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Original Article

Enhancement of Drought Tolerance in Trifoliate Orange by Mycorrhiza: Changes in Root Sucrose and Proline Metabolisms

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Abstract

Sucrose and proline metabolisms are often associated with drought tolerance of plants. This study was conducted to investigate the effects of two arbuscular mycorrhizal fungi (AMF) species (*Funneliformis mosseae* and *Paraglomus occultum*) on root biomass, lateral root number, root sucrose and proline metabolisms in trifoliate orange (*Poncirus trifoliata*) seedlings under well-watered (WW) or drought stress (DS). All the AMF treatments significantly increased root dry weight, taproot length, and the number of lateral roots in 1st, 2nd, and 3rd class under WW and DS. Mycorrhizal seedlings conferred considerably higher fructose and glucose concentrations but lower sucrose accumulation, regardless of soil water status. Under DS, *F. mosseae* treatment significantly increased root sucrose synthase (SS, degradative direction) and sucrose phosphate synthase (SPS) activity but deceased root acid invertase (AI) and neutral invertase (NI) activity, and *P. occultum* inoculation markedly increased root AI, NI, SS, and SPS activities. AMF treatments led to a lower proline accumulation in roots, in company with lower activities of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), δ-ornithine aminotransferase (OAT), Δ^1-pyrroline-5-carboxylate reductase (P5CR), and proline dehydrogenase (ProDH) in roots. It appears that the AM symbiosis induced greater root development and sucrose and proline metabolisms to adapt DS.

Keywords: arbuscular mycorrhizal fungi, citrus, invertase, osmotic adjustment, sugar

Introduction

Arbuscular mycorrhizal fungi (AMF), a kind of beneficial soil microorganism, can form mycorrhizal association with ~80% of land's plant species. Mycorrhizal symbiosis can help the host plant to absorb water and mineral nutrients from the soil to the fungal partner, and AMF get as many as 20% of photosynthetic carbon of the host plant (Parniske, 2008). Such AM symbiosis is a key component in resisting drought stress in various economic crops, including pepper (Davies, 2002), macadamia (Yooyongwech *et al*., 2013), citrus (Huang *et al*., 2014), and so on. The underlying mechanisms of mycorrhizal plants in enhancing drought tolerance have not yet been clearly elucidated. Possible mechanisms include (i) water and nutrient uptake directly by external hyphae (Zou *et al*., 2015), (ii) optimizing the root system architecture (Liu *et al*., 2016), (iii) greater osmotic adjustment capacity (Wu *et al*., 2007), (iv) enhancement of antioxidant defense systems (Huang *et al*., 2014), and (v) soil structure improvement by glomalin (Wu *et al*., 2008).

Drought stress (DS) invariably limits plant growth performance and yield to a series of irrigated plants including citrus (Abbaspour *et al*., 2012; Wu *et al*., 2013a). Under DS conditions, osmotic adjustment (OA) starts for the sake of inducing water potential of plants to maintain an optimal gradient for water flow from soils into roots. Sugars and proline as osmoprotectants, play key roles in accelerating water absorption and keeping the macromolecular structure and the sub cellular membrane under DS (Gomes *et al*., 2010). Studies showed that mycorrhizal plants exhibited higher sugar concentrations (Wu *et al*., 2007; Talaat and Shawky, 2014) due to mycorrhizal carbon cools for the fungal development in terms of sucrose cleavages (Franken, 2010) and AM-induced hydrolysis of starch to sugars in metabolic processes (Boldt *et al*., 2011). Moreover, accumulation of sugars in

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mycorrhizal plants would maintain both the membrane integrity and the osmoprotectant balance in plant cell under DS (Kapoor *et al.*, 2013). Prior to absorption and utilization by AMs, sucrose in roots must be cleaved into hexoses via either invertases or sucrose synthase (SS) (Schubert *et al.*, 2003). In general, sucrose invertases include acid invertase (AI) and neutral invertase (NI). Earlier studies showed that AMs notably decreased the activity of AI and NI in roots (Wu *et al.*, 2015). In addition, sucrose phosphate synthase (SPS), a kind of soluble enzyme observed in the cytoplasm, has the synergy with sucrose invertase in mediating long-distance transportation and metabolism in tissue sink of carbon in plants (Zhang and Li, 2002). Likewise, SS as a reversible type of glycosyl transferase existed in the cytoplasm that plays a key role in keeping the equilibrium between sugar metabolism and sink strength (Schäfer *et al.*, 2005).

