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Research article

Mycorrhizal symbiosis down-regulates or does not change root aquaporin expression in trifoliate orange under drought stress

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ABSTRACT

Arbuscular mycorrhizas absorb water from soil to host plants, while the relationship between mycorrhizas and aquaporins (AQPs, membrane water channel proteins, which function in water transport) in mycorrhizal plants is unclear. In this study, *Funneliformis mosseae*-colonized trifoliate orange (*Poncirus trifoliata*) seedlings were grown in pots fitted with 37-μm nylon meshes at the bottom of each pot to allow mycorrhizal hyphae absorb water from an outer beaker. The expression of seven plasma membrane intrinsic proteins (PIPs) genes, six tonoplast intrinsic proteins (TIPs) genes, and four nodulin-26 like intrinsic proteins (NIPs) genes were analyzed in roots of both well-watered (WW) and drought stressed (DS) plants. The six-week DS plants dramatically increased hyphal water absorption rate by 1.4 times, as compared with WW plants. Mycorrhizal plants exhibited greater plant growth performance, leaf water status (water potential and relative water content), and gas exchange under both WW and DS conditions. Mycorrhizal inoculation induced diverse expression patterns in these *AQPs* under WW: up-regulation of *PtNIP1;1*, *PtPIP2;1*, and *PtPIP2;5*, down-regulation of *PtNIP1;2*, *PtNIP6;1*, *PtPIP1;2*, *PtPIP1;5*, *PtPIP2;8*, *PtTIP1;1*, *PtTIP1;2*, *PtTIP1;3*, and *PtTIP5;1*, and no changes in other *AQPs*. However, the expression of *PtPIPs* and *PtNIPs* was down-regulated by mycorrhizal inoculation under DS, and *PtTIPs* was not induced by mycorrhizal colonization under DS. The expression pattern of AQPs in response to mycorrhizas under DS is a way of mycorrhizal plants to minimize water loss.

1. Introduction

Drought stress (DS) is one of the main environmental stresses severely limiting crop productivity and ultimately the food security (Fahad et al., 2017). The frequency, duration, and spatial extent of drought has further increased in recent years (Finnessey et al., 2016; Niu et al., 2018); therefore, it is indeed an urgent task to understand increasing drought tolerance in crop plants, to overcome this situation. Arbuscular mycorrhizal fungi (AMF) predominantly reside in the rhizosphere and can colonize roots of majority of terrestrial plants to establish mycorrhizal symbiosis (Smith and Read, 2008), in which host plants supply sugars and lipids for spore proliferation and subsequent completion of their life cycle (Keymer and Gutjahr, 2018), and AMF improves nutrient and water absorption of host plants (Cavagnaro et al., 2015; Basu et al., 2018). Earlier studies had confirmed a positive effect of AMF on enhancing drought tolerance of host plants by means of morphological adaptation, physiological responses in osmotic adjustment, nutrient and water uptake, antioxidant defense systems, and the molecular regulation of aquaporins (*AQPs*) genes, 14-3-3 genes, a binding protein, late embryogenesis, etc. (Allen, 2007; Birhane et al., 2012; Mirshad and Puthur, 2017; Xu et al., 2018; Zhang et al., 2018, 2019; He et al., 2019; Wu et al., 2019).

Extraradical hyphae of mycorrhizas on root surface can extend many-fold and thus proliferate beyond the rhizosphere nutrient- and water-depletion zones around plant roots (Cavagnaro et al., 2015). Zhang et al. (2018) estimated that water absorption rate of extraradical hyphae was 0.126–1.973 mg H_2O/h/mm, and DS treatment increased the hyphal water absorption rate by 2.3–6.6 times, indicating higher efficiency of mycorrhizal hyphae under dried soils than under saturated soils. Therefore, mycorrhizal hyphae act as highways with a direct pathway for water flow in arid soils (Allen, 2007). However, it is not clear whether under the condition of such water absorption of mycorrhizal hyphae, mycorrhizas affect *AQPs* expression levels in mycorrhizal plants subjected to DS.

Plant AQPs are cell membrane intrinsic proteins, which belong to a major intrinsic protein family, mainly including nodulin-26 like

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intrinsic proteins (NIPs), plasma membrane intrinsic proteins (PIPs), small basic intrinsic proteins (SIPs), tonoplast intrinsic proteins (TIPs), and X intrinsic proteins (XIPs) (Tian et al., 2018). These AQPs represent a high efficiency in water transport, as well as in ammonia, antimony, arsenite, boron, carbon dioxide, hydrogen peroxide, glycerol, formamide, lactic acid, silicon, and urea transport (Afzal et al., 2016; Kapilan et al., 2018). AQP activity is involved in a series of physiological processes including xylem water exit, stomatal aperture, gas exchange, and phloem loading (Shekoofa and Sinclair, 2018). Meanwhile, the effect of AQP (e.g., PIPs) activity on root hydraulic properties is usually high under drought (Grondin et al., 2016). Plants with overexpressed AQPs possess higher photosynthetic rates, faster plant growth, higher biomass and greater yield (Moshelion et al., 2015).

Earlier studies indicated a decrease in the expression of *PIPs* and *TIPs* in host plants by mycorrhizal colonization in saturated soils (Uehlein et al., 2007). Porcel et al. (2006) confirmed the down-regulation of *PIPs* from soybean and lettuce by mycorrhization under DS conditions. He et al. (2016) also reported the down-regulation of *TIP1;1* expression by AMF in *Robinia pseudoacacia* seedlings under DS conditions. The decreased expression of root *AQPs* during DS by mycorrhization potentially limits water loss from cells and prevents backflow of water to rhizosphere that is a drought avoiding behavior (Afzal et al., 2016; Ruiz-Lozano and Aroca, 2017).

