ABA, are involved in regulation of stomatal closure and therefore water loss by evapotranspiration (Hossain *et al.* 2011).

On the other hand, we have found that the expression of *GintAQP1* was increased in *def-1* AM plants grown under well-watered conditions, so *def-1* could regulate its expression. In contrast, we could not observe any difference in gene expression of fungal AQPs caused by drought treatment as it had been observed by Li *et al.* (2013b) and El-Mesbahi *et al.* (2012). As happens

with JA effects on AM establishment, the role of *R. irregularis* in plant AQPs regulation is not clear yet due to results showing contradictory effects. Therefore, some studies found an increase in AQP gene expression in AM plants under non stressed conditions (Alguacil *et al.* 2009, Aroca *et al.* 2008a, El-Mesbahi *et al.* 2012, Uehlein *et al.* 2007), other studies found opposite results (Aroca *et al.* 2008b, Ouziad *et al.* 2006, Porcel *et al.* 2006b, Ruiz-Lozano *et al.* 2009). Recently, Barzana *et al.* (2014) checked the expression of thirty-one plant AQPs in maize roots and they observed that thirteen of them were down-regulated and only three up-regulated in AM plants under well-watered conditions. In our work it was observed that only one out of eight genes decreased its expression in AM WT plants, while four genes were down-regulated in *def-1* plants, pointing toward an important implication of JA in gene expression. However, under drought condition, we found up regulation of three out of eight genes in AM WT plants, in accordance with results by Ruiz-Lozano *et al.* (2009) and El-Mesbahi *et al.* (2012), who found increased gene expression of several *PIP* AQPs.

Hormonal regulation

Previous studies have shown that several hormones are involved in the establishment and development of AM symbiosis. This includes ABA, which is involved in development of symbiosis and in the formation of arbuscules (Herrera-Medina *et al.* 2007, Martin-Rodriguez *et al.* 2010, Martin-Rodriguez *et al.* 2011). However, we could not find a significant increase of ABA in AM roots. SA is known to have a negative effect on AM symbiosis (Blilou *et al.* 1999, Herrera-Medina *et al.* 2003, Riedel *et al.* 2008). Nevertheless, it has been observed that SA was accumulated in AM barley roots (Khaosaad *et al.* 2008). In this study, we obtained higher SA concentration in AM roots than in non-AM roots under drought conditions. This increase could be due to a defensive response of AMF to the stress conditions rather than due to its implication in symbiosis development. Unlikely, we found that all AM treatments showed an increase of MeSA, regardless of water regime, as well as, a positive correlation with *L*. SA is known to reduce *L* under hydroponic conditions (Boursiac *et al.* 2008a), however nothing is known about the effects of MeSA on *L*.

In the same way, there are also studies under hydroponic conditions, which have found that IAA reduced the expression of aquaporins genes (Peret *et al.* 2012). Nevertheless, our outcomes showed a positive relationship between *L* and IAA concentration. In addition, we observed that this increase of *L* by IAA is due to presence of AMF. In fact, it is known that IAA is important for AM symbiosis (Hanlon & Coenen 2011) and in our study the IAA concentration was raised in AM treatments, except in *def-1* AM plants under well-watered condition, where the IAA levels were

constitutively high. This result shows a relationship between JA and IAA levels (Hentrich *et al.* 2013).

In conclusion, regardless of soil water status, the fungal colonization capacity was not affected by JA deficiency in the roots of *def-1* plants. Under well-watered conditions, *def-1* mutation was involved in regulation of aquaporins abundance and gene expressions. However, *def-1* plants behaved similarly to WT under drought conditions, indicating that under stress conditions, the AM fungi are able to find an alternative way to regulate plant water status independently of *def-1* phenotype

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Chapter 4: Effects of sodium nitropruside (a nitric oxide donor) and Nω-nitro-L-arginine methyl ester hydrochloride (a nitric oxide synthesis inhibitor) on the regulation of plant drought responses by the arbuscular mycorrizal symbiosis

Chapter 4: Effects of sodium nitropruside (a nitric oxide donor) and Nω-nitro-L-arginine methyl ester hydrochloride (a nitric oxide synthesis inhibitor) on the regulation of plant drought responses by the arbuscular mycorrizal symbiosis

Objective

The aim of the present study was to investigate the possible involvement of nitric oxide (NO) in regulating plant drought responses and *L* by AM symbiosis. For that, a donor of NO (sodium nitroprusside; SNP) and an inhibitor of NO generation (Nω-nitro-L-arginine methyl ester hydrochloride; NAME), were added exogenously to lettuce plants (*Lactuca sativa* L.) inoculated or not with the AMF *R. irregularis* and grown under well-watered or drought conditions. Plant growth, AMF colonization, stomatal conductance, water consumed, water status, *L* and PIP1 aquaporin expression and abundance were determined.

Experimental design

Plants of *Lactuca sativa* L. were used in an experiment following a combined factorial design with three factors: (1) biological factor, where plants were inoculated or not with the AMF *Rhizophagus irregularis*, (2) chemical factor formed by untreated plants, plants treated with 50 µM sodium nitroprusside (NO donor, SNP) and plants treated with100 µM Nω-nitro-L-arginine methyl ester hydrochloride (NO synthase inhibitor, L-NAME), (3) abiotic factor, in which plants were grown under well-watered or drought conditions. We had a total of 84 plants in 12 different treatments. Four replicates of each treatment were used for *L* measurement, and the other 3 replicates were frozen in liquid nitrogen immediately after harvest for later use in other molecular and biochemical determinations described in the Materials and Methods section. For all the treatments, physiological measurements and the collection of plant samples were carried out 3 h after sunrise, in order to avoid diurnal fluctuations in plant processes.

Growth conditions

The experiment lasted 13 weeks from seed germination and was conducted under greenhouse conditions with temperatures ranging 19 to 25°C, 16/8 light/dark photoperiod, a relative humidity of 50-60% and a photosynthetic photon flux density of 800 µE m^{-2} s^{-1}, as measured with a

light meter (LICOR; Lincoln, NE, USA model LI-188B). Before the beginning of drought treatment, all plants received 10 ml of 80% Hewitt`s nutrient solution (Hewitt 1952). Chemical treatments started after two months of growth and they lasted for one month. The different chemicals were applied twice a week. In the last week, plants underwent under two water regimes before harvesting. Then, half of the plants were subjected to drought (70% of field capacity) while the other half was maintained at field capacity. Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK).

Results

Mycorrhizal colonization and plant growth

Uninoculated plants did not show mycorrhizal colonization. On the other hand, untreated (NT) plants inoculated with *R.irregularis* presented 25% of mycorrhizal root length colonized. Treatment with SNP had no effects on mycorrhizal root length colonization (Fig. 4.1a). However, plants treated with NAME increased mycorrhizal root length colonization until 36%. Drought treatment had no effect on mycorrhizal root length colonization (Fig. 4.1a).

