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INCREASED TOLERANCE OF TRIFOLIATE ORANGE (*PONCIRUS TRIFOLIATA*) SEEDLINGS TO WATERLOGGING AFTER INOCULATION WITH ARBUSCULAR MYCORRHIZAL FUNGI

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ABSTRACT

Growth suppressive role of waterlogging (W_L) as an important abiotic stress to perennial plants is well known. A pot experiment was conducted to study a potential contribution of arbuscular mycorrhizal fungi (AMF, *Diversispora spurca*) on W_L tolerance of trifoliate orange (*Poncirustrifoliata* L. Raf.) seedlings on the basis of growth, root system architecture, and antioxidant enzymes. Four-month-old seedlings inoculated with or without *D. spurca* were subjected to W_L with non-waterlogging (NW_L) as control treatment. Root mycorrhizal (MY) colonization and vesicles notably decreased, but entry points significantly increased after 36 days of W_L. The W_L restricted plant growth performance and numbers of different order lateral roots, but the MY symbiosis exhibited theameliorating roles on plant growth, root morphology (total length, surface area, projected area, and volume) and numbers of different order lateral roots. The AMF inoculation significantly increased superoxide dismutase and catalase activities in leaf and root under both NW_L and W_L, thereby, resulting in lower oxidative damage in terms of malondialdehide concentration. These results suggest that the MY seedlings were biochemically and morphologically better prepared to tolerate W_L compared to the NMY seedlings.

Keywords: Antioxidant enzymes; Arbuscular mycorrhiza; Root system architecture; Trifoliate orange; Waterlogging

INTRODUCTION

Soil waterlogging (W_L), one of the important abiotic stresses, impairs with plant growth and productivity (Yin *et al*., 2009), by blocking the oxygen supply to the roots and affecting several metabolic processes of plants (Sairam *et al*., 2009).

Arbuscular mycorrhizal fungi (AMF) are the important component of crop production system as they can form mutualistic associations with roots of most terrestrial plants (Bainard *et al*., 2011). The characteristic of the mycorrhizal (MY) symbiosis is the transfer of sugars from the host plant to the fungus and of both nutrients and water from the fungus to the host plant partner (Fitter *et al*., 2011). Many studies have confirmed that inoculation with AMF increased the degree of tolerance in citrus plants exposed to various abiotic stresses like drought (Wu and Zou, 2010), high temperature (Wu, 2011), salt stress (Wu *et al*., 2010), and elevated CO_2 concentration (Jifon *et al*., 2002). Fougnies *et al*. (2007) earlier reported that colonization of *Pterocarpus officinalis* by *Glomus intraradices* enhanced its tolerance to W_L through improvements in plant growth and P-uptake. Better tolerance to $W_L$caused by AMF was previously reported in two semiaquatic grasses, *Panicum hemitomon* and *Leersia hexanda* (Miller and Sharitz, 2000), suggesting potential beneficial contribution of AMF inoculation to host plant possibly hold some promise for W_L tolerance.

Citrus in southern region of China is extensively grown under various soil habitats, including periodic soil waterlogged conditions. The waterlogged condition seriously inhibits tree growth and frequently declines in orchard productivity. However, the limited efforts have been made to understand the responses of citrus plants to waterlogged conditions through MY inoculation. In this background, the present study was carried out to study the effects of AMF on biochemical and morphological traits of citrus seedlings under W_L conditions.

MATERIALS AND METHODS

Experimental design: A total of four treatments in 2^2 randomized factorial design comprising MY inoculations (with or without *Diversispora spurca*) and waterlogged treatments (W_L and NW_L) were tested through a pot experiment under controlled greenhouse conditions .

Plant culture: Trifoliate orange (*Poncirus trifoliata* L. Raf.) was used as the experimental plant. Seeds of trifoliate orange were sown into the sterilised mixture of yellow-brown soil and vermiculite mixture (1:1, v/v). The five-leaf-old seedlings, without root MY colonization, were transplanted into the plastic pots(17.5 cm × 13 cm × 11 cm) containing a sterilized (121ºC, 0.11Mpa, 2 h) yellow-brown soil from a campus citrus orchard of

