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Reactive oxygen metabolism in mycorrhizal and non-mycorrhizal citrus (*Poncirus trifoliata*) seedlings subjected to water stress

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Summary

The effect of the arbuscular mycorrhizal (AM) fungus, *Glomus versiforme*, on growth and reactive oxygen metabolism of trifoliate orange (*Poncirus trifoliata*) seedlings was studied in potted plants under well-watered (WW) and water stressed (WS) conditions. Water stress significantly decreased root colonization. Shoot dry weight, plant height and stem diameter were higher in AM than in non-AM seedlings regardless of the water status. Inoculation with *G. versiforme* increased root dry weight and leaf number per plant of WW seedlings. There was less malondialdehyde (MDA) concentration in leaves and roots of AM seedlings, as well as lower hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$) concentrations in AM roots under WW and WS conditions. AM inoculation did not affect the H_2O_2 and $O_2^{\bullet-}$ concentrations of WW leaves. Whether WS or not, AM symbiosis notably increased the guaiacol peroxidase (G-POD) activity of leaves, glutathione reductase (GR) activity of leaves and ascorbate peroxidase (APX) activity of roots. AM infection also markedly increased the APX activity of WS leaves. Soluble proteins and glutathione (GSH) in leaves and roots and ascorbate (ASC) in leaves were higher in WW AM than in WW non-AM seedlings. AM infection also enhanced the ASC and GSH contents of leaves and roots in WS seedlings. Cross-tolerance might occur in AM plants and be enhanced by AM symbiosis. Our results suggest that the increased concentrations of antioxidant enzymes and non-enzymatic antioxidants found in AM plants may serve to protect the organism against oxidative damage, enhancing drought tolerance.

Abbreviations: AM, arbuscular mycorrhizal; AMF, arbuscular mycorrhizal fungi; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; G-POD, guaiacol peroxidase; GR, glutathione reductase; GSH, glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; $O_2^{\bullet-}$, superoxide anion radical; ROS, reactive oxygen species; SOD, superoxide dismutase; WS, water stressed; WW, well-watered.

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Introduction

When higher plants are subjected to environmental stresses, such as water stress and salinity, a variety of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) are induced (Elstner, 1982; Jung, 2004). The ROS may initiate destructive oxidative processes such as chlorophyll bleaching, lipid peroxidation, protein oxidation, and damage to nucleic acids (Herbinger et al., 2002). As a consequence, higher plants induce efficient antioxidant systems to protect them against oxidative injury (Asada, 1999). The antioxidant systems consist of antioxidant enzymes including superoxide dismustase (SOD), guaiacol peroxidase (G-POD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), and non-enzymatic antioxidants including ascorbate (ASC) and glutathione (GSH), which are designed to minimize the concentrations of $O_2^{\bullet -}$ and H_2O_2.

Ninety percent of the earth's land plant species form symbiotic associations with arbuscular mycorrhizal fungi (AMF) (Gadkar et al., 2001). Arbuscular mycorrhizal (AM) symbiosis can affect the water relations of many plants (Auge, 2001). The effect is often more pronounced in plants grown under water stressed (WS) conditions than under well-watered (WW) conditions (Sanchez-Diaz and Honrubia, 1994). Stomatal conductance, transpiration rates, hydraulic conductivity and leaf water potential were usually higher in AM plants under WS condition, indicating that AM plants maintained more normal water relations (Auge et al., 2004; Sanchen-Blanco et al., 2004).

The mechanisms by which AMF have enhanced the water relations of host plants are often not clear. Potential mechanisms included enhanced absorption of water by external hyphae (Faber et al., 1991; Ruiz-Lozano and Azcon, 1995), stomatal regulation through hormonal signals (Goicoechea et al., 1997), the indirect effect of improved phosphorus nutrition (Nelsen and Safir, 1982; Fitter, 1988), and greater osmotic adjustment in AM plants (Auge et al., 1986; Ruiz-Lozano, 2003). In addition, Auge (2004) suggested that mycorrhizal soil itself could somehow directly influence the water relations of plants growing in them.

