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RESEARCH ARTICLE

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Effect of *Glomus versiforme* inoculation on reactive oxygen metabolism of *Citrus tangerine* leaves exposed to water stress

Abstract In a potted greenhouse experiment, *Citrus tangerine* Hort. ex Tanaka was inoculated with arbuscular mycorrhizal (AM) fungus, *Glomus versiforme* (Karsten) Berch, or non-AM fungus as control. Arbuscular mycorrhizal and non-AM seedlings were grown under well-watered or water-stressed conditions after 97 days of acclimation. The reactive oxygen metabolism of *C. tangerine* leaves was studied in order to elucidate whether AM symbiosis affects enzymatic and non-enzymatic antioxidants. The results showed that water stress caused a decrement of 33% for the colonization of *G. versiforme* on *C. tangerine* roots. Under well-watered and water-stressed conditions, *G. versiforme* inoculation increased the leaf phosphorus (P) content by 45% and 27%, and decreased the leaf malondialdehyde and hydrogen peroxide contents by 25% and 21%, and 16% and 16%, respectively, compared with the control. Inoculation with *G. versiforme* enhanced the activities of leaf superoxide dismutase, peroxidase, catalase and ascorbate peroxidase, and increased the contents of leaf soluble protein, ascorbate and total ascorbate notably, regardless of soil moisture conditions. Under water-stressed conditions, *G. versiforme* inoculation decreased the leaf superoxide anion radical ($O_2^{\cdot-}$) content by 31%. It is concluded that drought resistance of *C. tangerine* leaves is enhanced due to the improvement of reactive oxygen metabolism after *G. versiforme* inoculation.

Keywords arbuscular mycorrhizal (AM) fungi, *Citrus tangerine*, water stress, reactive oxygen species

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1 Introduction

Under well-watered (W) conditions, the production and elimination of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals ($\cdot OH$) are kept in dynamic balance, thus maintaining the normal physiological function of higher plants. Once higher plants are subjected to water stress, the balance is broken, and then ROS are induced, resulting in activation of electron transfer reactions, which in turn results in lipid peroxidation, chlorophyll bleaching and protein oxidation (Noctor, 1998). As a consequence, higher plants evolve cellular adaptive responses like up-regulation of oxidative stress protectors and accumulation of protective solutes. Changes in enzymatic and non-enzymatic antioxidative systems may be used to alleviate the damage.

Arbuscular mycorrhizal fungi (AMF) are age-old and ubiquitous microbes in field soils. The arbuscular mycorrhizal (AM) symbiosis is probably the most important interaction between plants and microbes. More than 90% of vascular bundle plants form AM structure (Gadkar et al., 2001). It was well documented that AM colonization enhanced plant drought resistance through a set of mechanisms: an intensive absorption of phosphorus and water by external hyphae (Nelsen and Safir, 1982; Faber et al., 1991; Ruiz-Lozano and Azcón, 1995), the enlargement of active absorptive area of roots (Wu and Xia, 2004a), the improvement of osmotic adjustment (Wu and Xia, 2006), the changes of hydraulic conductivity of roots or plants (Levy and Krikun, 1980; Graham and Syvertsen, 1984), the regulation of stomatal conductance in response to hormonal signals (Duan et al., 1996), a low water conducted resistance channel (Wu and Xia, 2004b), and regulation of some resistance genes (Porcel et al., 2004; 2005; 2006). In addition, some reports on lettuce (Ruiz-Lozano et al., 1996) and soybean (Lambais et al., 2003; Porcel and Ruiz-Lozano, 2004) showed that the enhancement of plant drought resistance by AM symbiosis might be contacted with enzymatic antioxidative systems. However, these researches mainly focused on several enzymatic antioxidants

and lacked in the analysis of non-enzymatic antioxidants. At present, there are many experiments to understand reactive oxygen metabolism of fruit trees under water-stressed (S) conditions (Chen and Liu, 1998; Luo et al., 1999; Ramachandra et al., 2004), while the information about citrus is very deficient (Wu and Xia, 2005; Wu et al., 2006a).

The purpose of the present work was to evaluate the effect of AMF on reactive oxygen metabolism (enzymatic and non-enzymatic antioxidants) in leaves of *Citrus tangerin*e (a major citrus rootstock used in China) under W and S conditions.

2 Materials and methods

2.1 Plant materials

Air-dry seeds of tangerine (*C. tangerine* Hort. ex Tanaka) were sterilized with 70% alcohol for 5 min, rinsed four times with distilled water, and germinated on wet filter paper in Petri dishes at 28°C. The seven-day-old seedlings were transferred into plastic pots containing 3.371 kg of an autoclaved medium (0.11 MPa, 121°C, 2 h) of yellow soil, vermiculite and sphagnum (5 : 2 : 1, v/v/v), whose characteristics were pH 5.9, 1.3% organic matter, 29.97 mg/kg available phosphorus, 147.47 mg/kg alkali hydrolyzable nitrogen, and 140.89 mg/kg available potassium. Experimental pots were placed in a greenhouse without temperature regulating equipment in Wuhan.