Yangtze University. At the time of transplanting, 60 g of *Diversisporaspurca* inocula (12 spores/g) was inoculated into the rhizosphere of the citrus seedlings. The NMY seedlings received 60 g of autoclaved (121ºC, 0.11Mpa, 2 h) inocula plus 10 mL of 60 g inoculum filtrates through a 25 µm filter to account only the differences of *D. spurca* spores between MY and NMY seedlings. The mycorrhizal inocula were propagated through the use of *D. spurca* spores, based on pot culture, inoculated in white clover (*Trifoliumrepens*), and grew 13 w at a controlled growth charmber (PQX, Life Apparatus, Ningbo Life Science and Technology Ltd., China) under the conditions of 16:8 photoperiod, 25/19ºC day/night temperature, 80% relative humidity, and light intensity 1700 Lx. The mycorrhizal fungus was isolated from the rhizosphere of *Lycopersicon esculentum* in Shougguang, China, and exhibited better tolerance of trifoliate orange to salinity stress (Zou and Wu, 2011). All the seedlings were placed in a plastic greenhouse at the Yangtze University campus from May to October, 2011. No exogenous application of nutrients was undertaken.

Waterlogged treatment: One hundred and seventeen days after acclimation for establishing root mycorrhizal colonization, the seedlings were subjected to $W_L$and NW_L treatments. Waterlogging treatment was given by placing the pots into a larger plastic container (28 cm × 17 cm × 20 cm) filled with tap water to 2 cm above the pot. The seedlings of NW_L treatment maintained ~25% soil water content (corresponding to field water capacity) by gravimetry. Trifoliate orange seedlings were subjected to W_L and NW_L treatments for 36-days, and the W_L seedlings exhibited a small amount of leaf tip yellowing at 36 days.

Measurements of plant growth, root mycorrizal colonization, and root system architecture (RSA): All the seedlings were harvested 36 days after W_L and NW_L treatments, and fresh shoot and root weights (g), plant height (cm), and stem diameter (cm) were determined and recorded.

The taproot length per seedling was determined by the vernier caliper. The number of first, second, and third order lateral roots were manually mounted. The intact root system per seedling was scanned by an Epson Flatbed Scanner, Epson Perfection V700 Photo Dual Lens System (J221A, Indonesia). The image toeach root system was analyzed by a professional WinRHIZO software in 2007 version (Regent Instruments Inc., Quebec, Canada), automatically obtaining root morphological traits.

The root MY staining was carried out by the procedure of Phillips and Hayman (1970). The root MY colonization was assessed using the method as described by Wu *et al*. (2008).

Analysis of antioxidant enzymes and soluble protein: A 0.2 g frozen leaf or root sample was homogenized with 7 mL of 100 mM ice-cold phosphate buffer (pH, 7.8). The homogenate was centrifuged at 4,000 g for 10 min at 4ºC, and the supernatant was used in the determination of protein and antioxidant enzymes.

The soluble protein concentration of leaf and root was estimated using bovine serum albumin as a standard (Bradford, 1976). The leaf and root SOD activity assay was carried out by the method of Giannopolitis and Ries (1977). One unit of SOD activity was defined as the quantity of SOD that brought a 50% inhibition in photochemical reduction of nitro blue tetrazolium at 560 nm. The leaf and root CAT activity was assayed according to the potassium permanganate titrimetric method previously described by Wu *et al*. (2010).

Lipid peroxidation: Lipid peroxidation was assessed by measuring the amount of malondialdehide (MDA) concentrations, according to thiobarbituric acid reaction as described by Sudhakar *et al*. (2001).

Statistical analysis: Data were analyzed using two-way ANOVA carried out through SAS software (v8.1). Significant differences among means were assessed based on the Duncan's multiple range test at 5% level.

RESULTS AND DISCUSSION

Mycorrhizal development: Our study showed that the W_L treatment significantly decreased root colonization by 29% (Table 1), which is consistent with *Lotus glaber* colonized by native AMF exposed to 35 days of W_L (Mendoza and Garcia, 2007). On the other hand, the W_L significantly increased number of entry point by 95% but decreased number of vesicleby 78%. In contrast, earlier studies showed an increase of vesicles in roots of *Lotus glaber* subjected to W_L (Mendoza and Garcia, 2007). Interestingly, under W_L conditions, the hyphal density and root colonization of trifoliate orange by *Gigaspora margarita* were increased when *Paspalum notatum* was intercropped (Matsumura *et al*., 2008).

Growth performance: The present study showed that the W_L markedly restricted all the growth parameters viz., plant height, stem diameter, leaf number per plant and shoot and root fresh weights, but the AMF inoculation significantly increased these growth variables under both NW_L and W_L conditions (Table 1). The observation provides a strong clue about the role of MY inoculation in improvement of plant growth under W_L stress.