However, some AQP genes were up-regulated by mycorrhizal inoculation (Uehlein et al., 2007; He et al., 2016; Quiroga et al., 2017, 2019). Uehlein et al. (2007) reported the up-regulated expression of *MtNIP1* and *MtPIP2;1* in *Medicago truncatula* inoculated with *Funneliformis mosseae* under WW conditions. *RpTIP1;1* expression of *Robinia pseudoacacia* was induced by *Rhizoglomus irregularis* under WW, but was down-regulated under DS conditions (He et al., 2016). Quiroga et al. (2017) reported the induced expression of *ZmTIP4;*1 by mycorrhizal colonization in both drought-sensitive and drought-tolerant maize cultivars and also concluded that mycorrhizal inoculation generally down-regulated the expression of *AQPs* in the drought-sensitive than in the drought-tolerant genotype. In maize, ZmPIP2; 2 and ZmPIP2; 6 gene in maize plants had different expressed patterns to respond AMF inoculation: down-regulation under WW and up-regulation under DS (Quiroga et al., 2019). The changes in root *AQP* expression in response to AMF colonization can reflect the underlying event in altered water transport (Watts-Williams et al., 2019). In fact, mycorrhizal hyphae directly take part in water absorption of host plants under DS, suggesting a better water status in host plants (Allen, 2007; Zhang et al., 2018). Mycorrhizal plants under such relatively greater water status, possibly do not have to activate these *AQPs* expression in response to DS, thus, resulting in the down-regulation of *AQPs* expression. Such responses of host *AQPs* to drought under mycorrhization are dependent on host plant genotypes, soil water status, and AQP types. Analyzing AQP expression pattern in response to mycorrhizal colonization is possible to gain an overview of the complex regulation of water absorption in mycorrhizal plants (Watts-Williams et al., 2019). Therefore, more *AQP* members need to be noted, especially under DS conditions.

The present study analyzed the water absorption rate of mycorrhizal hyphae and changes in a series of roots *AQPs* expression in trifoliate orange (*Poncirus trifoliata* L. Raf.) under WW and DS conditions, in order to evaluate the relationship between host *AQPs* expression and mycorrhizas.

2. Materials and methods

2.1. AM fungal inoculums

Funneliformis mosseae (Nicol. & Gerd.) Schüßler & Walker was used because of a considerably higher capacity of drought tolerance in trifoliate orange exposed to DS (Wu et al., 2006). The AMF strain, *F. mosseae*, was procured from the Bank of Glomeromycota in China (BGC). The AM fungal strain was propagated in pots through identified spores using *Trifolium repens* as a host plant for three months. *Trifolium repens* was cut at the ground level; the roots were sliced to small fragments and mixed with the soil mass of the culture pots. The mycorrhizal inoculum including spores (15 spores/g), hyphae, infected root segments, and substrates of both soil and sand was used in this study.

2.2. Experimental device

The experimental device was described previously by Zhang et al. (2018) in details. Simply, the plastic pot with lots of holes at the bottom was laid by the 37-μm nylon meshes in the pot bottom to allow extraradical hyphae, but not roots, to pass through the mesh. A 2-mm bulge was established at the 3-cm place of the pot top. Subsequently, the pot was placed in a glass beaker (11 cm diameter and 13.5 cm height). Such combination provided a 2.5-cm air gap between the pot and the beaker to allow extraradical hyphae to absorb water from the beaker.

2.3. Plant culture

Two 4-leaf-old trifoliate orange seedlings without the colonization of arbuscular mycorrhizal fungi were transplanted in a plastic pot (11.5 cm upper diameter, 8.5 cm bottom diameter, and 14 cm height), where 1 kg of autoclaved (0.11 MPa, 121 °C, 2 h) soils and sand substrates (4 : 1, v/v) was supplied. The soil used in this experiment had pH 6.4, 8.5 mg/g organic carbon, 14.6 mg/kg Olsen-P, and 103.2 mg/kg available K. At the time of transplanting, AMF inoculation with 100 g inoculums of *F. mosseae* was used. Non-AMF treatment received 100 g sterilized (0.11 MPa, 121 °C, 2 h) inoculants plus 2 mL inoculum filtrates (25 μm filter). After transplanting, the inoculated and uninoculated plants were maintained in soil WW status with 75% of the maximum field water holding capacity for 12 weeks. And then, soil water status of plants was changed: half of the plants were continued to stay with soil WW status for 6 weeks; the other plants were changed from soil WW to soil DS (50% of the maximum field water holding capacity of the substrate, corresponding to a mild drought) for 6 weeks. The soil water status was controlled daily by weighting, and the soil water loss was compensated. During the entire experimentation, the pots were placed on the beaker with 1.5-cm-height water layer and 1-cm air gap. All the pots were randomized weekly to eliminate environmental effects. To decrease water evaporation of the beaker, a newspaper was packaged on the beaker.

2.4. Experimental design

The experiment was laid out in a 2^2 factorial completely randomized design with two soil water regimes (WW and DS) and two AMF inoculations (*F. mosseae* and non-AMF inoculations). Each of four treatments was replicated five times, for a total of 40 seedlings (2 seedlings per pot).

2.5. Determinations of plant growth and leaf water potential (Ψ)

At harvest, plant growth parameters such as plant height, shoot and root biomass, stem diameter, and leaf number per plant were determined. Leaf Ψ was measured by a PSΨPRO Water Potential System with a leaf hygrometer (L-51A-SF, WESCOR Inc., Logan, Utah, USA) according to the user's manual.