AM symbiosis might increase the drought tolerance of plants by promoting antioxidant enzymes (Ruiz-Lozano, 2003). However, information about activities of antioxidant enzymes in AM vs. non-AM plants is scarce. Host plants that have been examined include bean (Lambais et al., 2003), some shrub species (Alguacil et al., 2003), soybean (Porcel et al., 2003; Porcel and Ruiz-Lozano, 2004) and lettuce (Ruiz-Lozano et al., 1996). These studies focused mainly on antioxidant enzymes of leaves, including SOD, G-POD, CAT and APX. Non-enzymatic antioxidants and ROS of AM plants are not well understood. Moreover, little is known about the effects of mycorrhizal infection on antioxidant enzymes and non-enzymatic antioxidants of roots. The AM fungus, *Glomus versiforme*, has not been examined on citrus in regard to AMF and water stress.

The present study was undertaken to thoroughly evaluate reactive oxygen metabolism, including ROS, antioxidant enzymes and non-enzymatic antioxidants, in leaves and roots of trifoliate orange inoculated with *G. versiforme* under WW and WS conditions. Trifoliate orange is a major citrus rootstock used in China, and it has not been examine before when colonized by *G. versiforme* under WS conditions.

Materials and methods

Biological materials and material treatments

Seeds of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) were surface-sterilized with 70% alcohol for 5 min and germinated on wet filter paper in Petri dishes in darkness at 28 °C. Six 7-day-old seedlings were transplanted into a plastic pot (15 × 20 cm) and were inoculated with *G. versiforme* (Karsten) Berch or with a non-AM fungal control. Both AM and non-AM treatments received 30 g of their respective inocula. For each AM treatment, 30 g AM inoculum (approx. 2233 spores) were placed 5 cm below citrus seedlings in each pot at transplanting. The AM inoculum, which consisted of spores, soil, hyphae and infected jowar roots from a stock culture, were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences. The control was inoculated with a sterile growth substrate. Growth substrate in pots was an autoclaved (121 °C, 2 h) mixture of soil/vermiculite/sphagnum (5/2/1, v/v/v), with physical–chemical characteristics as follows: pH 5.9, 1.3% organic matter, 30 mg kg^{-1} available phosphorus, 147 mg kg^{-1} alkali hydrolysable nitrogen and 141 mg kg^{-1} available potassium. The soil was collected from Fruit Sample Garden, Huazhong Agricultural University (Wuhan, China). Each pot was filled with 3.371 kg of growth substrate. The experiment was conducted in a greenhouse under natural light conditions from March to September in 2004, where no temperature controlling equipment was available. The photon flux density ranged from 550 to 900 μmol m^{-2} s^{-1} during the water treatments. The average day/night temperature was 31/25 °C during the water treatments, and average relative humidity was 80%. AM and non-AM seedlings were not fertilized during the entire experiment.

Ninety-seven d after transplanting, AM and non-AM seedlings were subjected to WW and WS treatments. WW and WS pots were maintained at 75% of relative soil water content (−0.09 MPa) and 55% of relative soil water content (−0.40 MPa), respectively. Soil water content was determined gravimetrically. Each pot was weighed daily and re-supplied with sufficient water to maintain the target relative water contents. The soil water potential was measured by a pressure plate apparatus.

Growth parameters and root colonization analysis

After 80 d of water treatments, all seedlings were harvested and the growth substrate thoroughly removed from roots. Plant height, stem diameter and leaf number per plant were measured for all size plants in each pot, and shoot and root dry weights were measured for three plants in each pot. A small fraction of the root system were carefully washed in tap water, cut into 1 cm root pieces and fixed by formalin-acetic acid-alcohol solution (at least 24 h). These root samples were cleared with a 10% (w/v) KOH solution, stained with 0.05% (v/v) trypan blue in lactic acid (Phillips and Hayman, 1970), and microscopically observed for colonization by AM structures (Giovannetti and Mosse, 1980).