Proline is a compatible solute participating in OA in plants exposed to DS (Hassine *et al.*, 2008). Proline is synthesized not only by the glutamate synthetic pathway with Δ^1-pyrroline-5-carboxylate synthetase (P5CS) in cytoplasm or chloroplast but also by ornithine synthetic pathway with δ-ornithine aminotransferase (OAT) in mitochondria (Ashraf and Foolad, 2007; Szabados and Savoure, 2009). Proline dehydrogenase (ProDH) generally induces proline transformed into Δ^1-pyrroline-5-carboxylate (P5C) (Kiyosue *et al.*, 1996), while P5C can be processed by P5C reductase (P5CR) into proline (Hu *et al.*, 1992). Studies showed that plants inoculated with AMF had a lower or higher level of proline than non-mycorrhizal counterparts under DS conditions (Wu *et al.*, 2007; Abbaspour *et al.*, 2012; Asrar *et al.*, 2012; Zou *et al.*, 2013).

Trifoliate orange [*Poncirus trifoliata* (L.) Raf.], one of citrus rootstocks used in China, India and Japan, is strongly dependent on AMF in fields, due to less or no root hairs. Earlier studies have shown the positive effect of AMF on drought tolerance of trifoliate orange (Wu *et al.*, 2007, 2013a, 2015; Huang *et al.*, 2014), but information regarding sucrose and proline metabolisms are poorly known under DS. The objective of the present work was to clarify the effect of two AM fungal species (*Funneliformis mosseae* and *Paraglomus occultum*) on sucrose and proline metabolism of trifoliate orange seedlings under DS conditions.

Materials and Methods

Plant culture

Seeds of trifoliate orange were surface sterilized with 70% of ethanol for 15 min and then germinated in autoclaved (0.11 MPa, 121 °C, 2 h) sands in a growth chamber (28/20°C day/night temperature, 1200 μmol/m^2/s photon flux density, and 80% relative humidity). After three weeks, two four-leaf-old seedlings with uniform size were transplanted into a 1.18-L plastic pot, each filled with 3.0 kg autoclaved (0.11 MPa, 121 °C, 2 h) substrates of soils and sands (4:1, v/v).

Funneliformis mosseae (Nicol. & Gerd.) Schüßler & Walker and *Paraglomus occultum* (Walker) Morton & Redecker were used here, based on the research of Wu *et al.* (2015). The mycorrhizal inoculums were propagated through an identified spore with a host plant white clover (*Trifolium repens*) for 16 weeks under potted conditions. The inoculum consisted of AM-infected root segments, spores, extraradical hyphae and sands. The dosage of 1000 spores was mixed with the growth substance of the pot at the time of plant transplanting. Non-AMF treated pot received the same amount of antoclaved (0.11 MPa, 121 °C, 2 h) mycorrhizal inocula, and 3 mL inoculums filtrate (25 μm filter) was added into the non-AMF pot for other microbial communities. The pots were placed in a glass house with a photo flux density of 728-965 μmol/m^2/s, 20-35/15-26 °C (day/night) and a relative humidity of 70-95% from March 25 to August 30, 2013.

Experimental design

The experiment consisted of a randomized block design with three mycorrhizal inoculations (with *F. mosseae*, *P. occultum* and -AMF) and two water treatments (well-watered, WW, 75% of maximum water holding capacity of soils; DS, 55% of maximum water holding capacity of soils). Each treatment had five replicates, leading to a total of 30 pots.

