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ORIGINAL ARTICLE

Effect of *Rhizophagus irregularis* on osmotic adjustment, antioxidation and aquaporin PIP genes expression of *Populus* × *canadensis* 'Neva' under drought stress

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Abstract Drought is an abiotic stress that severely reduces plant growth. Responding to drought, plants would induce a series of physiological and biochemical changes. Colonization by arbuscular mycorrhizal (AM) fungi was reported beneficial in improving plants' drought tolerance. However, the effect of AM fungi in improving drought tolerance of widely planted *Populus* spp. was rarely reported. The effect of AM fungus (*Rhizophagus irregularis*) on malondialdehyde (MDA) content, proline and soluble proteins content, antioxidative enzymes activates, relative water content (RWC) and water use efficiency (WUE), and the aquaporin PIP genes expression of *Populus* × *canadensis* 'Neva' leaves under well-watered and drought-stressed condition was evaluated. *R. irregularis* could colonize more than 80 % of poplar roots, reduce MDA and proline content, lower antioxidative enzymes activates, and down-regulate the expression of *PIP2;1*, *PIP2;2*. Meanwhile, *R. irregularis* could increase soluble protein content, increase RWC and WUE, and up-regulate the expression of *PIP1;1*, *PIP1;3*, *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;2*, and *PIP2;3*. In conclusion, *R. irregularis* could improve drought tolerance of *P. canadensis* by increasing RWC via regulation of aquaporin genes expression, and consequently increased WUE, lowered accumulation of osmotic adjustment molecule, reduced ROS accumulation and oxidative damage. Further studies focusing on the influence of AM fungi on specific aquaporin PIP gene location, function and expression in plant responding to drought stress are needed.

Keywords *Rhizophagus irregularis* · Osmotic adjustment · Antioxidation · Aquaporins · Drought

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Introduction

Drought is a seriously abiotic stress which reduced plant growth and productivity. Responding to drought, plants induced a series of physiological and biochemical changes (Farooq et al. 2009). One important physiological reaction of plant was the accumulation of soluble substances, such as proline and solute protein, for osmotic adjustment. Proline accumulation could lower the osmotic potential of cell, attract water into cell and help the maintenance of turgor (Lin and Kao 2000). The accumulation of osmotic regulation substances could also improve the stability of cell membrane and the activity of the proteins and enzymes (Hessini et al. 2009).

One important biochemical change under drought stress is the reactive oxygen species (ROS) accumulation, which starts from plant stomatal closure and owes to the over-reduction of the photosynthetic electron chain (Ahmed et al. 2009). Drought often increased the level of ROS, including superoxide anion (O_2^-), hydroxyl radical (HO^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), and required protective mechanism to prevent ROS from interrupting normal metabolism and oxidizing proteins, DNA and lipids (Smirnoff 1993; Ahmed et al. 2009). For

instance, ROS could stimulate membranous lipid peroxidation and accumulate membranous peroxide product like malondialdehyde (MDA) (Lacan and Baccou 1998). In responding, plants would produce antioxidant enzymes and antioxidant molecules against oxidative stress to protect themselves (Liu et al. 2011). The most important antioxidant enzymes are superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC 1.11.1.7). Cooperation of SOD and POD scavenge O_2^- into H_2O (Wang et al. 2009; Wu and Zou 2009).

Arbuscular mycorrhizal (AM) fungi from the Glomeromycota could form symbiosis with more than 80 % terrestrial plants and improve their tolerance against biotic and abiotic stresses (Augé 2001; Smith and Read 2008; Augé et al. 2015). Ruiz-Lozano (2003) pointed out that an important mechanism of AM fungi in helping plant under drought stress was the improved ability of plant osmotic regulation, increased stability of cell membrane, and improved antioxidant enzyme activity. Due to the influence of AM fungi, the improved drought tolerance was documented in different plants, e.g., citrus, melon, pistachio, and *Knautia arvensis* (Huang et al. 2011; Abbaspour et al. 2012; Doubková et al. 2013; Zou et al. 2015).