Analysis of malondialdehyde (MDA) and ROS

Four hundred mg of fresh leaves or roots were homogenized in 5 mL of 5% (w/v) trichloroacetic acid and centrifuged at 3000*g* for 10 min. MDA of extracts was determined by the thiobarbituric acid reaction as described by Sudhakar et al. (2001). H_2O_2 and $O_2^{\bullet -}$ assays were performed as described by Wang and Jiao (2000) and Wang and Luo (1990), respectively.

Determination of antioxidant enzymes and soluble protein

Extracts for determination of soluble protein, SOD, G-POD and CAT were prepared from half a gram of fresh leaf or root tissue homogenized in 5 mL of 0.1 M phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1 mM ASC, 1 mM 1,4-dithiothreitol and 2% (w/v) polyvinylpyrrolidone and centrifuged at 4200*g* for 10 min, with the resulting supernatant used for assays. All steps of the extraction procedure were carried out at 4 °C. SOD activity was measured using the method of Giannopolitis and Ries (1977). One unit of SOD was defined as the amount of enzyme that inhibited 50% nitro blue tetrazolium by light, and SOD activity was expressed as SOD units per mg of protein. CAT activity was measured according to the method of Aebi (1984). G-POD activity was determined using the method of Chance and Maehly (1955). Soluble protein was evaluated by the method of Bradford (1976) using bovine serum albumin as the standard.

For determination of GR activity, half a gram of fresh leaf or root tissue was homogenized with 5 mL of 0.1 M Tricine-NaOH buffer (pH 7.8) and centrifuged at 15,000*g* at 4 °C for 15 min. The supernatant was collected and diluted properly for determination of GR activity according to the method of Chen and Wang (2002). The reaction mixture (1 mL) contained 1 mM NADPH, 0.1 M Tricine-NaOH buffer (pH 7.8), 5 mM oxidized glutathione and 0.2 mL enzyme extract. Absorbance decreased 0.01 at 340 nm during 1 min mg^{-1} protein was defined as an activity unit.

For determination of APX activity, half a gram of fresh leaf or root tissue was extracted with 5 mL of ice-cold 100 mM phosphate buffer (pH 7.0) containing 1 mM ASC and 1 mM EDTA, centrifuged at 15,000*g* at 4 °C for 15 min, and determined as described by Chen and Wang (2002). The assay mixture (3 mL) contained 100 mM phosphate buffer (pH 7.0), 15 mM ASC, 0.3 mM H_2O_2 and 0.1 mL enzyme extract. Absorbance decreased 0.01 at 290 nm was defined as an activity unit during 1 min mg^{-1} protein.

ASC and GSH assays

ASC and GSH were determined by extracting half a gram of fresh leaf or root tissue with 5 mL of 5% (w/v) trichloroacetic acid and centrifuging at 15,000*g* for 15 min at 4 °C. The supernatant was used for ASC and GSH assays. ASC was determined using the method described by Chen and Wang (2002). The ASC assay mixture (5 mL) contained 1.0 mL supernatant, 100 mM phosphate buffer (pH 7.7), 10% (w/v) trichloroacetic acid, 44% (v/v) H_3PO_4, 4% (w/v) 2, 2′-bipyridyl and 3% (w/v) $FeCl_3$. The final mixture was incubated in 37 °C for 60 min and cooled to room temperature. Absorbance of the colored solution was recorded at 525 nm. A modified method of Wang and Jiao (2001) was employed for the assay of GSH. The GSH assay mixture (3.7 mL) contained 1.0 mL supernatant, 100 mM phosphate buffer (pH 7.7) and 0.60 mM 5,5′-dithio-bis (2-nitrobenzoic acid). Absorbance was recorded at 412 nm.

Experimental design and data analysis

The experiment was a completely randomized 2 × 2 factorial design, with two mycorrhizal treatments, AMF and non-AMF, and two water treatments, WW and WS. Each of the four treatments had six replicates, for a total of 24 pots.

Experimental data were statistically analyzed using a two-factor analysis of variance (ANOVA) (SAS Institute Inc., 1996). The variance was related to the main treatments (AMF and water stress) and to the interaction between them. Probabilities of significant difference were used to test the significance among treatments and interactions, and Fisher's protected least significant differences ($p = 0.05$) were used to compare the means.