Before DS was begun, soil water of all these pots was kept in WW for 87 days. Subsequently, half of the seedlings were exposed to DS status for 71 days, and the other seedlings were still subject to WW status for 71 days. During water treatment periods, soil water status of the pot was determined daily through weighing and the quantity of water loss was re-supplied to keep the target soil water status at 6:00 PM. Meanwhile, 20 mL distilled water was weekly replaced with 20 mL Hoagland solutions for nutrients support.

Variable determinations

After 71 days of DS treatment, AM and non-AM seedlings were harvested. Roots of two seedlings from each replicate were collected as a sample. The taproot length was measured, and subsequently the number of different order lateral roots was counted. Dry weight of roots was determined after 75 °C for 48 h.

Fresh fine root segments (1 cm long) were cleared with 10% (w/v) KOH and stained with 0.05% (w/v) trypan blue by the protocol outlined by Phillips and Hayman (1970). Root mycorrhizal colonization was the percentage of AMF-colonized root length against total observed root length.

Root proline concentration (mg/g FW) was determined by the acid-ninhydrine method described by Bates *et al.* (1973). Root P5CS and ProDH activity was assayed according to Zou *et al.* (2013). One unit of P5CS was expressed as the enzyme amount of 1.0 μmol glutamate during 1 min from 1 g root (U/g FW), and one unit of ProDH activity was expressed as the absorbance of an increase of 0.01 at 600 nm in 1 min (U/g FW). Determination of root OAT activity was accorded to Kim *et al.* (1994), and OAT activity was defined as the amount of 1 nmol P5C during 1 min for 1 g fresh sample (U/g FW). Activity of root P5CR was assayed by the protocol of Chilson (1991), and one unit of P5CR activity was expressed as the enzyme amount of 1.0 μmol NADH during 1 min at 25°C (U/g FW).

Root sucrose, glucose and fructose concentration was determined according to the method of Wu *et al.* (2015). Activity of AI and NI in roots was assayed according to the protocol of Wu *et al.* (2013b). The activity of SS (degradative direction) and SPS was measured according to Lowell *et al.* (1989) and Hubbard *et al.* (1989), respectively.

Statistical analysis

Data (means ± SD, n = 5) were analyzed with two-factor variance (ANOVA) in SAS software (SAS Institute Inc., Cary, NC, USA), and the Duncan's multiple range test (P<0.05) was used to compare significant differences between treatments.

Results

Mycorrhizal colonization and root performance

DS treatment significantly reduced root colonization by 41% under *F. mosseae* conditions and by 30% under *P. occultum* conditions, respectively, as compared with WW treatment (Table 1). In addition, *P. occultum*-inoculation showed considerably greater root AMF colonization than *F. mosseae*-inoculation, regardless of WW or DS.

In general, AM seedlings represented significantly higher root biomass, taproot length and the number of lateral roots in the 1st, 2nd, and 3rd class than non-AM controls, irrespective of soil water status (Table 1). Meanwhile, treatment with *P. occultum* exhibited a relatively greater effect on these root variables than *F. mosseae* under WW and DS conditions.

Root fructose, glucose and sucrose concentrations

AMF colonization substantially increased root glucose concentrations than non-AMF treatment, regardless of soil water status and AMF species (Fig. 1b). Root sucrose concentrations were notably higher under non-AMF condition than under *F. mosseae* and *P. occultum* conditions, regardless of WW or DS, except a non-significant difference between non-AMF and *F. mosseae* under WW (Fig. 1c). AM seedlings represented markedly higher root fructose concentrations than non-AM seedlings, irrespective of soil water status and AMF species (Fig. 1a). There was a significant interacted effect of AMF and DS on root glucose and sucrose concentrations (Table 2).