Besides influencing plant physiological and biochemical reactions, AM fungi-regulated plant aquaporin genes expression was also reported to be beneficial for plant under drought stress (Aroca et al. 2007). Aquaporins are water channels that have the capacity of water permeation 10–100 times higher than that of diffusion (Agre et al. 2002). In plants, aquaporins could be separated into five subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the Nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), and the X-intrinsic proteins (XIPs) (Chaumont and Tyerman 2014). Under drought stress, mycorrhizal plants were observed to have better water status (higher root hydraulic conductivity and leaf water potential, and reduced leaf transpiration rate), which might be due to the AM fungi-regulated plant aquaporin genes (Ruiz-Lozano et al. 2006, 2009; Aroca et al. 2008; Maurel et al. 2008). With the help of AM fungi, relative water content of plant under drought stress was usually higher than their counterpart without AM fungi (Wu and Xia 2006; Huang et al. 2011, 2014). Along with improved plant photosynthesis and stomatal closure regulation by AM fungi, mycorrhizal plant usually had promoted water use efficiency (Birhane et al. 2012; Augé et al. 2015). Regulation by AM fungi would overall improve plant water status and drought tolerance (Aroca and Ruiz-Lozano 2009; Zhu et al. 2012).

Populus spp. and their hybrids were used as model plants in forest tree study due to their worldwide cultivation (Cooke et al. 2005). AM fungi could form symbiosis with different poplar species (Pallara et al. 2013; Li et al. 2015), increase their growth and tolerance to abiotic stresses, e.g., heavy metal (Cicatelli et al. 2010) and salinity (Wu et al. 2015). Drought could severely influence growth and physiological characteristics of *Populus* spp. (Han et al. 2013; Cao et al. 2014). However, the effect of AM fungi on *Populus* spp. drought tolerance was less investigated. Hence, this study aimed to explore the mechanism by which AM fungi increase drought tolerance of poplar through analyzing the influence of AM fungi on osmotic adjustment, antioxidation and expression of aquaporin genes.

Materials and methods

Growth substrate, plant material and AM fungal inoculum

Growth substrate, *Populus* × *canadensis* cuttings, and AM fungal inoculum (*Rhizophagus irregularis*) were prepared as described by Liu et al. (2015).

Experimental design and growth condition

The experiment consisted of a two-factorial design: (1) inoculation with either *R. irregularis* (5 g inoculum per pot) or not (5 g autoclaved inoculum with 10 mL inoculum filter drains); (2) water status, well watered (75 % of field capacity) or drought stressed (50 % of field capacity). Soil water content was controlled by controlling pot weights every day. There were four treatments with 30 replicates per treatment.

The growth condition was identical with Liu et al. (2015).

Mycorrhizal colonization

Twenty weeks after inoculation, roots of 6 seedlings from each treatment were collected, washed with tap water, and cut into 1 cm fragments. After staining with trypan blue (Phillips and Hayman 1970), root mycorrhizal colonization was measured by the method of Giovannetti and Mosse (1980).

Malondialdehyde (MDA) concentration, proline content, soluble protein content, POD activity and SOD activity

The fully expanded leaves of randomly selected six plants from each treatment were used to analyze malondialdehyde (MDA) content, proline content, soluble protein content, POD activity and SOD activity. MDA content was measured based on the method of Kramer et al. (1991). Proline

content was measured by the method of Bates et al. (1973). Soluble protein contents were measured by the method of Bradford (1976). POD activity was measured by the method of Kwak et al. (1995). SOD activity was measured by the method of Giannopolitis and Ries (1977).

Relative water content (RWC) and water use efficiency (WUE)

The RWC of poplar leaves was measured as described by Liu et al. (2015).

Water use efficiency (WUE) was calculated as the ratio between net photosynthetic rate (Pn) and transpiration rate (E). Each leaf of a seedling was numbered according to a leaf plastochron index (LPI) number, with the most recently appeared leaf (about 2 cm long) being LPI 0 (Larson and Isebrands 1971). Based on the method described by Liu et al. (2015), leaves of LPI 6 of 6 seedlings from each treatment were used to measure Pn and E.