Results

Colonization of seedlings by *G. versiforme* varied from 22% to 37% (Table 1). The WS treatment notably decreased root colonization, with WW seedlings having 66% higher root colonization than WS seedlings. No mycorrhizal colonization was observed in the roots of non-AM seedlings.

Water stress significantly decreased shoot dry weight, root dry weight, plant height and stem diameter (Table 1). AM inoculation markedly increased shoot dry weight, plant height and stem diameter of trifoliate orange seedlings under both WW and WS conditions. Leaf numbers per plant and root dry weight were higher in AM than in non-AM seedlings under WW conditions.

Water stress increased the MDA concentration of roots (Table 2). Compared with that of non-AM seedlings, the MDA concentration of AM leaves decreased by 33% and 18% and the MDA concentration of AM roots by 14% and 10% under WW and WS conditions, respectively.

AM roots had less H_2O_2 level than non-AM roots in both water treatments (Table 2). H_2O_2 concentration was 16% lower in AM than in non-AM leaves under WS conditions. AM inoculation did not affect the H_2O_2 concentration of leaves under WW conditions.

The $O_2^{\bullet-}$ concentration of roots was notably increased by water stress (Table 2). AM and

Table 1. Root and shoot characteristics of AM and non-AM *Poncirus trifoliata* seedlings subjected to water stress or kept well watered

Water status	AMF status	Root colonization (%)	Dry weight ($g\,plant^{-1}$)		Plant height (cm)	Leaf number per plant	Stem diameter (cm)
			Shoot	Root			
WW	AMF	36.98 ± 3.06a	1.16 ± 0.13a	0.49 ± 0.05a	50.97 ± 2.75a	42.5 ± 3.3a	0.43 ± 0.03a
	Non-AMF	0.00 ± 0.00c	1.00 ± 0.10b	0.42 ± 0.04b	40.22 ± 3.33b	38.7 ± 2.3b	0.36 ± 0.03b
WS	AMF	22.32 ± 8.11b	0.89 ± 0.10b	0.35 ± 0.04c	40.32 ± 4.10b	37.8 ± 3.5b	0.37 ± 0.05b
	Non-AMF	0.00 ± 0.00c	0.71 ± 0.07c	0.30 ± 0.06c	33.93 ± 3.89c	36.0 ± 3.2b	0.31 ± 0.03c
Significance							
WS		*	**	**	**	**	**
AMF		**	**	**	**	*	**
WS × AMF		*	NS	NS	NS	NS	NS

Note: Mean ± SD followed by the same letter within a column shows non-significant difference ($LSD_{0.05}$). $n = 3$ for root colonization, $n = 6$ for all other variables. Data are analyzed with ANOVA. NS, not significant; WW, well-watered; WS, water stressed. * $p < 0.05$. ** $p < 0.01$.

Table 2. Concentrations of reactive oxygen species in leaves and roots of AM and non-AM *Poncirus trifoliata* seedlings subjected to water stress or kept well watered

Water status	AMF status	MDA (μmol g^{-1} fwt.)		H_2O_2 (μmol g^{-1} fwt.)		$O_2^{\bullet -}$ (μmol g^{-1} fwt.)	
		Leaf	Root	Leaf	Root	Leaf	Root
WW	AMF	13.12±1.30c	9.88±0.80d	100.45±10.78b	18.19±1.65c	0.92±0.14c	0.48±0.09d
	Non-AMF	19.49±0.69ab	11.55±0.36c	105.94±10.60b	21.61±1.32b	0.99±0.11c	0.68±0.08c
WS	AMF	16.78±1.68b	14.44±1.21b	117.40±8.40b	21.90±0.37b	1.33±0.09b	1.19±0.08b
	Non-AMF	20.49±2.29a	16.07±0.48a	139.02±6.62a	25.43±1.54a	1.70±0.05a	1.60±0.13a
Significance							
WS		*	**	**	**	**	**
AMF		**	**	*	**	**	**
WS × AMF		NS	NS	NS	NS	*	NS