At each particular treatment, shoot dry weight of AM plants and non-AM plants was similar (Fig. 4.1b). The effect of chemical treatments on shoot growth was dependent on the water regime (Table 4.1). Concretely, both chemical treatments reduced shoot dry weight under well-watered conditions, the highest reduction being in SNP-treated plants. Non treated plants and non-AM plants treated with NAME decreased their shoot dry weight as a consequence of drought (Fig. 4.1b). Interestingly, plants treated with SNP were not further affected by drought, most probably because they were already the smallest ones.

Under well-watered conditions, the root growth was not significantly affected by chemical treatments in non-AM plants. However, AM plants treated with NAME displayed a root growth reduction. On the other hand, drought only reduced root growth in untreated plants (Table 4.1; Fig. 4.1c).

Stomatal conductance (gs)

Effects of AM symbiosis on gs were only observed in NAME-treated plants, where AMF presence reduced stomatal conductance under both water regimes (Fig. 4.2a). In the same way, drought stress decreased stomatal conductance in both NT and NAME plants. Interestingly, SNP treatment had a negative effect on gs under well-watered conditions. However, the stomatal

conductance of SNP-treated plants was not affected by drought conditions (Fig. 4.2a), because it was already low.

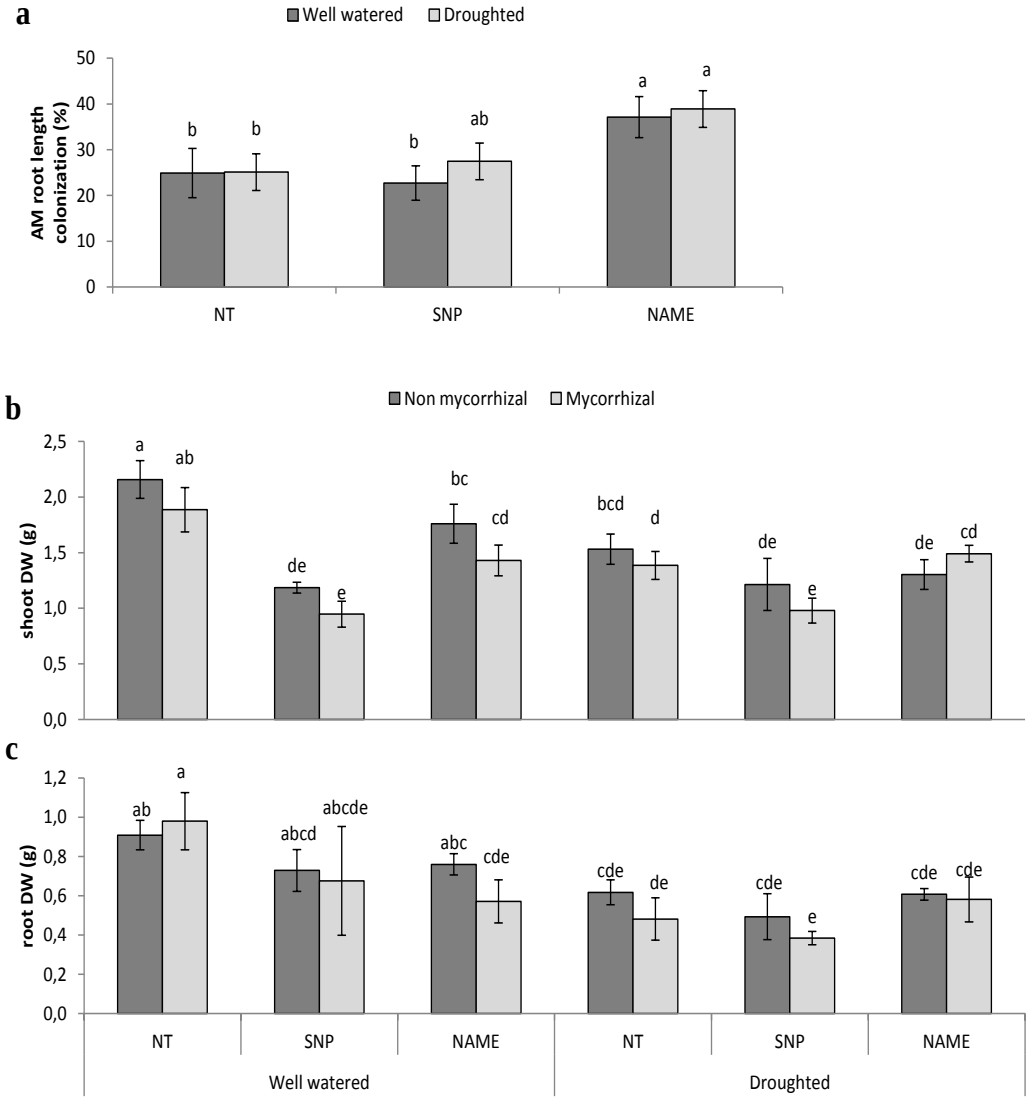


Figure 4.1. (a) Effect of water regime and SNP and NAME treatments on the percentage of mycorrizal root length colonization by *Rhizophagus irregularis* in lettuce roots. Ten plants of each treatment were analysed. Untreated plants (NT), plants treated with 50 µM SNP, and plants treated with 100 µM NAME. Well-watered plants (dark bars) and droughted plants (grey bars). Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test. Bars represent mean ± SE (n=10). **(b)** Dry weights of shoots and **(c)** roots of lettuce plants untreated (NT), treated with 50 µM SNP or 100 µM NAME. Plants were either uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars) and cultivated under well-watered or drought conditions. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test Bars represent mean ± SE (n=5).

Table 4.1. Significance of sources of variation after three-way ANOVA analyses for following parameters: shoot dry weight (SWD), root dry weight (RWD), stomatal conductance (gs), percentage of yellow leaves (YL), chlorophyll content (Chl), daily rate of root water consumption (WC), leaf relative water content (RWC), and root hydraulic conductivity (*L*). The sources of variation were AM symbiosis (AM), water regime (WR) and chemical treatments (CT), as well as their interactions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant effect.

	AM	WR	CT	AMxWR	AMxCT	WRxCT	AMxWRxCT
SWD	*	**	***	ns	ns	*	ns
RWD	ns	***	ns	ns	ns	ns	ns
gs	*	***	***	ns	**	**	ns
YL	*	***	***	ns	ns	*	ns
Chl	ns	ns	ns	ns	ns	ns	***
WC	***	ns	***	**	***	ns	***
RWC	ns	***	***	ns	ns	***	*
L	ns	*	ns	ns	ns	ns	ns

Chlorophyll content

Under well-watered conditions, NAME treated plants had similar chlorophyll content as non treated plants regardless of AMF presence (Fig. 4.2b). SNP treatment diminished chlorophyll content only in non-AM plants, but increased it in AM plants under well-watered conditions (Fig. 4.2b). On the other hand, SNP treatment increased chlorophyll content in non-AM plants under drought conditions, while NAME treatment had no effect (Fig. 4.2b). Furthermore, under drought stress conditions AMF increased chlorophyll content in NAME-treated plants. As shown in Table 4.1, these results were due to the interaction of the three factors.