RNA extraction and first-strand cDNA synthesis

Leaf sample was used to extract RNA using polysaccharide and polyphenol total RNA isolation kit (Bioteke Corporation, China) according to the manufacturer's instructions. RNA quality and quantity were evaluated by the A260/A280 ratio measured with NanoDrop 1000 spectrophotometer (Thermo-scientific, USA). The first-strand cDNA was synthesized using First-Strand cDNA Synthesis Kit (Sangon Biotech, China) from 0.5 μg RNA.

Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was performed to analyze the transcript accumulation of PIP family genes using Ubiquitin gene as the reference gene. Primers used are listed in Table 1 (Secchi et al. 2009). qRT-PCR was performed with SYBR Premix Ex Taq™ II (Perfect Real Time) (Takara Biotechnology Co., Ltd, China) according to the manufacturer's instructions. The amplifications were set at 20 μL reaction system including 10 μL SYBR Premix Ex Taq™ II, 0.8 μL forward and reverse primers, 7.4 μL nuclease-free water, and 1 μL cDNA. The reactions were performed on a Bio-Rad CFX96 real-time PCR instrument and the data were analyzed using Bio-Rad CFX Manager software, version 1.1 (Bio-Rad, USA).

The standard PIP genes PCR amplification was given as follows: initiated at 95 °C for 30 s, followed with 40 cycles of 95 °C for 5 s and 57 °C for 30 s. The fluorescence signal was recorded at each cycle end at 57 °C, and melting curve analysis was performed from 60 to 95 °C with fluorescence signal recorded every 0.5 °C during a 5 s hold. All qRT-PCRs were performed with three biological replications in three technical repetitions, and a maximum difference of 0.5 cycle between the CT of the duplicate samples was considered acceptable. Nuclease-free water was used as template in negative control and non-template controls were conducted for each primer pair to check significant levels of any contaminants.

C_T values obtained from qPCR were compared with $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) or relative expression software tool (REST©) (Pfaffl et al. 2002).

Statistical analysis

Analysis of variance (ANOVA) was performed by the SPSS 17.0 statistical program (SPSS Inc., IL, USA). Duncan's test was applied to compare means at 0.05 level of significance.

Results

AM colonization

No colonization was observed in non-mycorrhizal treatment. *R. irregularis* colonized 85.6 % poplar seedling roots under well-watered (WW) condition and 87.3 % poplar seedling roots under drought-stressed (DS) condition.

Table 1 Primers used in qRT-PCR

Names	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
PIP1;1	CAAGCCCAGTTTGTTCCATT	CAGCCAAACCCCTCAAACTA
PIP1;2	TTCGCCCTTTCAAGAATCAC	AGGGAGGGAATGAAGCAAAT
PIP1;3	GTGATGGAGGGCAAAGAAGA	ACAAGAAGGTGGCCATGAAC
PIP1;4	GTTTGGCTCTCAATTGTGTCTG	CCTTTCTGCAACACCTCACA
PIP1;5	CCCAATCAATGGATGTTTGA	GACGCAATTGAGAGCCAAAT
PIP2;1	TCGGATTATGATGGACCTTTC	ATGTGGTTGAGAAGGGAACG
PIP2;2	CCGCCAACTAAAGAGGAAAA	TGGGCAAAAGAAGAAAGGTC
PIP2;3	GTGAGCTTGGGCACTTGTTT	CGTGAATTTCCTTCCCTGAC
Ubiquitin gene	CAGCTTGAAGATGGGAGGAC	CAATGGTGTCTGAGCTCTCG

MDA

Both AM treatment and DS affected MDA content ($P < 0.01$). The interaction of AM treatment and DS showed no effect on the MDA content (Table 2). DS significantly promoted the MDA content in poplar leaves, while AM treatment decreased the MDA content in poplar leaves. The AM treatment had significantly reduced the leaves' MDA content by 34.3 % under WW condition, and 26.8 % under DS condition, compared with non-AM seedlings (Fig. 1a).