2.6. Determinations of mycorrhizal colonization and hyphal length

Plant roots for assessing mycorrhizal colonization were stained according to the procedure described by Phillips and Hayman (1970), and the extent of mycorrhizal colonization was expressed as the percentage of infected root lengths versus observed total root lengths. The hyphal lengths in nylon meshes were determined according to the methods outlined by Zou et al. (2015). The meshes collected were cut into

2 × 2 cm size and stained by 0.05% trypan blue in lactoglycerol for 2 min. The length of the mycorrhizal hyphae passed through nylon meshes was observed and measured in a microscope. Soil hyphal length was measured as per the protocol of Bethlenfalvay and Ames (1987). A 1.0 g soil sample was mixed with 50 mL of phosphate buffer solution (pH 7.0). After mixing with vortex, a 1.0 mL solution was incubated into 0.5 mL of 0.05% trypan blue in lactophenol at 70 °C for 20 min, and the sample was observed in slides by a microscope.

2.7. Determinations of hyphal water absorption rate

Hyphal water absorption rate was determined according to Zhang et al. (2018). Simply, in the morning hours (6:00 a.m. of the day), before plant harvest, all the beakers contained same amount of distilled water (130 g). Subsequently, water loss was measured every 2 h, until 6:00 a.m. of the next day. Water absorption rate of extraradical hyphae was calculated by the following formula (Zhang et al., 2018): hyphal water absorption rate (mg H_2O/h/cm) = $(WL_{AM} - WL_{NAM})/24/L_h$, where WL_{AM}, WL_{NAM}, 24, and L_h stand for water loss of the beaker in AMF treatment in a soil water regime (mg H_2O), water loss of the beaker in non-AMF treatment in the same soil water regime (mg H_2O), 24 h (h), and total extraradical hyphal length in entire meshes of the pot bottom (mm), respectively. Meanwhile, L_h = hyphal length in nylon meshes (mm/cm^2) × π × 4.25^2 (cm^2).

2.8. Leaf gas exchange

Net photosynthesis rate (Pn), transpiration rate (E), stomatal conduction (g_s), and leaf temperature (Lt) in the fifth leaf from the top were determined using a Li-6400 Portable Infrared Gas Exchange Analyzer (Li-COR, Lincoln, Nebraska, USA) during 9:00 a.m. to 11:00 a.m. Water used efficiency (WUE) denoted the proportion of Pn versus E.

2.9. Relative expression of AQPs

Root total RNA was extracted using an EASY spin Plus Plant RNA kit (RN38, Aidlab, Beijing, China) according to the user's protocol. RNA integrity was checked by the electrophoresis in 1.0% agarose gels, and RNA purity was estimated by the value of A_{260}/A_{280} ratio. RNA was reverse-transcribed to first-stand cDNA using the TRUEscruot 1st Stand Cdna Synthesis Kit with gDNA Eraser (PC5402, Aidlab, Beijing, China). According to the unigene sequences from our earlier transcriptome data (unpublished) and the genome data of *Citrus sinensis* (http://citrus.hzau.edu.cn), seven *PIP* genes (*PtPIP1;1*, *PtPIP1;2*, *PtPIP1;5*, *PtPIP2;1*, *PtPIP2;2*, *PtPIP2;5*, and *PtPIP2;8*), six *TIP* genes (*PtTIP1;1*, *PtTIP1;2*, *PtTIP1;3*, *PtTIP2;1*, *PtTIP2;2*, and *PtTIP5;1*), and four *NIP* genes (*PtNIP1;1*, *PtNIP1;2*, *PtNIP5;1*, and *PtNIP6;1*) were selected. The primer sequences of the genes were designed according to the Primer Premier 5 and shown in Table 1. The reactive components of qRT-PCR included 10 μL of 2 × AceQ SYBR Green qPCR Mix, 0.5 μL of each primer, 0.2 μL ROX Reference Dye, 2 μL of diluted cDNA template, and 6.8 μL ddH_2O. The amplification procedure of qRT-PCR reactions was denatured at 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The reaction was performed using the ABI StepOne Plus™ Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). Relative quantification was calculated by the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001) in which the reference gene β-actin was the control. The measured transcripts were normalized to the relative expression value in non-AMF plants under WW conditions. All the determinations of gene expression were replicated three times (biological replicates) with three technical replicates for each gene.

2.10. Statistical analysis

Data (means ± SD, n = 5) were analyzed by the two-way variance (ANOVA) with SAS (SAS Institute, Inc., Cary, NC, USA). Data of root AMF colonization were arcsine transformed prior to ANOVA analyses. The Duncan's Multiple Range Tests at 0.05 levels were utilized to compare significant differences among treatments.

3. Results

3.1. Mycorrhizal development and hyphal water absorption rate

Root mycorrhizal colonization was observed in AMF-inoculated seedlings, but not in non-AMF-inoculated seedlings. Mycorrhizal seedlings showed 46.0%–56.7% root colonization, 21.3–30.8 cm/g hyphal length in soils, and 1.18–1.64 cm/cm^2 hyphal length in nylon meshes under DS and WW conditions, respectively (Table 2). DS treatment drastically reduced root mycorrhizal colonization and hyphal length both in soils and nylon meshes by 18.8%, 31.2%, and 28.2%, respectively, as compared with WW treatment.

Hyphal water absorption rate was 0.125 and 0.302 mg H_2O/h/cm under WW and DS respectively, and was 2.4 times higher under DS than under WW (Table 2).

3.2. Plant growth performance

DS treatment drastically reduced plant growth performance of trifoliate orange plants as compared to WW treatment (Table 3). AMF inoculation significantly improved plant height, stem diameter, leaf number, shoot biomass, and root biomass: 60.8%, 12.2%, 46.2%, 70.8%, and 17.7% higher under WW and 42.1%, 10.1%, 28.2%, 63.2%, and 40.8% higher under DS. There was a significant interaction of plant height between AMF treatments and soil water regimes.