Percentage of yellow leaves

The percentage of yellow leaves is a measurement indicative of the stress symptoms caused by a given treatment such as drought (Aroca,Irigoyen & Sanchez-Diaz 2003). The highest percentage of yellow leaves was recorded in non-AM SNP-treated plants, regardless of the water regime (Fig. 4.2c). The increase of percentage of yellow leaves by SNP treatment was reduced by AM symbiosis only under well-watered conditions.

Drought treatment increased the percentage of yellow leaves in all plants, except in SNP treated plants which maintained similar values to SNP-treated plants under well-watered conditions

(Fig. 4.2c). Under drought conditions, the lowest values of yellow leaves were observed in NAME-treated plants (Fig. 4.2c).

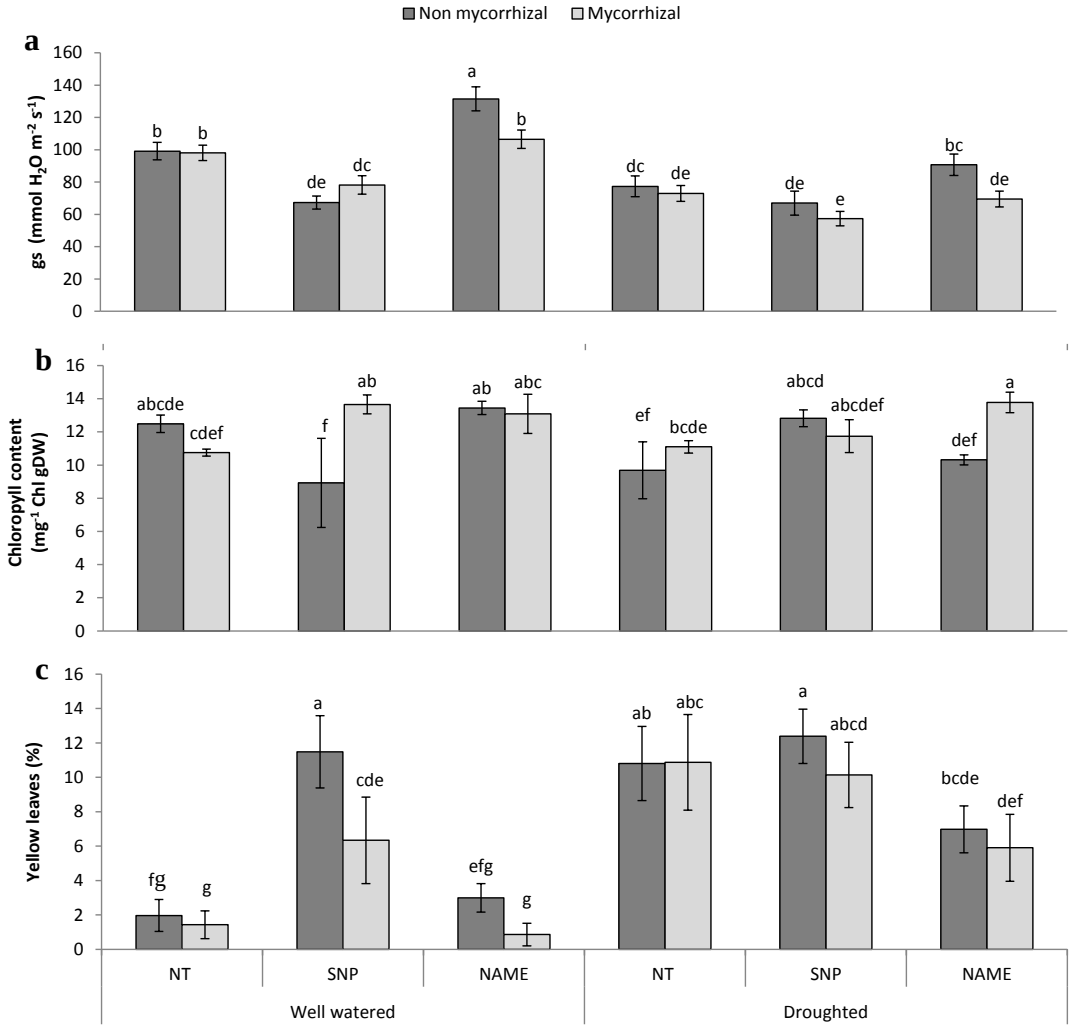


Figure 4.2. (a) Stomatal conductance (gs) (n=64), **(b)** chlorophyll content (n=6), **(c)** percentage of yellow leaves (n=5), of *L. sativa* plants. Plants were either uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars). Plants were untreated (NT), treated with 50 µM SNP or 100 µM NAME and cultivated under well-watered conditions or subjected to drought stress for one week. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test.