Free proline content

AM treatment affected the free proline content at $P < 0.05$ level. DS, the interaction of AM treatment and DS showed significant effect on the free proline content at $P < 0.01$ level (Table 2). Under WW condition, AM seedlings had higher free proline content than non-AM seedlings, but showed no significant difference. Under drought-stressed condition, AM inoculation decreased the free proline content by 13.5 % (Fig. 1b). Drought increased the free proline content of poplar seedlings, 72.5 % in non-AM seedlings while 39.3 % in AM seedlings (Fig. 1B).

Soluble protein content

The soluble protein content was increased by AM treatment and DS ($P < 0.01$). The interaction of AM treatment and DS showed no effect on the soluble protein content (Table 1). AM seedlings had higher soluble protein content than non-AM seedlings by 36.3 % under WW condition, and by 41.7 % under DS condition (Fig. 1c).

POD activity

AM treatment, DS and the interaction between AM treatment and DS significantly influenced POD activity ($P < 0.01$) (Table 2). AM seedling leaves had 36.6 % higher POD activity than non-AM seedling leaves under WW condition, while 5.7 % under DS condition. Drought increased the POD activity of poplar leaves, 89.8 % in non-AM seedlings while 47.0 % in AM seedlings (Fig. 1D).

Table 2 Effect of AM fungi treatments (AMF), drought stress (DS) and AMF × DS on the parameters of poplar seedlings

Parameters	AMF	DS	AMF × DS
MDA content	**	**	ns
Free proline content	*	**	**
Soluble protein content	**	**	ns
POD activity	**	**	**
SOD activity	**	**	**
Water use efficiency	**	ns	ns
Relative water content	**	**	**

ns Not significant

* $P < 0.05$; ** $P < 0.01$

SOD activity

SOD activity was influenced by AM treatment, DS and the interaction between AM treatment and DS ($P < 0.01$) (Table 2). Results showed that AM treatment had different effects on SOD activity of poplar seedling leaves under different water conditions. Under WW condition, AM treatment promoted the SOD activity by 24.2 %, but under DS condition, AM treatment decreased the SOD activity by 4.7 %. DS increased the SOD activity of non-AM poplar seedlings leaves, while had no effect on AM poplar seedlings leaves (Fig. 1E).

Water use efficiency

AM treatment significantly influenced WUE ($P < 0.01$) (Table 2). Under WW condition, AM inoculation had no effect on WUE of poplar seedlings. Under DS condition, AM inoculation showed positive effect on WUE of poplar seedlings, and increased 19.3 % compared with non-AM seedlings (Fig. 2a).

Relative water content

RWC was significantly influenced by AM treatment, DS and the interaction between AM treatment and DS ($P < 0.01$) (Table 1). DS decreased RWC of poplar seedlings. AM treatment had no effect on RWC of poplar seedlings under WW condition. However, the RWC of AM poplar seedlings was 10.2 % higher than non-AM poplar seedlings under DS condition (Fig. 2b).

Gene expression

AM treatment significantly influenced the expression of *PIP1;1*, *PIP1;3*, *PIP1;4*, *PIP1;5*, *PIP2;3* ($P < 0.01$). DS significantly influenced the expression of *PIP1;1*, *PIP1;3*, *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;2*, *PIP2;3* at $P < 0.01$ level and *PIP1;2* at $P < 0.05$ level. The interaction of AM treatment and DS significantly influenced the expression of *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;3* at $P < 0.01$ level, *PIP1;1*, *PIP2;2* at $P < 0.05$ level and showed no effect on the expression of *PIP1;2*, *PIP1;3* (Table 2). Under WW condition, AM treatment up-regulated the expression of *PIP1;1*, *PIP1;3*, *PIP1;5*, *PIP2;1* and *PIP2;3*, and had no effect on the expression of *PIP1;2*, *PIP1;4*, *PIP2;2*. Under DS condition, AM inoculation up-regulated the expression