Note: Mean±SD ($n = 3$) followed by the same letter within a column shows non-significant difference ($LSD_{0.05}$). Data are analyzed with ANOVA. MDA, malondialdehyde; H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide anion radical; NS, not significant; WW, well-watered; WS, water stressed. *$p<0.05$. **$p<0.01$.

non-AM leaves had similar concentration of $O_2^{\bullet -}$ under WW conditions, while under WS conditions the concentration of $O_2^{\bullet -}$ decreased by 22% in AM leaves. The $O_2^{\bullet -}$ concentration of AM roots was 30% and 26% less than that of non-AM roots under WW and WS conditions, respectively.

The SOD activity in leaves of AM and non-AM seedlings was also increased by water stress (Table 3). AM inoculation did not SOD activity of leaves in both water treatments. AM and non-AM roots had similar SOD activity under WW conditions, while AM roots had a lower SOD activity than non-AM ones under WS conditions.

The G-POD activity of leaves and roots was significantly enhanced by water stress (Table 3). In leaves, AM inoculation increased the G-POD activity of seedlings whether WS or not. Compared with that of non-AM leaves, the G-POD activity of AM leaves increased by 28% and 29% under WW and WS conditions, respectively. The G-POD activity of roots was similar in AM and non-AM seedlings.

WS caused a significant increase in the CAT activity of leaves or roots (Table 3). In leaves, AM colonization only increased CAT activity in WS seedlings. CAT activity was similar in AM and non-AM roots.

The APX activity of AM leaves, non-AM leaves and AM roots was increased by water stress (Table 3). The APX activity of WW leaves was similar in AM and non-AM seedlings, but under WS conditions the APX activity of leaves increased by 88% in AM seedlings compared with non-AM seedlings. AM roots had higher APX activity than non-AM roots regardless of water treatment: 192% higher in WW seedlings and 226% higher in WS seedlings.

The GR activity of AM leaves was markedly increased by water stress (Table 3). The GR activity of leaves was 46% and 83% higher in AM than in non-AM seedlings under WW and WS conditions, respectively. In contrast, no significant differences among treatments were found in the GR activity of roots.

Water stress markedly decreased the soluble protein content of leaves and roots, GSH content of leaves and roots, and ASC content of leaves (Table 4). The soluble protein content of WW leaves in AM seedlings was 4% higher than in corresponding non-AM seedlings, but AM inoculation did not affect the soluble protein content of WS leaves. The soluble protein content of roots was 13% and 22% higher in AM than in non-AM seedlings under WW and WS conditions, respectively. In spite of non-significant differences in the ASC content of roots between non-AM and AM seedlings under WW conditions, AM inoculation increased by 34% the ASC content of roots under WS conditions. AM seedlings had 11% and 51% higher the ASC content of leaves than non-AM seedlings under WW and WS conditions, respectively. The GSH content of leaves in AM seedlings increased by 67% and 101% and the GSH content of roots by 36% and 81% under WW and WS conditions when compared with that in non-AM seedlings, respectively.

Summarizing all the observations (Table 5), the main results of reactive oxygen metabolism from the experiment are as follows: (1) For ROS, AM inoculation markedly decreased the concentrations of MDA, H_2O_2 and $O_2^{\bullet -}$ in WW roots, WS leaves and WS roots. (2) The effects of AM symbiosis on five antioxidant enzymes were dissimilar in leaves and roots under WW and WS conditions. (3) For three non-enzymatic antioxidants, AM seedlings showed notably higher soluble protein, ASC and GSH in WW leaves and WS roots.