3.3. Leaf Ψ and RWC

When compared with WW plants, DS treated plants significantly reduced leaf Ψ and RWC (Fig. 1a and b). On the other hand, mycorrhizal seedlings recorded higher leaf Ψ and RWC than non-mycorrhizal seedlings: 12.2% and 6.8% higher under WW and 13.3% and 11.0% higher under DS. There was no significant interaction of leaf Ψ and RWC between AMF treatments and soil water regimes (Table 4).

3.4. Gas exchange

DS treatment caused a significant reduction in gas exchange in leaves (Table 5). Mycorrhizal seedlings exhibited greater Pn, g_s, and E than non-mycorrhizal seedlings, which were 53.6%, 35.2%, and 8.5% higher under WW and 111.0%, 31.6%, and 18.0% higher under DS conditions, respectively. Mycorrhizal treatment did not alter Lt under WW conditions, but decreased Lt under DS conditions by 0.40%, as compared with non-mycorrhizal treatment. In addition, DS treatment did not alter WUE, while AMF colonization significantly increased WUE. There was no significant interaction of gas exchange between AMF treatments and soil water regimes.

3.5. Expression of root AQPs

DS treatment significantly induced the expression of root *PtPIP1;1*, while down-regulated the expression of root *PtPIP1;2*, *PtPIP1;5*, *PtPIP2;2*, and *PtPIP2;8* (Table 4; Fig. 2a, b, 2c, 2e, 2g). AMF treatment dramatically regulated the expression of root *PtPIP1;1*, *PtPIP1;2*, *PtPIP1;5*, *PtPIP2;1*, *PtPIP2;2*, and *PtPIP2;8* (Table 4; Fig. 2a–e, 2g). AMF inoculation in WW plants significantly down-regulated the expression of root *PtPIP1;5* and had no effects on root *PtPIP1;1* expression, when compared with non-AMF inoculations (Fig. 2a and c). AMF colonization up-regulated the transcript levels of root *PtPIP1;1* and *PtPIP1;5* than non-AMF treatment by 42.1%, 26.9% and 69.7% under DS, respectively. Under WW, AMF inoculation induced *PtPIP2;1* and *PtPIP2;5* expression, and did not affect *PtPIP2;2* (Fig. 2d–g). Under DS, *PIP2*

Table 1
Gene-specific primer sequences used in this study for qRT-PCR.

Genes	Gene ID	Primer sequence (5'→3')	
PtPIP1;1	Cs7g31420.1	F:TGAGGGACTAGCAGAAATGGAAGGT	R:GTAAAACGACCAAGACTTGAGCTCG
PtPIP1;2	Cs5g03460.1	F:GATGCGAAGAGAAACGCCAGAGACT	R:GGCATGGTCTTTGTTGAAGATGATG
PtPIP1; 5	Cs7g31410.1	F:GTAGCAAGATCAAACAGCTACACGC	R:CCTCTCAGTGAACTTATTGGCTCCT
PtPIP2;1	Cs8g16640.1	F:TCTTGGATCCTTCAGGAGCAATGCT	R:GGCCTTCGTAACAGACTTGCACTCC
PtPIP2;2	XM_006428723.1	F:AATTCATCCTCAGAGCATCTGCTTC	R:GCTTGACAGCCATTGATTAATCCTC
PtPIP2;5	Cs7g25610.1	F:ACCAATTCATCTTGAGAGCTGGAG	R:TAACCCCATGAAGATGTGGATAATC
PtPIP2;8	Cs8g02530.1	F:GAAGAAGGACAAACTCACCGCCATC	R:ACTGACGTAGAGGAAGAGGAGAGTG
PtTIP1;1	Orange1.1t03005.1	F:TCGCTAATCCACTGCTAACCAACTT	R:TGGTCTCCACTTCTCAACTAGGAGC
PtTIP1;2	Cs7g28650.1	F:GATCGTGGAGAGAATTGAAGTTGTG	R:GCAAGACATAAATCCATCCACTCCT
PtTIP1;3	Cs8g17900.1	F:TAAAAAATGCCCGGAATTGCTATCG	R:TCCATTGTCCGTGAGCTTGCTGTAA
PtTIP2;1	Cs1g15440.1	F:CCTTCAAGGCCTATCTTGCTGAGTT	R:CCTGATGGATCAAGTGCTGCATCTG
PtTIP2;2	Cs5g08710.1	F:CTAGTGAGCAACAGAAATGGTGAAG	R:ACAAAAAGAAGCGTGGCGATGAACT
PtTIP5;1	Cs9g14770.1	F:AGTCCAGACGCAGCATCAAATACAT	R:ACGGTGATGTGTCCACTCACTGCCT
PtNIP1-1	Cs3g18230.1	F:CCCCTAAACATACACTGCCTACGAA	R:AACAATCTTTTGGAAATGAGACGGC
PtNIP1-2	Cs2g04370.1	F:AGGGTTTGTGGATATACATTGTGGC	R:GAGGCACTCTTTGTAATCTCTCGCA
PtNIP5;1	Cs3g20790.1	F:TTCCAGTTCCAAATGTCTCTCTCAC	R:GTACTTCTGGTTCACTATGGGTCCT
PtNIP6;1	Cs1g11140.1	F:CTTGCTAGAAAGGTCGGTGCTGAAT	R:CAATTAGGGTTTCTGAGCCTTGTGT
β-Actin	Cs1g05000	F:CCGACCGTATGAGCAAGGAAA	R:TTCCTGTGGACAATGGATGGA

Table 2
Root colonization, hyphal length, and hyphal water absorption rate of *Poncirus trifoliata* by *Funneliformis mosseae* under well-watered (WW) and drought stress (DS) conditions.