Root system architecture: RSA, so-called the spatial configuration throughout the root system, determines the acquisition of nutrients and water from soil (Hodge *et al*., 2009). In the present work, the W_L induced no significant change in RSA properties viz., taproot length, root total length, root projected area, root surface area, and root volume, but significantly decreased the numbers of first-, second-, and third-order lateral roots (Table 2). On the other hand, the seedlings colonized by AMF recorded significantly higher total root length, root projected area and surface area, root volume, and numbers of first-, second-, and third-order lateral roots than the non-AMF seedlings, regardless of W_L and NW_L. Since RSA is a major factor to affect tolerance to abiotic stresses (Remans *et al*., 2012), greater RSA of the MY seedlings in the present study would benefit nutrient uptake of plants, thereby increasing tolerance of the MY seedlings to W_L.

Andioxidant enzyme system: W_L stress usually induces root oxygen deficiency (axonsis), triggering further the photoxidative damage via an increased generation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide (Yin *et al*., 2009). In our study, the W_L significantly increased MDA concentration of leaf and root in the AMF and the non-AMF seedlings, whereas the AMF seedlings recorded significantly lower MDA concentration: 17% in leaf and 23% in root under the NW_L and 16% in leaf and 16% in root under the W_L as compared with the non-AMF controls (Fig. 1; Table 3). The result implies that oxidative damage was higher in the W_L than in the NW_L seedlings, but lower oxidative damage was in the MY than in the NMY seedlings.

Table 1: Effects of W_L treatment on MY development and plant growth of trifoliate orange (*Poncirus trifoliata*) seedlings

Waterlogged treatment	AMF status	Mycorrhizal attributes			Plant growth parameters				
		Root colonization (%)	Entry points (num./cm root)	Vesicles (num/cm root	Height (cm)	Stem diameter (cm)	Leaf number of plant	Shoot fresh weight (g)	Root fresh weight (g)
NW_L	Non-AMF	0±0c	0±0c	0±0c	21.5±0.2c	0.26±0.01c	21.8±0.8b	1.6±0.1b	1.4±0.1b
	AMF	48.9±0.0a	4.2±1.8b	7.7±0.9a	28.9±1.1a	0.32±0.01a	27.2±0.8a	1.9±0.0a	1.7±0.1a
W_L	Non-AMF	0±0c	0±0c	0±0c	19.0±0.3d	0.23±0.01d	15.7±2.8c	1.2±0.1c	1.1±0.0c
	AMF	34.7±0.1b	8.2±2.3a	1.7±0.8b	27.2±0.2b	0.29±0.01b	22.8±1.5b	1.7±0.1b	1.4±0.1b
Significance									
W_L		**	*	**	**	**	**	**	**
AMF		**	**	**	**	**	**	**	**
W_L ×AMF		**	*	**	NS	NS	NS	*	NS

Means±SE (*n*=3) with different letters are significantly different (P<0.05). NS – not significant. $^{*}P$< 0.05. $^{**}P$< 0.01.

The antioxidant enzymes such as SOD, CAT, and peroxidase play scavenging effect toward ROS under W_L stress (Ahmed *et al*., 2002). The SOD activity of leaf and root and CAT activity of leaf decreased, but CAT activity of root increased on account of W_L stress (Fig. 2, 3; Table 3), suggesting that the MY and NMY seedlings suffered from a serious burst of ROS under W_L, and the antioxidant enzymes could not absolutely eliminate excess ROS. Our results also showed that in leaf, SOD activity was 25% and 38% higher in the AMF than in the non-AMF seedlings under the NW_L and W_L conditions; respectively (Fig. 2; Table 3). While in root, 12% and 15% higher SOD activity was observed in the AMF seedlings than in the non-AMF seedlings under the NW_L and W_L conditions, respectively. Under the NW_L conditions, the AMF infection significantly decreased CAT activity of leaf by 8% but increased CAT activity of root by 14% (Fig. 3; Table 3). Under the W_L conditions, inoculation with AMF significantly increased CAT activity of leaf and root by 27% and 15%, respectively. Wu (2011) reported similar results in SOD and CAT activity of *Glomus mosseae*-colonized trifoliate orange exposed to high temperature. In an experiment performed by Fester and Hause (2005), hyphae and arbuscules of mycorrhizas could accumulatea certain amount of ROS such as hydrogen peroxide (H_2O_2) in roots. On the other hand, MY symbiosis (colonized by *G. intraradices*) in combination with drought stress considerably increased the expression of the *Mn-sod II* gene (Ruiz-Lozano *et al*., 2001). The higher SOD and CAT activities of leaf and root caused by AMF are further accountable to both MY accumulation and the expression of specific genes. Our study also indicated that the AMF colonization significantly increased soluble protein concentration in leaf by 28% and 21% and in root 27% and 30% by under the NW_L and W_L conditions (Fig. 4; Table 3), respectively, suggesting that some stressed proteins are strongly induced to tolerate W_L conditions. With regard to these reactions, further studies are needed to unravel the mechanism involved at cellular and molecular levels.