Table 3. Superoxide dismutase (SOD), guaiacol peroxidase (G-POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities of leaves and roots in AM and non-AM *Poncirus trifoliata* seedlings subjected to water stress or kept well watered

Water status	AMF status	SOD ($U\,mg^{-1}$ protein)		G-POD ($U\,mg^{-1}$ protein)		CAT ($U\,mg^{-1}$ protein)		APX ($U\,mg^{-1}$ protein)		GR ($U\,mg^{-1}$ protein)	
		Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
WW	AMF	10.41±0.63b	17.01±2.28c	3.90±0.27c	2.97±0.39b	7.87±0.78c	12.87±2.10bc	5.99±1.37c	11.61±0.56b	12.84±1.70b	30.16±9.85a
	Non-AMF	9.80±0.85b	15.59±1.19c	3.04±0.23d	2.48±0.14b	6.46±0.54c	11.02±1.32c	6.59±0.50c	3.98±0.89c	8.81±2.12c	20.20±5.38a
WS	AMF	20.88±0.43a	33.45±3.04b	5.90±0.28a	5.86±0.54a	20.05±0.57a	22.64±3.96a	21.14±0.88a	18.35±3.84a	17.41±2.74a	13.24±5.86a
	Non-AMF	19.87±0.33a	40.12±4.39a	4.57±0.12b	5.96±0.52a	17.50±1.64b	18.16±3.19ab	11.23±0.14b	5.63±1.35c	9.52±1.10bc	22.06±5.23a
Significance											
WS		**	**	**	**	**	**	**	**	*	NS
AMF		*	NS	**	NS	**	NS	**	**	**	NS
WS × AMF		NS	*	NS	NS	NS	NS	**	NS	NS	*

Note: Mean±SD ($n = 3$) followed by the same letter within a column shows non-significant difference ($LSD_{0.05}$). Data are analyzed with ANOVA. NS, not significant; WW, well-watered; WS, water stressed. * $p<0.05$. ** $p<0.01$.

Discussion

AM symbiosis increased growth of trifoliate orange seedlings in both the presence and absence of water stress, confirming earlier findings (Graham and Timmer, 1985; Shrestha et al., 1995; Fidelibus et al., 2001; Wu and Xia, 2004; Wu et al., 2005). AM effect on host plant growth during water stress has often been related to improved phosphorus nutrition (Nelsen and Safir, 1982; Johnson and Hummel, 1985), which though not measured, was probably the case with our plants, as well.

In higher plants, ROS are continuously produced in chloroplasts, mitochondria and peroxisomes (Apel and Hirt, 2004). Production and removal of ROS are strictly controlled under amply watered conditions. When higher plants are subjected to water stress, the equilibrium between production and scavenging of ROS is broken, resulting in oxidative damage to proteins, DNA and lipids. MDA is one of the byproducts and could reflect the degree of the peroxidation of membrane lipid (Lacan and Baccou, 1998). In the present study, water stress increased MDA in leaves and roots, with increases related to the production H_2O_2 and $O_2^{\bullet -}$. H_2O_2 and $O_2^{\bullet -}$ were higher in WS than in WW seedlings, causing the membrane lipid peroxidation. AM inoculation notably decreased H_2O_2 and $O_2^{\bullet -}$ in roots and WS leaves, indicating a lower accumulation of ROS in AM seedlings. Salzer et al. (1999) used the diaminobenzidine staining technique to examine the accumulation of H_2O_2 in the roots of *Medicago truncatula* colonized by *G. intraradices*, showing that H_2O_2 accumulated in clumped and less branched arbuscules. Moreover, H_2O_2 accumulated around hyphal tips penetrating a host cell. However, H_2O_2 accumulation was not observed in hyphal tips, appressoria and vesicles. Subsequently, Fester and Hause (2005) observed that the intracellular accumulation of H_2O_2 was found in the cytoplasm of *Zea mays* colonized by *G. intraradices* close to intact and collapsing fungal structures, whereas intercellular H_2O_2 was located on the surface of fungal hyphae. This suggested that in the root cells of AM roots, locally induced accumulations of H_2O_2 were limited both in the intracellular AM hyphae and at the intercellular hyphal surface.