Water regimes	AMF inoculation	Root colonization (%)	Hyphal length		Hyphal water absorption rate (mg H_2O/h/cm)
			Soil (cm/g)	Nylon mesh (cm/cm^2)	
WW	Non-AMF	0c	0c	0c	0c
	F. mosseae	56.65 ± 4.73a	30.83 ± 4.69a	1.64 ± 0.14a	0.125 ± 0.031b
DS	Non-AMF	0c	0c	0c	0c
	F. mosseae	46.03 ± 2.01b	21.25 ± 2.16b	1.18 ± 0.19b	0.302 ± 0.061a
DS		**	**	**	**
AMF		**	**	**	**
DS × AMF		**	**	**	**

Data (means ± SD, $n = 5$) followed by different letters among treatments represent significant differences at the 5% level. $^{**}P < 0.01$.

genes, including *PtPIP2;1*, *PtPIP2;2*, and *PtPIP2;5* was down-regulated by mycorrhizal treatment.

DS caused rapid decline in all the six *PtTIPs* gene expression, and mycorrhizal fungal inoculation regulated the expression of root *PtTIP1;1*, *PtTIP1;2*, *PtTIP1;3*, *PtTIP2;2*, and *PtTIP5;1* (Table 4; Fig. 3a–f). In addition, the transcript expression of root *PtTIP1;1*, *PtTIP1;2*, and *PtTIP1;3* was down-regulated by AMF colonization under WW by 23.5%, 26.5%, and 84.1%, and was not changed by AMF colonization under DS.

DS treatment caused a significant down-regulation of root *PtNIP1;1*, *PtNIP1;2*, and *PtNIP5;1* and the inducement of root *PtNIP6;1* expression (Table 4; Fig. 4a–d). Mycorrhizal treatment distinctly altered the expression pattern of root *PtNIP1;1*, *PtNIP1;2*, *PtNIP5;1*, and *PtNIP6;1*. Under WW conditions, inoculation with *F. mosseae* significantly increased root *PtNIP1;1* expression, reduced root *PtNIP6;1* expression, and did not alter root *PtNIP5;1* expression. Under DS conditions, AMF colonization dramatically reduced the transcript expression of *PtNIP1;1*, *PtNIP5;1*, and *PtNIP6;1*.

4. Discussion

The high reduction of symbiotic development (including root colonization and hyphal length in soil and nylon mesh) was found under DS than under WW treatment, which is in agreement with earlier studies in robinia (He et al., 2016), trifoliate orange (Zhang et al., 2018; He et al., 2019), maize (Quiroga et al., 2017), and tomato (Xu et al., 2018). This inhibition in symbiotic development under DS versus WW conditions may be due to the reduction of mycorrhizal hyphal growth, spore germination, and carbohydrate supplement from host plants (Wu et al., 2013).

In this study, the 6-week DS treatment considerably inhibited the symbiotic development, whereas DS application still increased hyphal water absorption rate by 2.4 times. It is in agreement with Zhang et al. (2018). Even so, the experiment device still has shortcomings in

Table 3
Effect of *Funneliformis mosseae* on plant growth performance of *Poncirus trifoliata* under well-watered (WW) and drought stress (DS) conditions.

Water regimes	AMF inoculation	Plant height (cm)	Stem diameter (mm)	Leaf number (#/plant)	Shoot biomass (g FW/plant)	Root biomass (g FW/plant)
WW	Non-AMF	14.78 ± 0.99c	2.50 ± 0.11bc	15 ± 1c	1.07 ± 0.15c	1.03 ± 0.10b
	F. mosseae	23.75 ± 2.34a	2.80 ± 0.17a	21 ± 2a	1.83 ± 0.13a	1.22 ± 0.09a
DS	Non-AMF	12.54 ± 1.47c	2.30 ± 0.11c	14 ± 2c	0.86 ± 0.08d	0.85 ± 0.03c
	F. mosseae	17.81 ± 0.91b	2.53 ± 0.11b	18 ± 1b	1.40 ± 0.05b	1.20 ± 0.11a
DS		**	**	**	**	NS
AMF		**	**	**	**	**
DS × AMF		*	NS	NS	NS	NS

Data (means ± SD, $n = 5$) followed by different letters among treatments represent significant differences at the 5% level. $^{*}P < 0.05$. $^{**}P < 0.01$. NS, not significant.

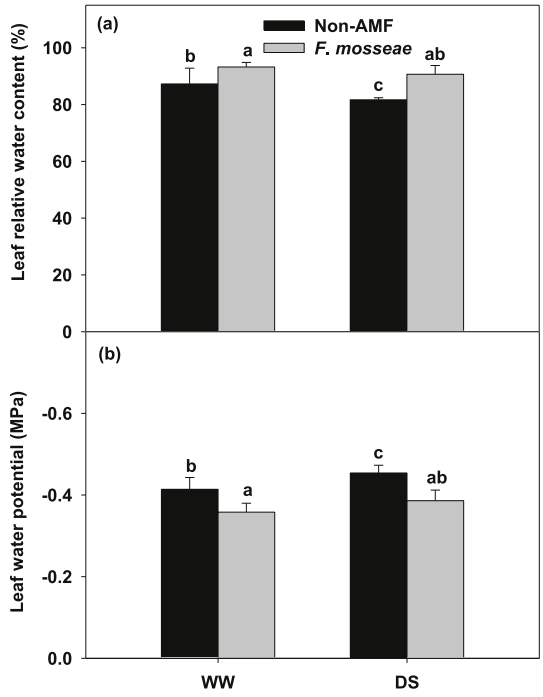

Fig. 1. Effects of *Funneliformis mosseae* on leaf relative water content (a) and water potential (b) of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, $n = 5$) followed by different letters above the bars among treatments represent significant differences at the 5% level.

Table 4
Significance of variables between trifoliate orange (*Poncirus trifoliata*) seedlings inoculated with *Funneliformis mosseae* under well-watered and drought stress (DS) conditions.