Table 1. Mycorrhizal colonization, root dry weight, taproot length and number of lateral roots of *Funneliformis mosseae*- and *Paraglomus occultum*-colonized trifoliate orange (*Poncirus trifoliata*) seedlings grown under well-watered (WW) and drought stress (DS) conditions

Water status	AMF status	Root AMF colonization (%)	Root dry weight (g DW/plant)	Taproot length (cm)	Number of lateral roots (#/plant)		
					1st	2nd	3rd
WW	*F. mosseae*	57.54±5.53b	0.93±0.07b	42.0±1.9a	76±4a	156±6b	27±3b
	P. occultum	70.53±5.60a	1.37±0.11a	40.6±1.8a	78±1a	210±5a	70±3a
	-AMF	0±0e	0.63±0.03d	35.9±1.4b	63±3bc	105±6c	9±2d
DS	*F. mosseae*	34.19±2.98d	0.65±0.10cd	33.9±1.4b	61±4c	91±3d	21±4c
	P. occultum	49.71±5.11c	0.75±0.05c	35.2±2.0b	67±3b	108±8c	28±3b
	-AMF	0±0e	0.52±0.04e	30.4±1.5c	55±3d	79±3e	6±2d
Signification							
DS		**	**	**	**	**	**
AMF		**	**	**	**	**	**
DS×AMF		NS	**	NS	NS	**	**

Note: Data (means ± SD, n=5) followed by different letters between treatments indicated significant differences at 5% level. NS: not significant. *P<0.05; **P<0.01

Table 2. Significance of variable variations between AM and non-AM colonized trifoliate orange (*Poncirus trifoliata*) seedlings under well-watered (WW) and drought stress (DS) conditions

Variables	AMF	Drought stress (DS)	AMF×DS
Root glucose	**	**	**
Root sucrose	**	**	**
Root fructose	**	**	NS
Root AI	**	**	**
Root NI	**	**	**
Root SS	**	**	**
Root SPS	**	**	*
Root proline	**	**	**
Root P5CR	**	**	NS
Root OAT	**	**	**
Root ProDH	**	NS	**
Root P5CS	**	**	NS

Note: *P<0.05; **P<0.01. Abbreviations: AI, acid invertase; AMF, arbuscular mycorrhizal fungus; NI, neutral invertase; NS, not significant; OAT, ornithine-δ-aminotransferase; ProDH, proline dehydrogenase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; SPS, sucrose phosphate synthase; SS, sucrose synthase