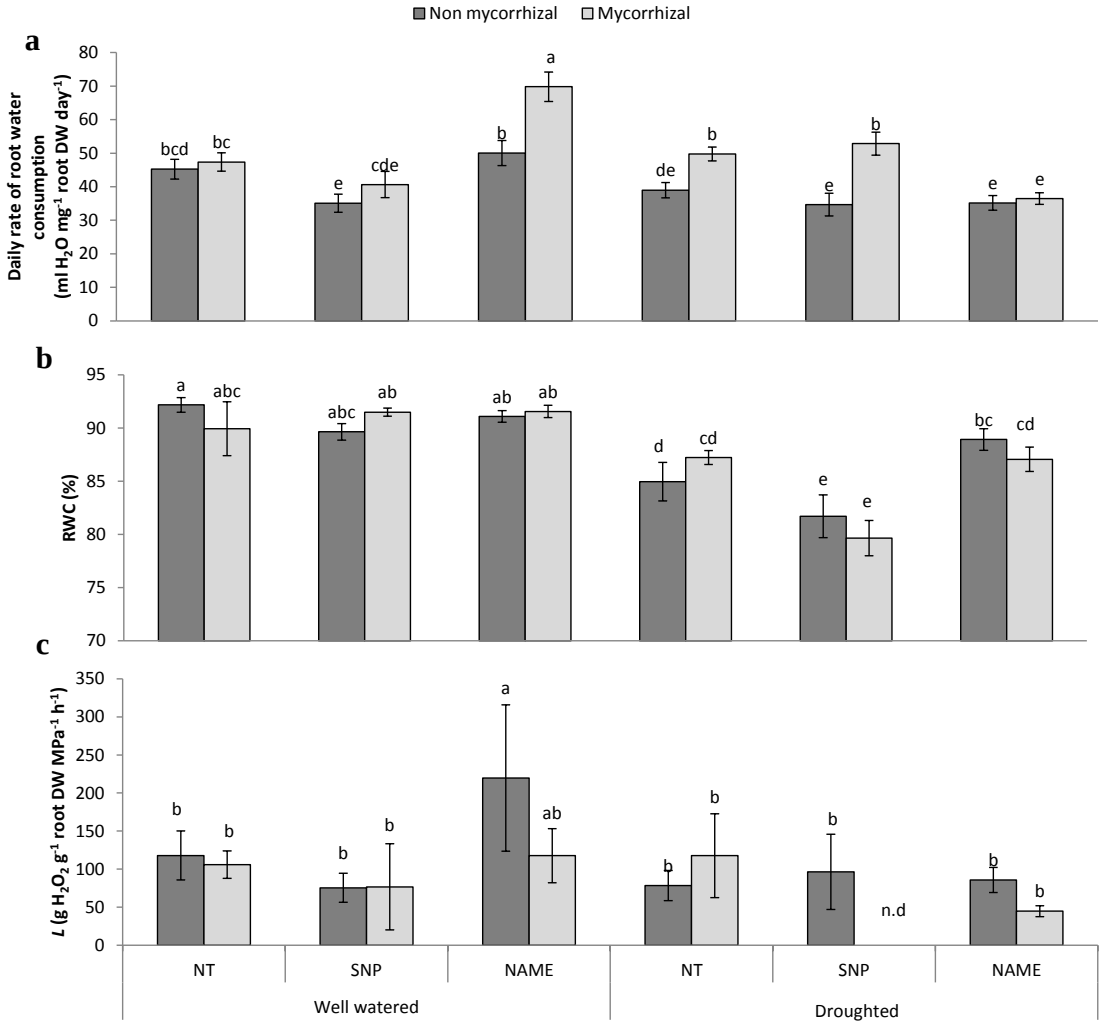


Figure 4.3. (a) Daily rate of root water consumption (n=50), **(b)** leaf relative water content (RWC) (n=5), **(c)** osmotic root hydraulic conductivity (*L*) (n=4) of *L. sativa* plants. Plants were either uninoculated (dark bars) or inoculated with *Rhizophagus irregularis* (grey bars). Lettuce plants treated with 50 µM SNP, plants treated with 100 µM NAME and control untreated plants (NT). The plants were cultivated under well-watered conditions or subjected to drought stress for one week. The *L* value of SNP-treated plants and inoculated could not be detected (n.d) under drought condition. Bars represent mean ± SE. Different letters mean significant differences ($p < 0.05$) after ANOVA and LSD test.

Daily rate of root water consumption

SNP treatment had no effect on the rate of root water consumed regardless of inoculation or water regime treatments, except in non-AM plants which decreased their root water consumption under well-watered conditions (Fig. 4.3a). By contrast, NAME treatment increased it under well-water conditions in AM plants (Fig. 4.3a). On the other hand, under drought conditions, AM plants enhanced the daily water consumed, except NAME treated plants which keep similar results to non-AM plants. On the contrary, AM plants treated with SNP increased daily water consumption as compared to well-watered conditions, while AM plants treated with NAME decreased it (Fig. 4.3a).

Relative Water Content (RWC)

No effects of chemical treatments or AM symbiosis on RWC were observed under well-watered conditions (Fig. 4.3b). Drought decreased RWC in almost all plants, except in non-AM plants treated with NAME and in AM plants untreated. The presence of AMF did not have an effect on RWC. Non-AM plants treated with NAME and subjected to drought increased their RWC as compared to untreated non-AM plants. Moreover, the lowest RWC were obtained in plants treated with SNP (Fig. 4.3b).

Root hydraulic conductivity (*L*)

Under well-watered conditions, plants of all treatments showed similar *L* values, except non-AM plants treated with NAME, which had the highest *L* value and AM plants treated with SNP under drought conditions for which *L* was negligible (Fig. 4.3c).

***LsPIP1* gene expression and protein abundance**

LsPIP1 gene expression was regulated by both AMF and chemical treatments. AM plants showed lower *LsPIP1* expression than non-AM plants, regardless of water regime and chemical treatment. Secondly, SNP and NAME treatments reduced its expression under well-watered conditions, especially in AM plants. The highest *LsPIP1* expression was observed in untreated non-AM plants under well-watered conditions (Fig. 4.4a). The figure 4.4b shows the PIP1 protein abundance in lettuce roots. PIP1 proteins accumulated mostly in SNP treated roots under well-watered conditions, although this effect was reduced by AMF. Under drought conditions, non-AM plants displayed more PIP1 protein abundance than AM plants, excepted in NAME-treated plants where the opposite effect was observed. Also, AM plants treated with SNP showed the lowest PIP1 protein abundance.

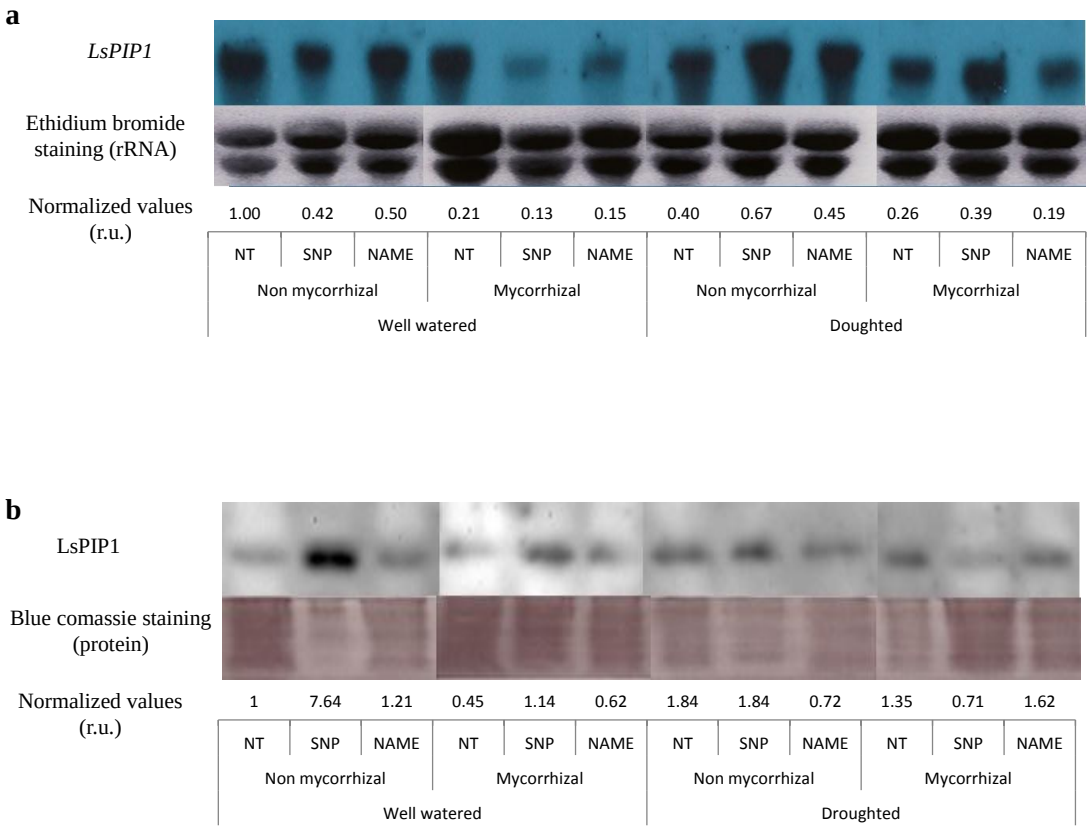


Figure 4.4. (a) Northern blot analyses of *LsPIP1* expression (n=3), with lower panel showing the amount of rRNA loaded for each treatment (ethidium bromide staining), **(b)** western blot analysis showing the abundance of PIP1 aquaporins (n=3), with lower panel showing the amount of total protein loaded for each treatment (comassie brilliant blue staining). Lettuce plants were not inoculated (non-AM) or inoculated (AM) with the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, and were treated with 50µM SNP, 100 µM NAME or control untreated plants (NT), under well-watered conditions or under drought conditions. Numbers represent the relative gene expression (after normalization to rRNA; figure (a) and relative abundance of protein (after normalization to total protein content; figure (b) as compared to the values for non-AM plants grown under well-watered conditions without chemical treatment (NT).