The higher antioxidant enzyme activities of the MY seedlings were associated with lower levels of ROS in the plant tissues, thus, decreasing extent of membrane lipid peroxidation, namely, lower MDA concentration. These results suggested metabolically better MY plant preparedness to combat W_L stress.

Table 2: Effects of AMF and waterlogged stress on root morphological traits and numbers of different order lateral roots of trifoliate orange seedlings

W_L treatment	AMF status	Root morphology parameters					Lateral roots characteristics		
		Taproot length (cm)	Total Length (cm)	Projected area (cm^2)	Surface area (cm^2)	Root volume (cm^3)	First order	Second order	Third order
NW_L	Non-AMF	31.1±7.6a	256.8±4.3b	13.9±0.1b	43.7±0.5b	0.43±0.01c	40±1b	152±6b	21±2b
	AMF	34.0±2.8a	503.9±15.0a	20.3±0.6a	63.6±1.8a	0.64±0.02a	45±1a	186±5a	28±1a
W_L	Non-AMF	33.5±2.9a	321.1±15.1b	13.9±0.4b	43.7±1.2b	0.48±0.01b	37±1c	107±4c	14±0c
	AMF	33.3±0.5a	499.1±37.1a	20.0±1.4a	62.7±4.2a	0.63±0.04a	41±1b	155±13b	20±1b
Significance									
W_L		NS	NS	NS	NS	NS	**	**	**
AMF		NS	**	**	**	**	**	**	**
W_L ×AMF		NS	NS	NS	NS	*	NS	NS	NS

Means±SE (*n*=3) with different letters are significantly different ($P<0.05$). NS – not significant. $^{*}P< 0.05$. $^{**}P< 0.01$.

Table 3: Significance of the sources of variation for biochemical variables in mycorrhizal and non-mycorrhizal trifoliate orange seedlings exposed to waterlogged stress

Sources of variation	Soluble protein		SOD		CAT		MDA	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
W_L	**	**	**	**	**	**	**	**
AMF	**	**	**	**	NS	**	**	**
W_L ×AMF	NS	NS	NS	NS	**	NS	NS	NS

NS – not significant. $^{*}P< 0.05$. $^{**}P< 0.01$.

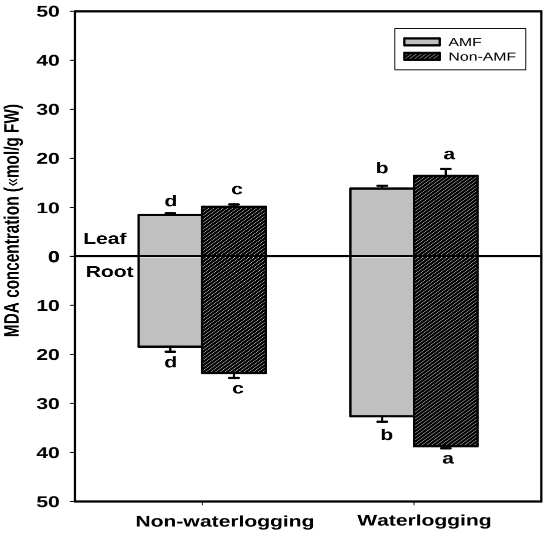

Figure 1: Effect of AMF and W_L stress on malondialdehide (MDA) concentration of leaf and root of trifoliate orange seedlings. Data were means ± SE (*n*=3) followed by different letters above the bars, which are significantly different ($P<0.05$).