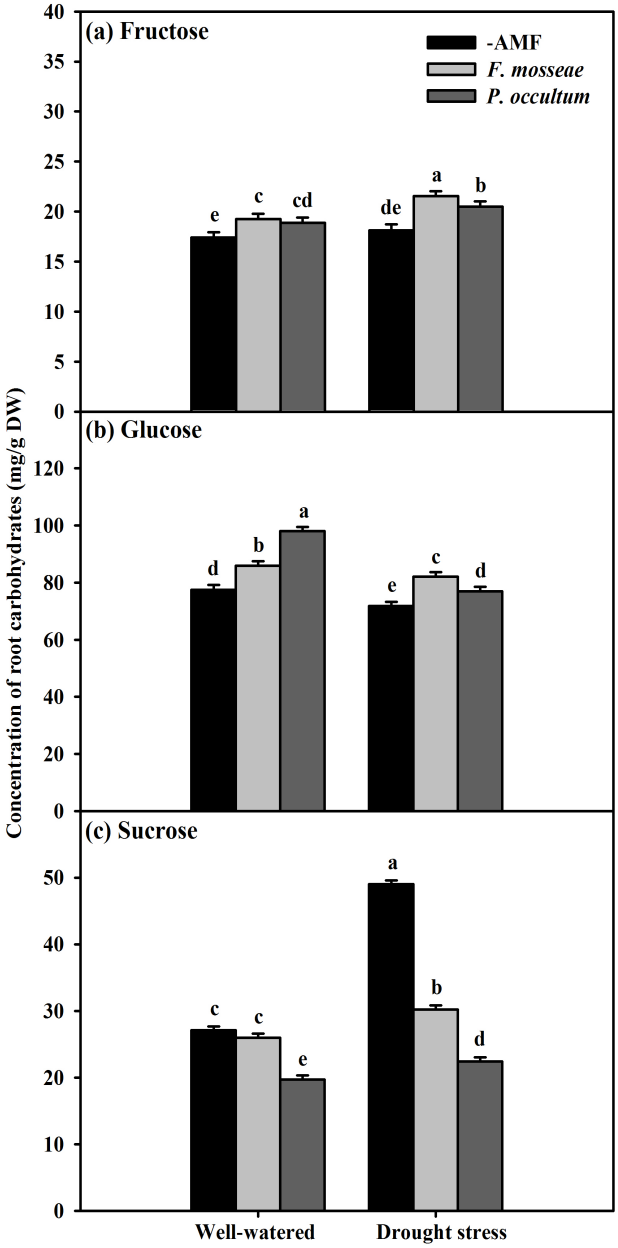

Fig. 1. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on root fructose (a), glucose (b), and sucrose (c) concentrations of *Poncirus trifoliata* seedlings under well-watered (WW) and drought stressed (DS) conditions. Data (means ± SD, n = 5) followed by the same letter above the bars are not significantly different among treatments at P<0.05

Root sucrose-metabolized enzyme activities

The seedlings inoculated with *F. mosseae* significantly increased the activity of AI, NI, and SPS in roots by 19%, 52%, and 70% under WW condition, compared to non-AMF-inoculated seedlings (Fig. 2a, 2b, 2d; Table 2). Under DS, *F. mosseae* inoculation also notably increased the activity of root SS and SPS by 117% and 82% (Fig. 2c, 2d; Table 2), whereas *F. mosseae* markedly decreased the activity of root AI and NI by 37% and 37% (Fig. 2a, 2b; Table 2). Compared with non-AMF seedlings, *P. occultum*-inoculated seedlings significantly increased the activity of root AI, NI, SS, and SPS by 38%, 50%, 210%, and 19% under DS condition (Fig. 2a-2d; Table 2). Under WW conditions, *P. occultum* treatment significantly increased the root SPS activity by 23% but decreased the root AI, NI, and SS activity by 8%, 21%, and 50%, respectively.

Root proline concentration

Compared with non-AMF treatment, *F. mosseae* inoculation notably decreased the root proline concentration by 32% and 30%, and *P. occultum* inoculation significantly reduced the root proline concentration by 32% and 13.33% under WW and DS, respectively (Fig. 3). There was a significant interaction in root proline concentration between AMF and DS treatment (Table 2).

Root proline-metabolized enzyme activities

Mycorrhizal seedlings showed considerably lower activities of root OAT, P5CR, ProDH, and P5CS than non-mycorrhizal seedlings: 60%, 52%, 53%, and 30% lower under *F. mosseae* and 34%, 48%, 63%, and 17% lower under *P. occultum* under WW; 18%, 46%, 72%, and 28% lower under *F. mosseae*, and 1%, 41%, 32%, and 28% under *P. occultum* under DS (Fig. 4a-4d). Root OAT and ProDH activity was markedly interactively affected by AMF and DS (Table 2).

Discussion

The 71-day DS treatment markedly decreased the root colonization of trifoliate orange plants by *F. mosseae* and *P. occultum*. This is in agreement with the reports of Huang *et al.* (2017), who found that trifoliate orange seedlings had significantly lower root AMF colonization under DS than under WW condition after inoculation with *F. mosseae*. Possibly, the negative effects of DS on root mycorrhizal colonization ascribe to the inhibition in both the germination of spores and the spread of soil hyphae (Huang *et al.*, 2014; Zhang *et al.*, 2014). Even so, such root mycorrhizal colonization still stimulated the enhancement of root biomass, taproot length, and the number of different order lateral roots under DS condition. The greater root biomass and morphology in AM seedlings would enlarge the contacted area of roots to soils, thus, potentially enhancing water and nutrient absorption under DS conditions (Remans *et al.*, 2012; Wu *et al.*, 2013a).

In this work, AMF treatment significantly increased root glucose and fructose concentrations while notably decreased root sucrose concentration under WW and DS, regardless of AMF species. The changes can be interpreted that in roots, AMs need hexoses, especially glucose, for its formation and development, which is originated from sucrose cleavage (Schubert *et al.*, 2003).