Discussion

In the last decade, the number of studies dealing with the role of NO as an anti-stress agent has increased considerably (Arasimowicz-Jelonek *et al.* 2009b, Xiong *et al.* 2012). Several studies have observed an improvement of plant drought tolerance by SNP treatment (Arasimowicz-Jelonek *et al.* 2009a, Garcia-Mata & Lamattina 2001, Lei,Yin & Li 2007a, Tian & Lei 2007, Xiong *et al.* 2012). At the same time, several studies found that establishment of AM symbiosis also improved the tolerance of plants to drought stress (Abbaspour *et al.* 2011, Barzana *et al.* 2012, Doubkova,Vlasakova & Sudova 2013). In this work, we have focused mainly on the involvement of NO in regulating plant responses to AM symbiosis and water relations. For that, AM and non-AM lettuce plants were grown under both well-watered and drought conditions and treated with a NO donor (SNP) or an inhibitor of NO synthesis (NAME).

SNP and NAME effects on well-watered and droughted plants

First, we tested the effects of SNP (a NO donor; Garcia-Mata & Lamattina 2001) and NAME (an inhibitor of NO generation; Fan & Liu 2012) under well-watered conditions. Our results showed that SNP treatment caused a decrease in SDW and chlorophyll content, and an increase in the percentage of yellow leaves. It would mean that the SNP treatment could cause a nitrosative stress (Corpas *et al.* 2008). However, such damage was partially avoided by AM symbiosis as it is shown for chlorophyll content and percentage of yellow leaves. So it is possible that AM symbiosis enhanced tolerance to nitrosative stress as it has been described for oxidative damage (Li *et al.* 2013c). Indeed, NAME application improved gs and *L*, though reduced SDW. Hence, water transport was improved by NAME, while NO could be inhibiting water transport as was reported for ROS (Boursiac *et al.* 2008a). However, the improvement of global water transport was different in AM and non-AM plants, most probably because AM symbiosis alters the proportion of water flowing through different pathways (cell-to-cell and apoplastic) (Barzana *et al.* 2012, Plamboeck *et al.* 2007).

Under drought conditions SNP treatment decreased RWC and increased concentration of total chlorophyll. By contrast, application of NAME caused an increase in gs and RWC. Fan *et al.* (2012) noted in orange plants that leaves under drought conditions showed an increase in total chlorophyll content after treatment with SNP, just as we have observed here. Instead, they obtained a reduction of total chlorophyll concentration after application of NAME, which was not obtained in our work. In relation to stomatal conductance, similar results to ours were obtained by Xiong *et*

al. (2012), who observed that rice plants subjected to water stress and treated with SNP decreased their gs. Instead, an application of 2-(4-carboxyphenyl)-4,4,5,5,-tetramethyllimidazoline-1-oxyl-3-oxide (cPTIO), NO scavenger, increased gs. On the other hand, despite the fact we observed that SNP-treated plants decreased their RWC, several studies have reported the opposite results (Arasimowicz-Jelonek *et al.* 2009a, Garcia-Mata & Lamattina 2001, Xiong *et al.* 2012). The fact that SNP and NAME caused opposite results regarding RWC could be due to a RWC reduction under drought conditions mediated by NO. However, it is also probable that here we used a toxic SNP concentration for lettuce plants, which together with the NO generation under drought conditions (Xiong *et al.* 2012), would cause a higher dehydration, avoided by the NAME application.

Interaction between NO and the AM symbiosis

Several reports have noted an increase in the concentration of NO in roots of AM plants, suggesting a possible role of NO as a molecule involved in the establishment and functioning of the symbiosis (Calcagno *et al.* 2012, Li *et al.* 2013c, Yamasaki & Sakihama 2000). In our experiment, the percentage of mycorrhizal colonization was increased by NAME treatment, regardless of the water regime. These results indicate that certain descent in the levels of NO could increase AM root colonization. So, although NO could be essential for the establishment of the AM symbiosis, it could be not beneficial for the subsequent spread of root colonization. A similar dual role is thought for JA, which has been described as being involved in the symbiosis establishment rather than in the recognition between partners or in the beginning of interaction (Hause *et al.* 2002).

Regulation of *L* by AMF and NO

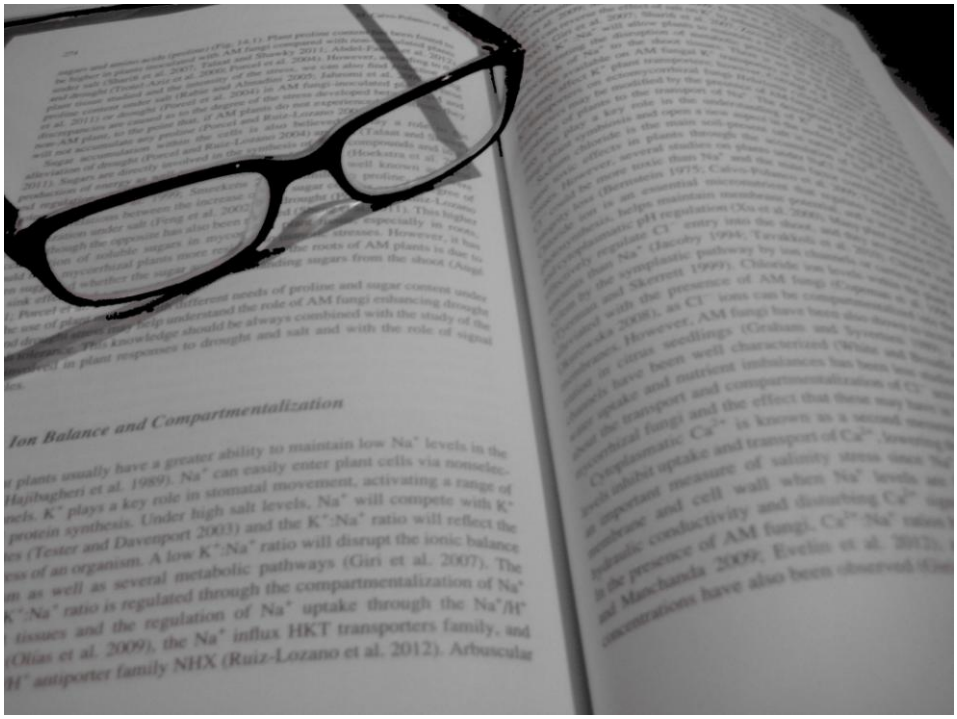
There is very little information about the involvement of NO in regulating aquaporins expression. Liu *et al.* (207) reported that SNP and GSNO (another NO donor) applications stimulated the transcription of four PIP genes in germinating rice seeds. Here, although we could observe a decrease in *LsPIP1* gene expression in SNP- and NAME-treated plants under well-watered conditions, we also noticed an increase in PIP1 protein abundance in the SNP treatment under well-watered conditions. The discrepancy between PIP gene expression and protein abundance has been observed before (Aroca *et al.* 2005, Marulanda *et al.* 2010), it could be due to an autoregulation of gene expression by the synthesized protein. Also, the accumulation of PIP1 proteins by SNP did not translate into increased *L*. This divergence may be explained by an internalization of aquaporins in subcellular localization as it was demonstrated by Boursiac *et al.* (2008b), when H_2O_2 treatment decreased *L* and caused an accumulation of PIPs in intracellular