Variables	DS	AMF	DS × AMF	Variables	DS	AMF	DS × AMF
Leaf RWC	*	**	NS	*PtTIP1;1*	**	**	**
Leaf water potential	*	**	NS	*PtTIP1;2*	**	**	**
PtPIP1;1	**	**	**	*PtTIP1;3*	**	**	**
PtPIP1;2	**	**	NS	*PtTIP2;1*	**	NS	NS
PtPIP1;5	**	**	**	*PtTIP2;2*	**	*	NS
PtPIP2;1	NS	*	**	*PtTIP5;1*	**	*	NS
PtPIP2;2	**	*	*	*PtNIP1;1*	**	**	**
PtPIP2;5	**	NS	**	*PtNIP1;2*	**	*	NS
PtPIP2;8	**	**	NS	*PtNIP5;1*	**	**	**
				PtNIP6;1	**	**	**

*$P < 0.05$; **$P < 0.01$; NS: not significance.

measuring hyphal water absorption rate. There was not an anoxia effect on the extraradical hyphae in contact with the beaker of water. The estimated mycorrhizal hyphae passing through the nylon mesh were not necessarily involved in water absorption from the beaker. But, this device could be considered to roughly estimate how the hyphae behave in response to water deficit. This further implies that mycorrhizal roles in water absorption might be more important for host plants under dried soils than that of the saturated soils. Mycorrhizal hyphae possess hydrophilic behavior near hyphae tips, thereby, to absorb water, or to increase the thickness of water "bundles" by protecting the extraradical hyphae from the outside drying soil pores (Allen, 2007). Such hyphal behavior of mycorrhizas for water absorption is critical to water managing of irrigated agriculture (Allen, 2011).

In this study, *F. mosseae*-inoculated trifoliate orange seedlings possessed significantly higher leaf RWC and Ψ under both WW and DS conditions, and the AMF effect was considerably higher under DS than under WW conditions. This was in line with the changes of hyphal water absorption rate under WW and DS conditions. As a result, mycorrhizal hyphal functioning for water absorption was greater under DS than under WW conditions. On the other hand, AMF inoculation significantly increased Pn, g_s, Lt, and E, which is in agreement with Birhane et al. (2012) in *Boswellia papyrifera* seedlings colonized by *Glomus* sp. The enhancement of gas exchange by mycorrhization may be the result of increased concentration or activity of photosynthetic enzymes by improved nutritional status (Querejeta et al., 2003). Better gas exchange in AM plants under DS means greater transpiration demand for providing water transport power from root to leaf, which is a strategy to adapt to arid environment (Querejeta et al., 2003).

A substantial decrease in most of root AQP activity in response to DS was found in the present study, which is in accordance with earlier studies (Rodriguez-Gamir et al., 2011; Gambetta et al., 2017). Plant AQPs play important roles in water balance and WUE (Li et al., 2015). Down-regulated expression of root *AQPs* could facilitate water maintenance in the root cells and thus provide more water into leaves by long-distance transport (Rodriguez-Gamir et al., 2011), resulting in better gas exchange in AM seedlings under DS.

The present work showed different expression patterns of different *PtPIP1* and *PtPIP2* genes in response to DS treatment. It is in agreement with Lian et al. (2006) in rice. In fact, PIPs and TIPs undertake the regulation of root hydraulics under environmental stress conditions, whereas the changes in *AQP* expression are highly variable among AQP isogenes (Aroca et al., 2012), due to the heterotetramers of AQPs and the tissue-specific expression patterns (Gambetta et al., 2017). Due to the complex of AQPs, mycorrhizal inoculation induced different expression patterns under WW: down-regulation of *PtPIP1;5* and, up-regulation of *PtPIP2;1* and *PtPIP2;5*, and no changes in *PtPIP2;2*. However, under DS, all the *PtPIPs* expression was down-regulated by the AMF treatment. As proposed by Ruiz-Lozano and Aroca (2017), overexpression of *PIPs* may cause the fast wilting of plants. Therefore, such down-regulation of *PtPIPs* in response to AMF inoculation under DS is a way to minimize water loss from cells in a drought-sensitive plant (e.g., trifoliate orange) (Porcel et al., 2006; Quiroga et al., 2017; Ruiz-Lozano and Aroca, 2017). In addition, Quiroga et al. (2019) observed different expressed patterns of *PIPs* in response to water regimes: the down-regulation of *ZmPIP2;2* and *ZmPIP2;6* in AM maize plants under WW and the up-regulation of *ZmPIP2;2* and *ZmPIP2;6* in AM

Table 5
Effect of *Funneliformis mosseae* on leaf gas exchange of *Poncirus trifoliata* under well-watered (WW) and drought stress (DS) conditions.

Water regimes	AMF inoculation	Net photosynthesis rate (μmol $CO_2/m^2/s$)	Transpiration rate (mmol $H_2O/m^2/s$)	Stomatal conduction (mol $H_2O/m^2/s$)	Leaf temperature (°C)	Water used efficiency (μmol CO_2/mmol H_2O)
WW	Non-AMF	1.89 ± 0.12b	2.32 ± 0.11b	0.054 ± 0.003c	37.37 ± 0.02c	0.84 ± 0.07b
	F. mosseae	2.90 ± 0.12a	2.51 ± 0.07a	0.073 ± 0.004a	37.26 ± 0.09c	1.14 ± 0.04a
DS	Non-AMF	0.98 ± 0.05c	1.50 ± 0.10d	0.048 ± 0.003c	37.69 ± 0.11a	0.69 ± 0.05c
	F. mosseae	2.07 ± 0.19b	1.76 ± 0.15c	0.063 ± 0.007b	37.54 ± 0.07b	1.20 ± 0.09a
DS		**	**	**	**	NS
AMF		**	**	**	**	**
DS × AMF		NS	NS	NS	NS	NS

Data (means ± SD, $n = 5$) followed by different letters among treatments represent significant differences at the 5% level. **$P < 0.01$. NS, not significant.