Water stress elicits other biochemical responses in higher plants that minimize its deleterious effects. One important component of protective systems is enzymatic defense. SOD catalyses the dismutation of $O_2^{\bullet -}$ to H_2O_2, CAT dismutates H_2O_2 to oxygen and water, and APX reduces H_2O_2 to water by ASC as specific electron donor (Gara et al.,

Table 4. Soluble protein, ascorbate (ASC) and glutathione (GSH) contents of leaves and roots in AM and non-AM *Poncirus trifoliata* seedlings subjected to water stress or kept well watered

Water status	AMF status	Soluble protein (mg g^{-1} fwt.)		ASC (μmol g^{-1} fwt.)		GSH (μmol g^{-1} fwt.)	
		Leaf	Root	Leaf	Root	Leaf	Root
WW	AMF	63.53±1.68a	34.10±1.45a	10.35±0.22a	2.57±0.22a	3.85±0.12a	0.75±0.04a
	Non-AMF	61.23±1.14b	30.29±0.82b	9.32±0.10b	2.37±0.13a	2.30±0.09c	0.55±0.06b
WS	AMF	45.70±0.75c	30.59±1.25b	9.15±0.59b	2.31±0.31a	2.87±0.37b	0.58±0.07b
	Non-AMF	45.25±0.20c	25.13±1.95c	6.04±0.23c	1.73±0.27b	1.43±0.08d	0.32±0.01c
Significance							
WS		**	**	**	*	**	**
AMF		NS	**	**	*	**	**
WS × AMF		NS	NS	*	NS	NS	NS

Note: Mean±SD ($n = 3$) followed by the same letter within a column shows non-significantly different ($LSD_{0.05}$). Data are analyzed with ANOVA. NS, not significant; WW, well-watered; WS, water stressed. *$p<0.05$. ** $p<0.01$.

Table 5. Effects of the AM fungus, *Glomus versiforme*, on malondialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$), superoxide dismutase (SOD), guaiacol peroxidase (G-POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), soluble protein, ascorbate (ASC) and glutathione (GSH) in *Poncirus trifoliata* seedlings under well-watered (WW) and water stressed (WS) conditions

Parameter	WW				WS			
	Leaf	*Significance*	Root	*Significance*	Leaf	*Significance*	Root	*Significance*
MDA	<	*	<	*	<	*	<	*
H_2O_2	<	NS	<	*	<	*	<	*
$O_2^{\bullet-}$	<	NS	<	*	<	*	<	*
SOD	>	NS	>	NS	>	NS	<	*
G-POD	>	*	>	NS	>	*	<	NS
CAT	>	NS	>	NS	>	*	>	NS
APX	<	NS	>	*	>	*	>	*
GR	>	*	>	NS	>	*	<	NS
Soluble protein	>	*	>	*	>	NS	>	*
ASC	>	*	>	NS	>	*	>	*
GSH	>	*	>	*	>	*	>	*

Note: < or > means that AM symbiosis decreases or increases the parameter, respectively. * or NS means the significant or non-significant differences ($p<0.05$) in the parameter, respectively.

2003). In our citrus seedlings, G-POD activities of leaves, CAT activity of WS leaves, APX activities of WS leaves and roots, and GR activity of leaves were higher in AM than in non-AM seedlings (Table 3). This agrees with previous reports obtained from the shoots of three shrub species inoculated with *G. claroideum* in a degraded semi-arid soil (Alguacil et al., 2003), from the shoots of *Glycine max* colonized by *G. etunicatum* subjected to NaCl salinity (Ghorbanli et al., 2004), and from the leaves of trifoliate orange seedlings colonized by *G. mosseae* during drought (Wu et al., 2005). The higher G-POD, CAT, APX and GR activities in AM seedling would partly explain the lower H_2O_2 concentration in AM seedlings, protecting the organism against oxidative damage, in turn enhancing drought tolerance.