The present study showed lower AI, NI and SS activities in roots of *P. occultum*-inoculated seedlings under WW condition, which was in agreement with previous works in trifoliate orange inoculated with five AMF species (Wu *et al.*, 2015). Such reductions of sucrose-cleaved enzyme activities in mycorrhizal seedlings were due to the fact that sucrose was cleaved in phloem apoplasts unloading in roots, in order to maintain a suitable sucrose concentration

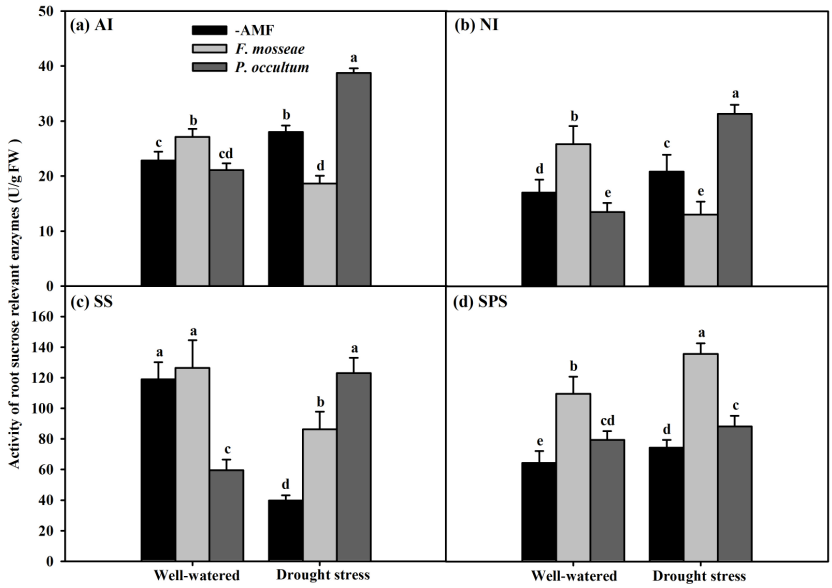

Fig. 2. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on activities of acid invertase (AI) (a), neutral invertase (NI) (b), sucrose synthase (SS, degradative direction) (c), and sucrose phosphate synthase (SPS) (d) in roots of *Poncirus trifoliata* seedlings under well-watered (WW) and drought stressed (DS) conditions. Data (means ± SD, n = 5) followed by the same letter above the bars are not significantly different among treatments at P <0.05

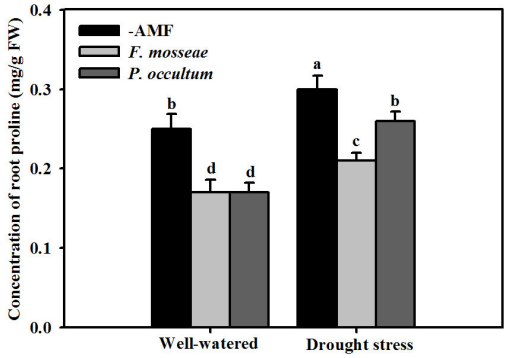

Fig. 3. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on root proline concentration of *Poncirus trifoliata* seedlings under well-watered (WW) and drought stressed (DS) conditions. Data (means ± SD, n = 5) followed by the same letter above the bars are not significantly different among treatments at P <0.05