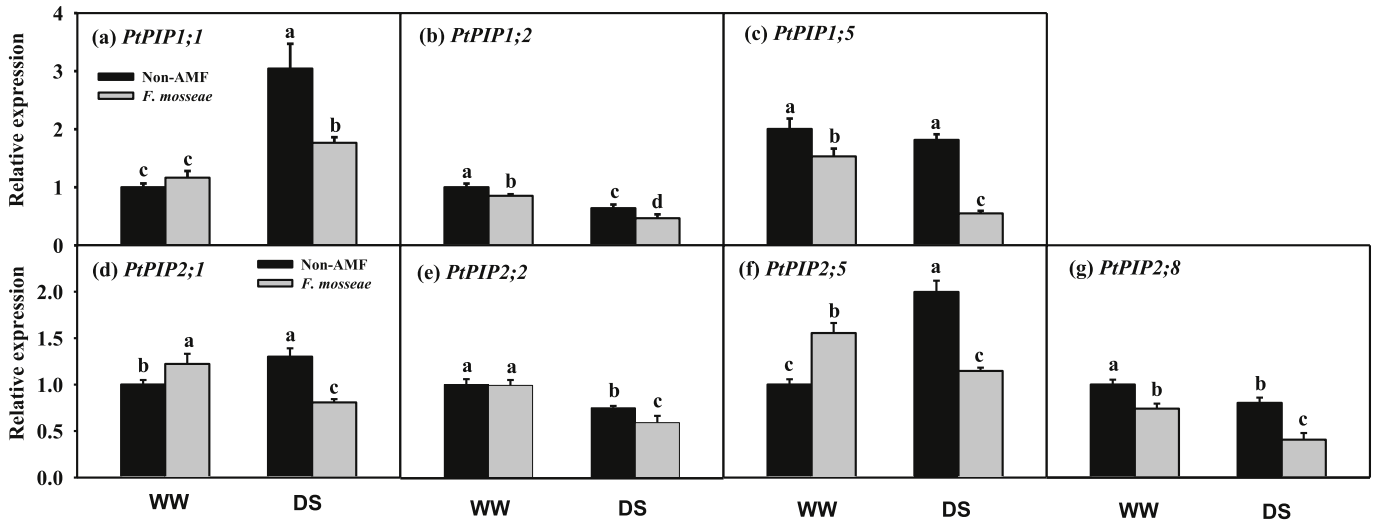

Fig. 2. Effects of *Funneliformis mosseae* on relative expression of *PtPIP1;1* (a), *PtPIP1;2* (b), *PtPIP1;5* (c), *PtPIP2;1* (d), *PtPIP2;2* (e), *PtPIP2;5* (f), and *PtPIP2;8* (g) in roots of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, $n = 3$) followed by different letters above the bars among treatments represent significant differences at the 5% level.

maize plants and the fungal *GintAQPF2* under DS. The response of PIPs to mycorrhization is dependent on host plant species, water deficit degree and AM fungal species. Even so, Aroca et al. (2009) proposed that the expression of an *AQP* gene cloned from an arbuscular mycorrhizal fungus *Glomus intraradices* was regulated as a compensatory way for the host plant *AQP* expression under stressed conditions. It seems that fungal and plant *AQP* genes strictly cooperate to mediate the mycorrhizal response to DS (Chitarra et al., 2016). On the other hand, under long-term (i.e., 6 weeks) drought stressed conditions, AM symbiosis may restrict most of the physiological processes in which these *AQPs* are involved (Ruiz-Lozano and Aroca, 2017).

In trifoliate orange, the DS treatment reduced the transcript levels of all the six *PtTIPs* expression, which is in accordance with Boursiac et al. (2005) in *Arabidopsis* roots exposed during 2–4 days to 100 mM NaCl. It indicates that in drought-sensitive trifoliate orange, *PtTIP* family members responded DS by down-regulation to avoiding water loss and preventing drought damages (Quiroga et al., 2017). In addition, such down-regulation of *PtTIPs* after DS was accompanied by a reshaping of vacuole structure (Takano et al., 2017). In this study, root *PtTIP1;1*, *PtTIP1;2*, and *PtTIP1;3* were down-regulated under WW conditions in response to AMF inoculation, while these *PtTIPs* expression was unaltered by mycorrhization under DS conditions. He et al. (2016) reported the up-regulation of *RpTIP1;1* in *Robinia pseudoacacia* after colonized by *R. irregularis* under WW and the down-regulation of *RpTIP1;1* under DS. *ZmTIP4;1* in maize was strongly induced by AMF treatment, irrespective of drought-sensitive and drought-tolerant cultivars (Quiroga et al., 2017). It indicated that AMF-modulated *TIP1* expression patterns were dependent on host plants and soil water status. Although TIPs can maintain cellular osmotic balance by controlling the exchange of water between vacuole and cytosol, mycorrhizal extraradical hyphae in AM plants take part in water uptake from soil pores inaccessible to roots, which are important to improve water supply to the plant (Ruiz-Lozano and Aroca, 2017). More evidence needs to be done in next works involved on AQP protein abundance and phosphorylation state to analyze the important of PIPs and TIPs on plant water relation.