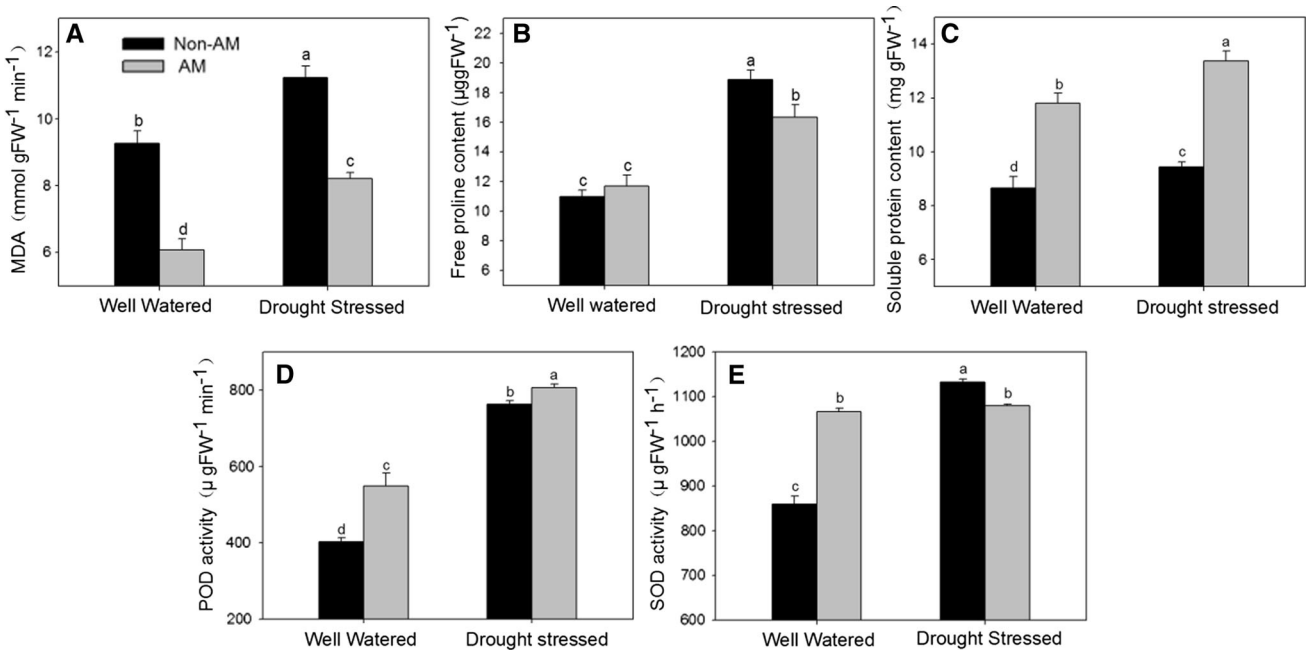

Fig. 1 Effect of AM fungi on the MDA content (**a**), free proline content (**b**), soluble protein content (**c**), POD activity (**d**) and SOD activity (**e**) of poplar seedlings under drought. Data were shown as mean ± SD ($n = 6$). *Different letters* on which column indicated significant differences at $P < 0.05$ level