structures. On the other hand, the role of AMF in aquaporins regulation has been controversial as there are conflicting reports suggesting that it can either increase aquaporin expression (Aroca *et al.* 2008a, Ruiz-Lozano *et al.* 2009) or decrease it (Porcel *et al.* 2006a). Although more recently, Barzana *et al.* (2014) showed a general down regulation of eleven aquaporin genes in maize roots caused by AM symbiosis under well-watered conditions. In this work it was clearly observed a reduction of expression and abundance of PIP1 in AM plants regardless of chemical treatment and water regime. Finally, the interaction between NO and AMF was evidenced by an increased water absorption by NAME treatment in AM plants and by a decrease of PIP1 abundance by SNP treatment in AM plants under well-watered conditions. As commented above, since AM symbiosis may regulate NO levels (Calcagno *et al.* 2012), it is expected that the response to NAME and SNP were different between AM and non-AM plants.

It is also remarkable that SNP treatment in AM plants under drought conditions reduced completely *L*. Here *L* was measured under atmospheric pressure, so only water moving through the cell to cell pathway was determined (Steudle & Peterson 1998). However, these plants have similar gs and higher rate of root water consumption to non-AM plants. So, it is possible that SNP treatment increased the proportion of water circulating by the apoplastic path. In fact, a lower PIP1 abundance was observed. Since it was demonstrated that AM symbiosis enhances the water circulating by the apoplastic path (Barzana *et al.* 2012), it is possible that SNP stimulates such enhancement.

To sum up, SNP treatment caused a stress in well-watered plants, especially in non-AM plants, and overall water transport was ameliorated by NAME treatment. It seems also that AM plants were more resistant to the stress caused by SNP treatment and that NO interferes with AM symbiosis activity. On the other hand, the combination of drought stress and SNP treatment caused extra leaf dehydration, while NAME treatment overcame partial dehydration caused by drought. Thus, NO could be involved in the leaf dehydration caused by drought. Results also suggest that SNP treatment could enhance the water circulating by the apoplastic path in AM roots.

General discussion



General Discussion

The aim of the different studies included in this Thesis was to investigate, both at molecular and physiological levels, the involvement of signaling molecules such as NO- and JA-related compounds in the regulation of root hydraulic conductivity (*L*). Also, it was investigated how the AM symbiosis modulates the effects of these molecules on *L* under drought conditions.

Effect of MeJA on *L*

There were experimental evidences showing that MeJA modulates stomatal aperture (Akter *et al.* 2012, Hossain *et al.* 2011), but its effects on *L* had not been studied yet. Bean, tomato and Arabidopsis plants grown hydroponically were treated with MeJA, and all of them increased their *L* after treatment. It is noteworthy that mutant plants deficient in JA (*def-1*; Howe *et al.* 1996) had less *L* than WT plants, but increased their *L* after application of MeJA. However they failed to restore the WT, most probably due to a dose effect.

Thereafter the effects of MeJA on plants cultivated in soil and subjected or not to drought were checked. The results obtained under soil conditions did not follow the same pattern as those obtained previously under hydroponic conditions. Specifically, under well-watered conditions, the application of MeJA had no effect on *L* of bean plants and in the case of tomato; our results were completely opposite to the previous ones, because JA-deficient plants showed higher *L* than WT. This may be as a result of changes in the anatomy of the root, as was demonstrated by Matsuo *et al*. (2009), who found different *L* ranking among rice varieties depending on whether the plant were grown hydroponically or in a solid substrate. It is also known that plants grown in soil may have suberin deposits in roots. Hence, water must flow by the cell-to-cell pathway (Vandeleur *et al.* 2009, Zimmermann & Steudle 1998). For instance, suberin contents are formed in plants treated with salt, whereby a decrease in apoplastic pathway was observed (Krishnamurthy *et al.* 2011, Ranathunge *et al.* 2011, Schreiber *et al.* 2005) and plants subjected to low temperatures decreased *L* as a result of suberin deposits in their roots (Lee *et al.* 2005). Consequently, root water uptake is modified as well as the water movement inside roots.

Another point to be considered is that the methods used to measure *L* were different in both kind of experiments. In beans growing in soil, the free exudate method was used and we quantified only the cell-to-cell pathway because transpiration rate was eliminated when the steams were cut (Steudle and Peterson 1998). In tomato experiment we used the HPFM method by which we

quantify *L* taking into account water flowing through the apoplastic and the cell-to-cell routes (Steudle and Peterson 1998). However, under hydroponics conditions, these different methods did not affect the results of *L*. This can be explained because both routes contributed equally to the whole water flow or because in plants growing hydroponically the apoplastic pathway did not contribute enough to the water transport. However, in plants grown in soil, the reliability of the methods was not checked. Recently, Barzana *et al.* (2014) observed that depending on the method used, the *L* patterns can change in the same plant.

Under drought conditions, we noted that bean plants treated with MeJA increased *L*, while JA deficient tomato plants exhibited the same values as WT. This prompted the question whether JA is involved in several signaling pathways and whether its effect on *L* is the sum of all of them. In fact, JA dependent and independent regulatory pathways for other plant processes have been observed (Hossain *et al.* 2011).

Another cause why values from experiments of plants grown under greenhouse and under hydroponics conditions cannot be compared is related to the way of application of the chemical treatment. In the case of bean plants grown in soil the MeJA was applied several times a long a week. In contrast, under hydroponic conditions only one application was done and plants were harvested 1 or 24 hours after application. Therefore, we consider that the results obtained in plants grown in soil were due to an application effect on these plants rather than to the immediate effect that MeJA may cause on *L*. Thus, under drought conditions we observed an increase of *L* due to MeJA application, and we consider that it may be due to an increase in plant resistance to the stress as has been previously reported (Anjum *et al.* 2011, de Ollas *et al.* 2013). Concretely, Anjum *et al.* (2011) observed that MeJA application in soybean leaves increase the SOD, POD and CAT activities and proline content under drought conditions in addition to improved RWC, making plants more tolerant to drought. The absence of MeJA effect on *L* under well-watered conditions, may be due to the fact that *L* was not measured immediately after MeJA application. Hence, what we observed were its cumulative effects over time. Thereby, MeJA-treated plants may be better prepared to support stress conditions although the treatment did not affect *L*. Evidence for that was that plants grown under drought conditions and treated with MeJA were able to maintain *L* similar to plants grown under well-watered conditions.