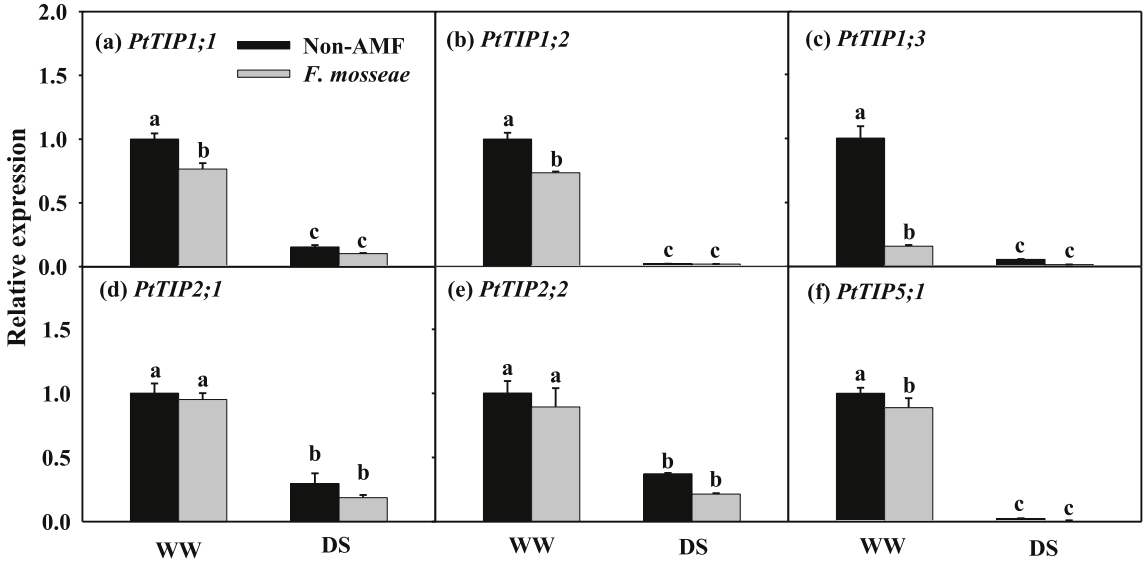

Fig. 3. Effects of *Funneliformis mosseae* on relative expression of *PtTIP1;1* (a), *PtTIP1;2* (b), *PtPIP1;3* (c), *PtTIP2;1* (d), *PtTIP2;2* (e), and *PtPIP5;1* (f) in roots of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, $n = 3$) followed by different letters above the bars among treatments represent significant differences at the 5% level.

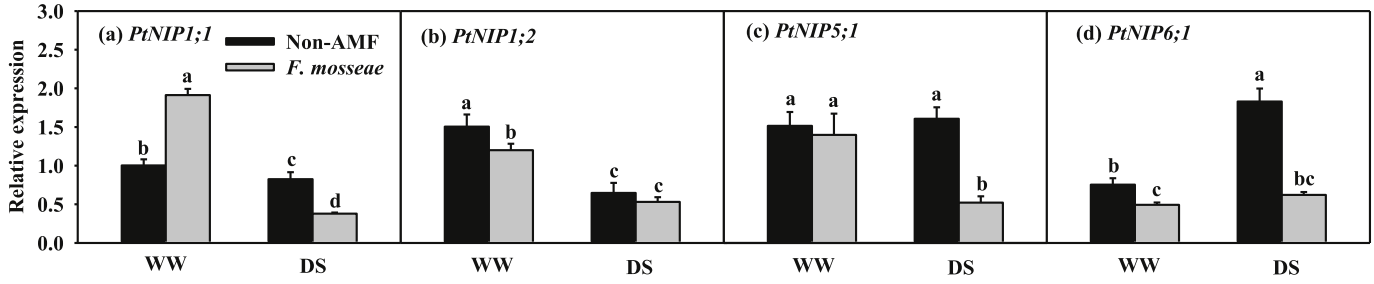

Fig. 4. Effects of *Funneliformis mosseae* on relative expression of *PtNIP1;1* (a), *PtNIP1;2* (b), *PtNIP5;1* (c), and *PtNIP6;1* (d) in roots of trifoliate orange seedlings under well-watered (WW) and drought stress (DS) conditions. Data (means ± SD, $n = 3$) followed by different letters above the bars among treatments represent significant differences at the 5% level.

Nodulin-26 like intrinsic proteins (NIPs) are found in the plasma membrane and the endoplasmic reticulum in the non-legume plants, where some of NIPs transport small uncharged solutes such as boric acid and silicic acid (Takano et al., 2017). In this study, most of *PtNIPs* expression was reduced by DS, and mycorrhizal inoculation dramatically reduced root *PtNIP1;2* and *PtNIP6;1* expression levels under WW conditions and root *PtNIP1;1*, *PtNIP5;1*, and *PtNIP6;1* expression levels under DS conditions. We speculated that mycorrhizal hyphae might provide the direct boron (B) to the host plant, and the down-regulation of *NIPs* avoided the toxicity of excess B accumulation in the host plant (Ruiz-Lozano and Aroca, 2017). In addition, Si mechanically hinders the penetration of fungi, and the reduced expression of *PtNIPs* associated with Si transport by mycorrhization is possibly expected (Ruiz-Lozano and Aroca, 2017).

5. Conclusions

A higher hyphal water absorption rate was found under DS than under WW conditions, indicating an important hyphal behavior in arid environment. qRT-PCR revealed diverse expression patterns of root *AQPs* under WW conditions and no changes or down-regulation of root *AQPs* under DS conditions. The expression pattern of *AQPs* in response to mycorrhizas should be a way of mycorrhizal plants to minimize water loss. Mycorrhiza-mediated drought tolerance in host plants could ascribe to the complex regulation at transcriptional levels of *AQPs*.

Author contribution

YNZ and QSW designed the work. HHW performed the experiment and collected data. HHW, YNZ and BG analyzed and interpreted the data. YNZ, QSW and BG wrote this manuscript. KK revised the manuscript. All authors approved the final manuscript.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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