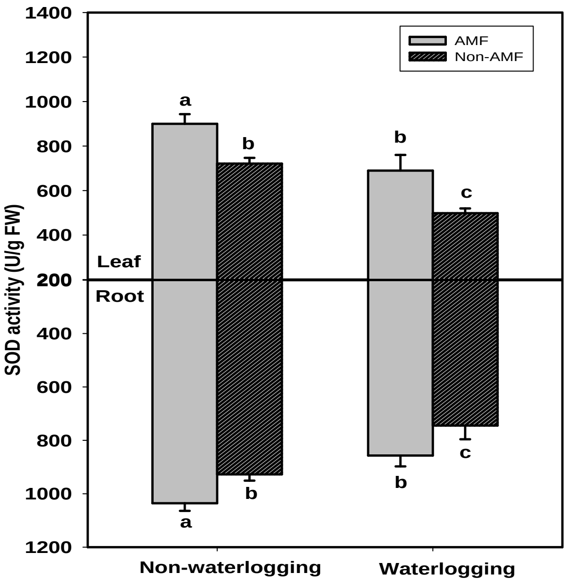

Figure 2: Effect of AMF and W_L stress on superoxide dismutase (SOD) activity of leaf and root of trifoliate orange seedlings. Data were means ± SE (*n*=3) followed by different letters above the bars, which are significantly different ($P<0.05$).

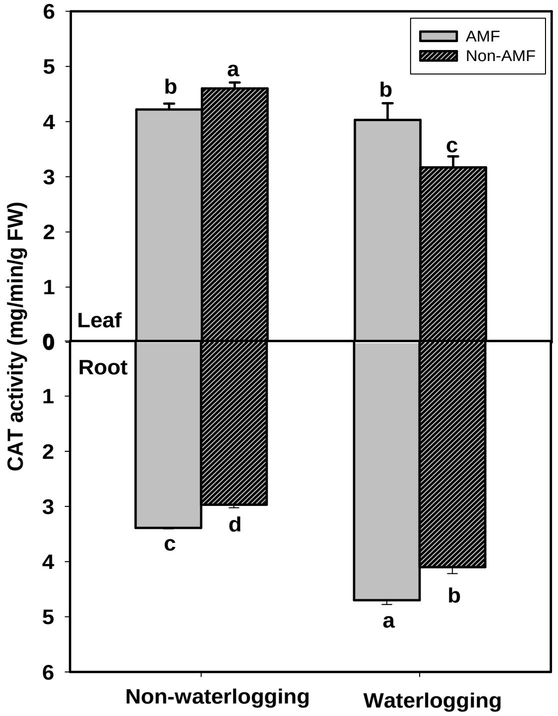

Figure 3: Effect of AMF and W$_L$ stress on catalase (CAT) activity of leaf and root of trifoliate orange seedlings. Data were means ± SE (*n*=3) followed by different letters above the bars, which are significantly different (*P*<0.05).

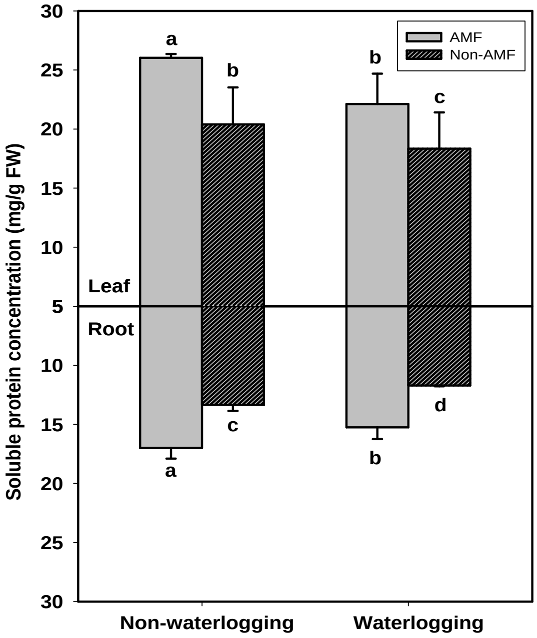

Figure 4: Effect of AMF and W$_L$ stress on soluble protein concentration of leaf and root of trifoliate orange seedlings. Data were means ± SE (*n*=3) followed by different letters above the bars, which are significantly different (*P*<0.05).

Conclusion: The W$_L$ stress restricted the plant growth, RSA, and SOD and CAT activities of the trifoliate orange seedlings. These adverse effects were significantly alleviated by MY inoculation enhancing SOD and CAT activities, and collectively imparting higher tolerance of MY seedlings under W$_L$ stress.

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