AM inoculation did not affect the SOD activity of our citrus leaves (Table 3). In roots, AM infection markedly decreased SOD activity, but only in WS plants. Porcel and Ruiz-Lozano (2004), who had similar findings, showed that AM colonization significantly decreased the SOD activity of shoots in WW soybean. However, SOD activity was higher in shoots and roots of *Lactuca sativa* colonized by *G. mosseae* than in non-AM *Lactuca sativa* under WS conditions (Ruiz-Lozano et al., 1996). Moreover, three SOD isozymes, including Mn-SODI, Cu, Zn-SODI (M, 31000) and Cu, Zn-SODII (M, 34300) were found in leaves and roots of *Trifolium pratense*, while a Cu, Zn-SOD (M, 40500) was found in the spores of *G. mosseae* (Palma et al., 1993). Two new isozmyes, Mn-SODII (M, 37800) and mycCu, Zn-SOD (M, 33300) were observed in *G. mosseae*-colonized roots in that study. This suggests that AMF may induce some new SOD isozymes in mycorrhizal roots. Additionally, AM symbiosis increased the expression of the Mn-SODII gene in WS lettuce plants, and this correlated well with plant tolerance to water stress (Ruiz-Lozano et al., 2001). The response of AM colonization to SOD warrants more study.

AM infection increased APX activity of citrus roots and GR activity of citrus leaves under both amply watered and stressed conditions (Table 3). In a prior work, *G. intraradice*-colonized soybean plants had lower APX activity in roots and shoots, and lower GR activity in shoots, than non-AM plants before and during drought (Porcel and Ruiz-Lozano, 2004).The differences in AM influence on soybean were perhaps due to different AMF, experimental conditions, plant health and phenology than ours, resulting in the dissimilar behaviors of several antioxidant enzymes. Moreover, in the presence of rhizobium, *G. mosseae*-inoculation notably decreased GR activity of roots in WW and WS soybean plants and of nodules in WW soybean (Porcel et al., 2003), suggesting that rhizobium might have affected AMF-induced changes in antioxidant enzymes of plants.

Non-enzymatic antioxidants include the major cellular redox buffers, ASC and GSH, which are the crucial antioxidants in ASC-GSH cycle (Apel and Hirt, 2004). This cycle, occurring in higher plants, serves the removal of ROS and is an important antioxidant defense. Up to 30% of the photosynthetically produced electrons are dissipated by the ASC-GSH cycle under WS condition and 2–5% under normal conditions (Polle, 2001). In our work, *G. versiforme*-inoculation markedly enhanced the ASC and GSH contents of leaves under WW and WS conditions, GSH content of roots under WW and WS conditions, and ASC content of WS roots (Table 4). The higher APX activity and ASC content in AM plants would result in faster removal of H_2O_2 through the ASC–GSH cycle, helping to alleviate oxidative damage. The greater GSH content of AM plants was related to GR activity, because the ASC–GSH cycle closed with GR converting glutathione disulphide back into GSH. In addition, GSH occurs in the glutathione peroxidase cycle, converting H_2O_2 into water using reducing equivalents from GSH. Therefore, the higher ASC and GSH contents of leaves and roots in AM seedlings would help the host plant in dissipating the photosynthetically produced electrons and in alleviating oxidative damage.

Plants that are resistant to one stress are often more resistant to other stresses, a phenomenon known as cross-tolerance (Bowler and Fluhr, 2000). Water stress can cause an oxidative stress in higher plants. In our work, mycorrhizal inoculation changed reactive oxygen metabolism (ROS, antioxidant enzymes, non-enzymatic antioxidants, etc.) in trifoliate orange seedlings, at least partly alleviating oxidative stress under WS conditions. ROS are central components controlling cross-tolerance, at least at the cellular level. Therefore, AM colonization might influence the cross-tolerance of host plants. We observed greater osmotic adjustment in mycorrhizal trifoliate orange seedlings during drought stress in a previous work, suggesting that AM symbiosis might also alleviate osmotic stress under WS conditions (Wu and Xia, 2004). It appears likely that cross-tolerance (e.g. alleviate osmotic stress and oxidative stress) might occur in AM plants and be enhanced by AM symbiosis. However, further experiments will be necessary to elucidate how and when AMF confer cross-tolerance to their hosts under WS conditions.

In conclusion, *G. versiforme*-inoculation had a positive effect on reactive oxygen metabolism of trifoliate orange seedlings, decreasing the concentrations of ROS in leaves and roots and increasing both antioxidant enzymes and non-enzymatic antioxidants. The lower oxidative damage in AM plants appeared due to higher antioxidant enzymes and non-enzymatic antioxidants.

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