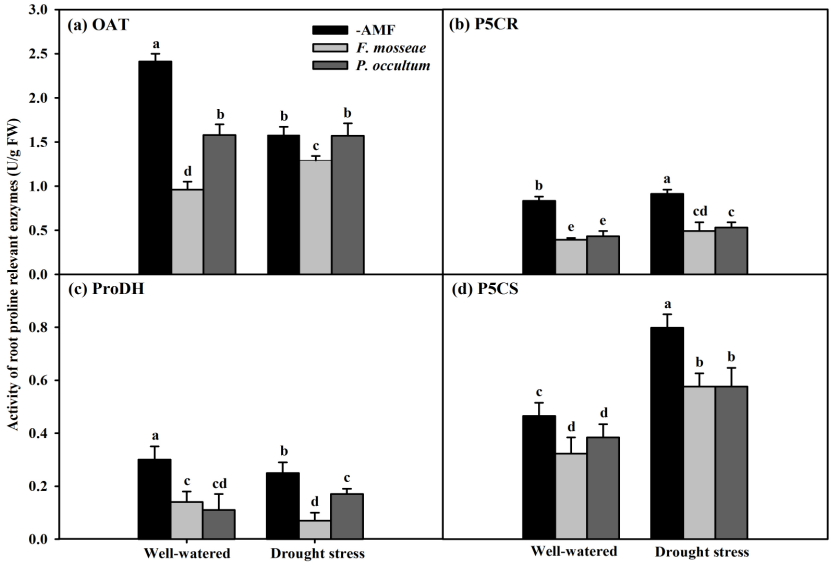

Fig. 4. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on activities of ornithine-δ-amino transferase (OAT) (a), Δ^1-pyrroline-5-carboxylate reductase (P5CR) (b), proline dehydrogenase (ProDH) (c), and Δ^1-pyrroline-5-carboxylate synthetase (P5CS) (d) in roots of *Poncirus trifoliata* seedlings under well-watered (WW) and drought stressed (DS) conditions. Data (means ± SD, n = 5) followed by the same letter above the bars are not significantly different among treatments at P <0.05

gradient between the source and the sink (Schaarschmidt *et al.*, 2007). In contrast, the seedlings inoculated with *F. mosseae* revealed higher root AI and NI activities relative to non-AM seedlings under WW condition, suggesting that the responses of root AI and NI activities to mycorrhization are mycorrhizal fungal dependent (Wu *et al.*, 2015). The present study also showed that root SS (degradative direction) and SPS activities were significantly increased by AMF inoculation under WW and DS conditions, except the similar SS level between non-AMF and *F. mosseae* and a lower SS level between non-AMF and *P. occultum* under WW. These results indicated that mycorrhization induced a higher root SPS activity to synthesize sucrose and also modulated diverse changes in sucrose-cleaved enzymes for greater hexose concentration in roots, which is beneficial for OA.

The present work showed that root proline concentration was significantly lower in AMF-treated trifoliate orange seedlings than in non-AMF colonized seedlings under WW and DS conditions, which is independent on AMF species. Similar results were found in *Macadamia tetraphylla* plants (Yooyongwech *et al.*, 2013). The lower proline accumulation under mycorrhization suggests less damage to AM seedlings exposed to DS conditions, resulting in a successful avoidance of DS in AM plants (Subramanian and Charest, 1995; Wu *et al.*, 2007; Zou *et al.*, 2013).

On the other hand, the significantly lower activity of P5CS, P5CR and OAT in roots was found in the AM seedlings than in non-AM plants under WW and DS conditions, except the similar root OAT activity between non-AMF and *P. occultum* under DS. The similar result was also reported by Zou *et al.* (2013). It is concluded that the lower proline accumulation under mycorrhization may be due to the inhibition of AMF-mediated glutamate and ornithine synthetic pathways (Szabados and Savoure, 2009). In addition, a significantly lower activity of root P5CR and ProDH was exhibited in mycorrhizal trifoliate orange plants under WW as well as DS conditions than non-mycorrhizal plants. It is well known that proline catabolism is another factor involved in the net proline accumulation under DS. Δ^1-pyrroline-5-carboxylate (P5C) is transformed into proline by P5CR, and in return proline is catabolised by ProDH into P5C (Szabados and Savoure, 2009). The activity of root ProDH was decreased by AMs in this work, in company with a decrease of root P5CR, suggesting that P5CR may work together with ProDH in regulation of proline accumulation. In a word, AMs made the trifoliate orange root exhibiting greater functions in proline degradation than in proline accumulation, no matter plants were grown under DS or not.

Conclusions

Inoculation with *F. mosseae* and *P. occultum* considerably increased root biomass and lateral root formation under either WW or DS condition. Mycorrhizas induced greater hexose accumulation and lower sucrose accumulation in roots, which is closely related to the change in sucrose-cleaved enzyme activities. Mycorrhizal plants represented lower root proline accumulation, which was significantly positively correlated with root P5CS, OAT, P5CR and ProDH activity.

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