Finally, JA-deficient plants have higher *L* than WT plants under well-watered conditions. By contrast, under drought conditions no differences in *L* between the two plants were observed.

We suggest that this result was due to *def-1* mutant characteristics could cause a stress situation in the plant similar to the state of stress caused by drought in WT plants. In fact, some particular stresses may cause a rise on *L* (Calvo-Polanco,Sanchez-Romera & Aroca 2014b).

MeJA signaling pathway

In this study we have tried to elucidate for first time the signaling pathway involved in the regulation of *L* by MeJA. We have observed that MeJA acts together with ABA and calcium in regulating *L*, as was previously described for stomatal regulation (Hossain *et al.* 2011, Islam *et al.* 2010, Munemasa,Mori & Murata 2011b). Similar to MeJA application, exogenous ABA application is known to increase *L* in several plant species (Aroca *et al.* 2008b, Kudoyarova *et al.* 2011, Mahdieh & Mostajeran 2009, Ruiz-Lozano *et al.* 2009). However, other studies showed opposite results (Beaudette *et al.* 2007). Nevertheless, *L* is also co-regulated by other hormones such as ethylene whose results are also contradictory (Islam *et al.* 2003), SA which inhibited *L* (Boursiac *et al.* 2008a) or IAA whose effect has been observed at aquaporins level (Peret *et al.* 2012).

Furthermore, previous studies have described the joint action of ABA and JA in several plant processes such as defense against pathogen attack and stomatal regulation (Garcia-Andrade *et al.* 2011, Hossain *et al.* 2011). It was in the latter study where it was found that the response to JA was the result of two signaling pathways acting simultaneously. One of which was dependent on ABA and the other one was independent on ABA (Hossain *et al.* 2011). The same kind of regulation has been observed here. MeJA effect on *L* was in part regulated by ABA and in part independent of ABA. However, De Ollas *et al.* (2013) noted that JA content must be accumulated previously to take place an increase of the ABA concentration in roots under drought conditions.

On the other hand, Sun *et al*. (2009b) observed an increase in cytosolic calcium concentration in response to exogenous application of MeJA in order to start a signaling pathway that could result in increased gene expression, protein synthesis or a post- translational modification of proteins. In the same way, our results showed that the enhancement of *L* caused by MeJA could be mediated by mobilization of internal calcium stores, concretely from those dependent on IP_3 calcium channels, which are localized in endoplasmic reticulum and tonoplast and are inhibited by heparin (Poutrain *et al.* 2009, Sun *et al.* 2009b, White 2000).

Role of AMF in plant water status

Symbiotic association between AMF and plants provides several advantages to survive under adverse conditions. AM fungi are able to get water from the soil that is inaccessible to plants due to better soil exploration through their hyphae (Marulanda *et al.* 2003), to reduce oxidative damage caused by stress (Garmendia *et al.* 2004, Palma *et al.* 1993, Ruiz-Lozano *et al.* 1996) and regulating RWC of plants (Barzana *et al.* 2012, El-Mesbahi *et al.* 2012). AM symbiosis has been widely studied in a great variety of plants and under various abiotic stress conditions. It is known that *L* could be increased by AM symbiosis under drought conditions (Barzana *et al.* 2012).

In our experiments, it was first noted that neither JA nor drought affected the percentage of mycorrhizal colonization; however inhibitors of NO formation (NAME) increased the effectiveness of fungal colonization. It was previously found that root NO levels increased during initial phase of AMF root colonization (Calcagno *et al.* 2012). However, this increase may be transient and later on it could cause an inhibition of AM hyphae proliferation, since we added NAME during a long period of time. Despite the lack of response to MeJA in our work, other studies show mixed results. On one hand there are studies where it has been observed an increase in the concentration of JA in AM plants and, specifically, into the cells containing arbuscules (Hause *et al.* 2002). Conversely, Isayenkov *et al.* (2005) found that low levels of JA decreased mycorrhization rate and arbuscules formation and Ludwing-Muller *et al.* (2002) observed a reduction of fungal colonization after exogenous MeJA application and in other case, fewer arbuscule number (Herrera-Medina *et al.* 2008). Then, we thought that JA is involved in the establishment and development of symbiosis, but its effect can vary depending on the type of plant and other environmental cues.

At the biomass level, the three plant species analysed showed no significant growth enhancement by AM symbiosis. In fact, AM tomato plants grown under drought conditions were smaller than non-AM ones. So, in the present thesis, the growth promotion effect of AM symbiosis, usually observed was not recorded here, as happened in other studies (El-Mesbahi *et al.* 2012, Ruiz-Lozano *et al.* 2009). It is possible that no nutritional limitation took place in the growing substrate and by that reason the AM symbiosis did not show its potential. For instance, it has been seen that high P concentration reduces fungus colonizing ability, which improve when soil P concentration is decreased (Smith & Read 2008).

In relation to *L*, AMF increased it in tomato plants (WT) and decreased it in bean plants under well water conditions, although we must take into account that the method of measurement

was different in both experiments. However, AMF improved *L* in both plants under drought conditions, as it has been described before (Barzana *et al.* 2012, El-Mesbahi *et al.* 2012). On the other hand, in lettuce plants AMF had no effects on *L*, although SNP treatment inhibited completely the water transport by the cell-to-cell pathway under drought conditions in AM plants. Therefore, endogenous NO level could affect the AMF colonization, as well as, its regulation of *L*.

AQP regulation by MeJA, AMF, drought, and NO

The regulation of aquaporins by the different treatments followed a complex pattern. In the case of bean plants grown in hydroponics, only three out of six aquaporins were regulated by the application of MeJA, two of them were down-regulated and one was up-regulated.In bean plants grown in soil under well-watered conditions, five out of six aquaporin genes were unaffected by treatment with MeJA, and five out of eight genes were unchanged in *def-1* tomato plants. Similarly in plants subjected to drought, four out of six genes in bean plants and six out of eight genes in tomato were left unchanged. Then, we could say that the role of JA is limited in affecting aquaporin gene expression.

By contrast, the abundance of aquaporins was more responsive to MeJA. It was observed that Arabidopsis plants expressing aquaporins labeled with GFP showed a decrease in PIP1;1 abundance in the plasma membrane after MeJA application and an increase in PIP1,2 and PIP2;2 abundances. Previous studies have demonstrated that the presence of heterotetramers with PIP1;2 and PIP2;5 were able to increase Pf values in *Xenopus laevis* oocytes. On the contrary, PIP1;1 tends to internalize in vesicles in the endoplasmic reticulum membrane under stress conditions or when it is inactive. In general, PIP1;1 has less water transport capacity than PIP1;2 in affecting PIP2;5 activityproteins (Fetter *et al.* 2004).

It is noteworthy that MeJA acts mainly in the regulation of the phosphorylation state at Ser-280 in PIP2 proteins (PIP2PH). It is known that aquaporins are able to transport more water when this residue is phosphorylated (Johansson *et al.* 1998). Under hydroponics conditions, PIP2PH abundance was higher in MeJA-treated plants. In beans grown under greenhouse conditions, PIP2PH abundance decreased under well-watered conditions, although it increased under stress conditions after MeJA addition. In the case of tomato, under well-watered conditions JA-defective plants reduced their abundance of PIP2PH. Therefore, these results suggest 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 *L*.