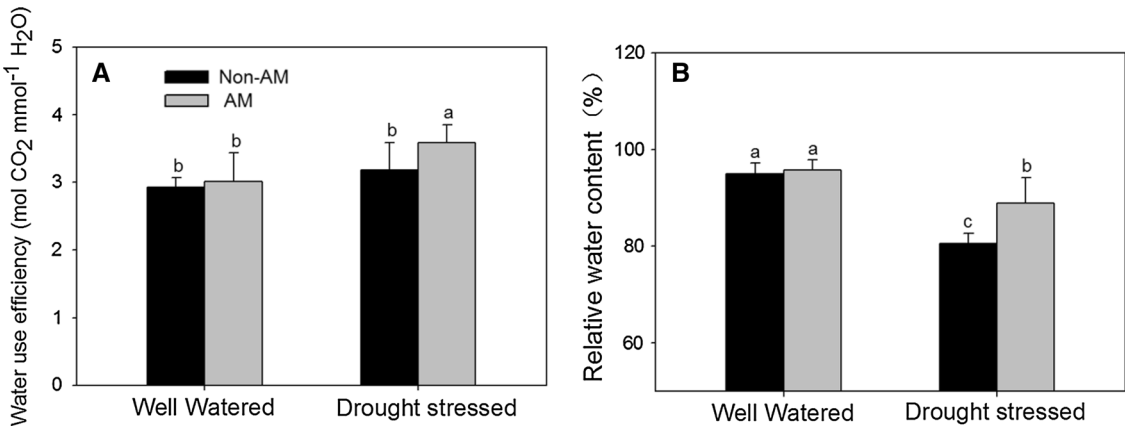

Fig. 2 Effect of AM fungi on the water use efficiency (**a**) and relative water content (**b**) of poplar seedlings under drought. Data were shown as mean ± SD ($n = 6$). *Different letters* on which column indicated significant differences at $P < 0.05$ level

of *PIP1;1*, *PIP1;2*, *PIP1;3*, *PIP1;4*, *PIP1;5* and *PIP2;3*, reduced the expression of *PIP2;1* and *PIP2;2* (Fig. 3).

Discussion

Plants developed their own defense systems to protect themselves from drought (Chaves and Oliveira 2004). Forming symbiosis with arbuscular mycorrhizal fungi was reported to be beneficial for plant in drought resistance (Augé 2001). In this study, *R. irregularis* colonized more than 80 % of poplar seedling roots in 20 weeks. This was consistent with the study of Liu et al. (2014) and set the basis for the study of AM fungus in helping poplar in drought resistance.

Under drought stress, plants accumulate some small molecules, such as proline, for osmotic adjustment (Augé 2001). In this study, drought increased proline content in poplar leaves, but the proline content in mycorrhizal plant was lower than in non-mycorrhizal plant. This was consistent with previous studies (Wu and Xia 2006; Monoharan et al. 2010; Asrar et al. 2012). However, mycorrhizal plants containing higher proline content than non-mycorrhizal plants were also observed in other studies (Fan and

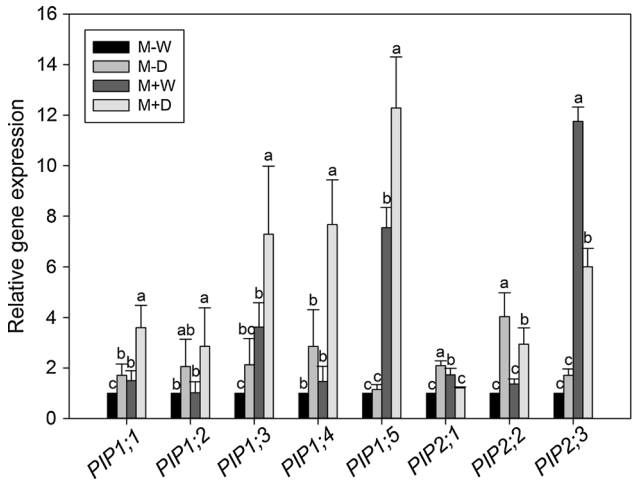

Fig. 3 Effects of AM fungi on the expression of the aquaporin PIP genes of poplar seedlings under drought. Data are shown as mean ± SD ($n = 3$). Different letters on which column indicated significant differences at $P < 0.05$ level. $M - W$ non-mycorrhizal and well-watered treatment; $M - D$ non-mycorrhizal and drought-stressed treatment; $M + W$ mycorrhizal and well-watered treatment; $M + D$ mycorrhizal and drought-stressed treatment

Liu 2011; Yooyongwech et al. 2013). Different plant response induced by AM fungi might be due to the different drought stress time. In short-time drought stress, the mycorrhizal plants responded fast and strong by accumulating more proline for osmotic adjustment as AM fungi induced 'priming' in response to disease (Jung et al. 2012). In long-time drought stress, AM fungi improved plant leaves' water status, which was observed in this study, lowered the accumulation of proline. Contrast to the proline content, the soluble protein in mycorrhizal plant leaves was higher than in non-mycorrhizal plant leaves. This might be owed to the fact that AM fungi-improved plant photosynthesis accumulated proteins (Wu and Xia 2006; Wu et al. 2010; Zhu et al. 2012) and AM fungi induced/enhanced specific plant protein under drought stress (Fan and Liu 2011).