Nevertheless, AM symbiosis is recently known to decrease the expression and abundance of most plant aquaporins (Barzana *et al.* 2014), in spite of that some previous studies found an increase in AQP gene expression in AM plants under non stressed conditions (Alguacil *et al.* 2009, Aroca *et al.* 2008a, El-Mesbahi *et al.* 2012, Uehlein *et al.* 2007) and other studies found opposite results (Aroca *et al.* 2008b, Ouziad *et al.* 2006, Porcel *et al.* 2006b, Ruiz-Lozano *et al.* 2009). In our study we could observe that PIP2 and PIP2PH concentrations were reduced in AM WT tomato plants and PIP2PH in AM bean plants under well-watered conditions. Regarding gene expression, *PvPIP1;2* in bean and *SlPIP2;4* and *SlPIP2;6* in tomato were down-regulated by the AM symbiosis under well-watered conditions. However, six aquaporin genes in tomato and five aquaporin genes in bean remained unchanged. By contrast, different results were obtained under drought stress conditions. For instance, there was no change in the AQP gene expressions in bean and three out eight genes were up-regulated in AM tomato plant. In lettuce experiment, we observed that both the expression and the abundance of PIP1 were reduced by AMF, regardless of water regimen. So, as it has been reported before (Aroca *et al.* 2006b), it seems that each aquaporin gene has a specific response to AM symbiosis and to a particular environmental condition

On the other hand, three different fungal AQP have been described in *Rhizophagus irregularis* (Aroca *et al.* 2009, Li *et al.* 2013b). In our experiment we observed that the expression of two of them were increased under drought conditions in beans plants as it has been describe previously (Aroca *et al.* 2009, Li *et al.* 2013b). Whereas different results were obtained in WT and *def-1* tomato plants where drought treatment did not have any effect on them. Only we can stand an increased expression of one gene *(GintAQP1)* in *def-1* tomato plants under well-watered conditions which could be regulated by JA endogenous levels.

Chemical products applied to change the endogenous NO concentration (NAME and SNP) also had an effect on the abundance and expression of aquaporins. The most remarkable effects were that both chemical products reduced the expression of *LsPIP1* and SNP increased PIP1 abundance under well-water conditions. Liu *et al.* (2007) observed an up-regulation of four PIP genes after SNP and GSNO applications but there is no more information about NO and aquaporin expressions so far.

Cross-talk between hormones

This is one of the few reports where a variety of hormones have been analysed simultaneously in order to understand the interactions established among them. Considering all the

results obtained, we can emphasize some aspects that had never been described previously in the literature. The first is the increase in IAA levels observed in bean and tomato (WT) AM plants, which could be related to the decreased expression of aquaporins that is often linked to the presence of AMF (Barzana *et al.* 2014, Porcel *et al.* 2006a), as well as, to exogenous IAA application (Peret *et al.* 2012). Thus, an increase of IAA could be a possible strategy used by AM plants to reduce radial water flow through the cell-to-cell pathway and to increase it through the apoplastic one, favoring the switching between both pathways according to the environmental conditions (Barzana *et al.* 2012).

After performing the regression analysis, we noted that there was a positive linear correlation between the concentration of MeSA and *L*, which has never been previously described. Similarly, we noted that an increase of MeSA concentration may negatively regulate the abundance of PIP1 aquaporins, group of aquaporins that have lower water transport capacity than PIP2 aquaporins (Fetter *et al.* 2004). These results suggest the necessity of conducting specific experiments to understand the role of MeSA in regulating *L* and aquaporins.

Finally, we discuss the observed effect on SA. The most important role of SA is its involvement in systemic acquired resistance against biotic stresses (Donovan,Nabity & DeLucia 2013, Forchetti *et al.* 2010) and, to a lesser extent, against abiotic stresses (Farooq *et al.* 2009, Kang *et al.* 2013). Moreover, SA is considered a hormone antagonistic to JA, despite both hormones act together in several processes (Proietti *et al.* 2013). Here we observed that bean and tomato plants (WT) subjected to drought showed higher levels of SA than plants that were under non stressed conditions. In the case of bean plants, a negative correlation was observed between the SA concentration and *L* values. This interaction can be explained by the internalization of aquaporins in vesicles caused by SA, as Boursiac *et al.* (2008a) described before, and this could be avoided by both AM symbiosis and MeJA treatment.

Conclusions

Conclusions

1 - The application of 0.1 mM MeJA increases *L* in bean, tomato and arabidopsis plants under hydroponic conditions.

2 - The ABA and cytosolic calcium concentration are partially involved in the signaling pathway implicated in the JA regulation of *L*.

3 - JA down-regulates gene expression of most aquaporins. Conversely, JA increases the concentration of the active state of aquaporins by enhancing phosphorylation of serine residue at position 280.

4 - The degree of root colonization by AMF is not affected by *def-1* mutation. However the use of a NO scavenger enhanced the degree of AMF colonization.

5 - An increase in the concentration of IAA in mycorrhizal roots may be responsible for the decrease in the expression and abundance of aquaporins associated with the AM symbiosis, and the increase of the water circulating through the apoplastic path.

6 - Both MeJA-treated plants and plants inoculated with *Rhizophagus irregularis* improved *L* values under drought conditions, most probably as a result of the descent in SA contents caused by drought.

7 - A possible positive role of MeSA in the regulation of *L* and in the descent of PIP1 protein amounts is postulated for the first time.

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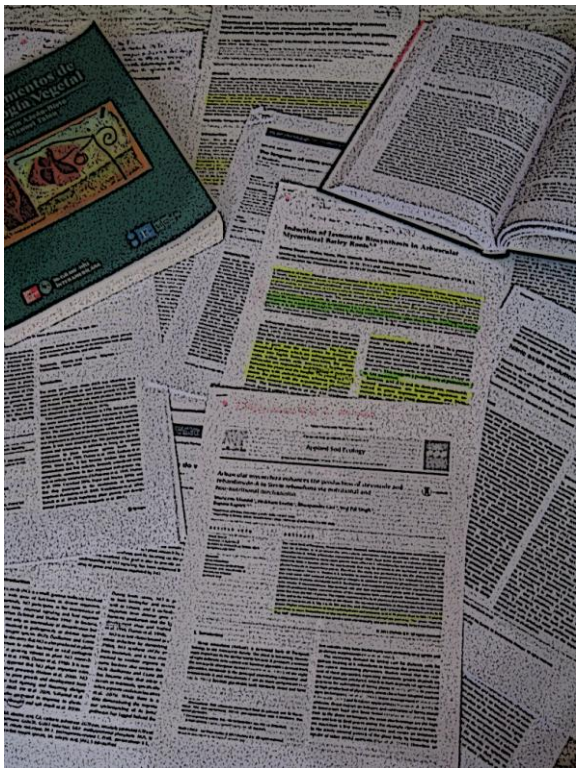

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