Plant could strictly control ROS production and removal under well-watered condition, but lose its control under drought stress, which might result in proteins, DNA and lipids oxidation. Generated by peroxidation of membrane lipid, MDA could reflect the ROS circulation (Lacan and Baccou 1998). In this study, *R. irregularis* significantly reduced the MDA accumulation which indicated less injury during drought in poplar seedling leaves. This was consistent with studies that analyze the AM fungal influence on plant drought tolerance (Porcel and Ruiz-Lozano 2004; Ruiz-Sánchez et al. 2010). Efficient scavenge of ROS needs the cooperation of several antioxidative enzymes. Increased antioxidative enzymes activity, resulting in limited oxidative damage, had been reported in mycorrhizal plants (Wu and Zou 2009; Huang et al. 2014; Zou et al. 2015). In this study, drought increased the SOD and POD activity in leaves of non-mycorrhizal poplars and increased only POD activity in leaves of mycorrhizal poplars. This indicated that less ROS was accumulated in leaves of inoculated poplars, further confirmed by limited MDA accumulation. In leaves of inoculated poplars, inhibited oxidative damage was probably due to the improved water status observed in this study.

Plant water status and plant photosynthesis, and metabolic processes are closely related. Compared with non-mycorrhizal plants, higher RWC and WUE were observed in mycorrhizal plants (Kaya et al. 2003; Wu and Xia 2006; Huang et al. 2011; Birhane et al. 2012; Huang et al. 2014), and it is believed to be beneficial for stomatal open and high plant metabolism maintenance (Zhu et al. 2012). In this study, AM fungi significantly improved RWC and WUE of poplar leaves under drought stress. This result, on one hand, indicated that *R. irregularis* could improve poplar seedlings' water absorption and regulate plant water homeostasis. On the other hand, the improved RWC explained the limited accumulation of antioxidative enzymes, MDA and proline in leaves of inoculated poplars. Improved RWC and WUE by AM fungus might be due to the expanded water absorption area and systemically modified plant gene expression (Augé 2001; Cervantes-Gámez et al. 2016).

Drought stress and AM fungal inoculation-regulated aquaporin genes expression had been documented in previous studies (Porcel et al. 2005, 2006; Bárzana et al. 2012). In this study, *R. irregularis* differentially regulated poplar PIP genes under drought stress. This result was consistent with previous studies (Aroca et al. 2007; He et al. 2016), and it might be due to the different functions that each PIP genes played from the same gene family (Almeida-Rodriguez et al. 2010). With the help of AM fungi, the water demand might be partially satisfied under drought-stressed condition. In accordance with the improved water status discussed above, 6 PIP genes that were up-regulated in mycorrhizal poplar were used to improve water permeability (Chaumont and Tyerman 2014). Down-regulation of the *PIP2;1* and *PIP2;2* may be a water protection mechanism to avoid the plant dehydration under drought stress (Alexandersson et al. 2005). Another possibility was the plant-modulated gene localization to facilitate water absorption in other tissue (He et al. 2016). The specific function of each PIP gene under drought stress needed further investigation.

In conclusion, our results indicated that *R. irregularis* could improve poplar RWC under drought stress via regulation of aquaporin genes expression, and consequently increased WUE, lowered accumulation of osmotic adjustment molecule, reduced ROS accumulation and oxidative

damage. Further studies are needed to illustrate the influence of AM fungi on specific aquaporin PIP gene location, function and expression in plant responding to drought stress.

Author contribution statement Ting Liu carried out the experiment, gathered the data, and drafted the manuscript. Zhen Li gave assistance in qRT-PCR, tables and figures preparation in this manuscript. Hui Chen, Ming Tang and Haoqiang Zhang helped interpretation of the results, prepared and revised the manuscript.

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