2.2 Mycorrhizal inocula

The mycorrhizal inocula including spores, soils, hyphae and infected jowar root fragments from a stock culture of *Glomus versiforme* (Karsten) Berch, were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences.

2.3 Experimental design

The experimental treatments consisted of two soil water regimes (W and S) and two mycorrhizal inoculations (*G. versiforme* and non-mycorrhizae) and arranged in a completely randomized block design (four seedlings per pot) with eight replicates for each treatment to give a total of 32 pots.

The mycorrhizal inocula were placed 5 cm below roots of transplants. Inoculated dosage was 30 g of inocula per pot containing approximately 2 233 spores. Non-mycorrhizal treatments received 30 g of autoclaved medium per pot. According to the method of Hsiao (1973), water treatments began in the presence or absence of *G. versiforme* after 97 days of acclimating seedlings in greenhouse conditions, when W pots were controlled at the range of 75%−80% of maximum field moisture capacity and 55%−60% for S treatment. Seedlings were harvested 80 days after water treatments.

2.4 Parameter measurements

Root fragments for the determination of AM colonization were cleaned with 10% KOH and stained with 0.05% Trypan Blue in lactophenol as described by Phillips and Hayman (1970). The AM colonization was calculated by the following formula: AM colonization (%) = 100 × root length infected / root length observed.

Phosphorus was analyzed by vanadate-molybdate-yellow method (John, 1970). The malondialdehyde (MDA) content, the soluble protein content and the activities of superoxide dismutase (SOD) and peroxidase (POD) were measured using the methods of Li (2000). The assays for $O_2^{\cdot-}$ and H_2O_2 were performed as described by Wang and Luo (1990) and Lin et al. (1988), respectively. The activities of catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were assayed according to the methods of Chen and Wang (2002).

Non-enzymatic antioxidants were determined by extracting the leaf sample with 5 mL 5% TCA and centrifuging at 15 000 g at 4°C for 15 min. The supernatant was collected for determination of ascorbate (ASC), total ascorbate (TASC), glutathione (GSH) and total glutathione (TGSH) using the previously described methods by Wu et al. (2006b). Dehydroascorbate (DHA) was calculated by the following formula: DHA = TASC − ASC. Oxidized glutathione (GSSG) was determined by subtraction of GSH from TGSH.

2.5 Statistical analysis

Experimental data were assessed by the analysis of variance (ANOVA) using the Statistical Analysis System (SAS) software. Fisher's protected least significant difference (LSD) ($P < 0.05$) was used to compare means. General linear model (GLM) procedure was used to test the significance of interactions, and correlation (CORR) procedure was used to analyze the correlation among the variables.

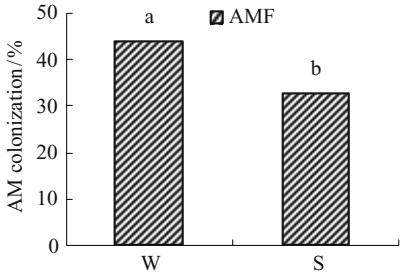
3 Results

3.1 AM colonization

No AM colonization was observed in the roots of non-AM seedlings. *G. versiforme* successfully infected the tangerine roots (Fig. 1). Water stress decreased the colonization of *G. versiforme* at tangerine roots significantly, with a decrement of 33%.

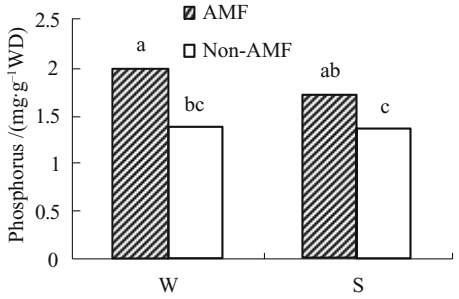
3.2 Effect of AMF on the P content of *C. tangerine* leaves under W and S conditions

Water stress reduced the P content of leaves in both W and S seedlings (Fig. 2). The P status of leaves showed that P uptake was stimulated by AM inoculation, independent of soil water status. Arbuscular mycorrhizal inoculation increased P



Note: W stands for well-watered, and S for water-stressed; different small letters mean significant differences within the treatments at 0.05 level by LSD.

Fig. 1 Effect of water stress on AMF colonization of *C. tangerine*



Note: W stands for well-watered, and S for water-stressed; different small letters mean significant differences within the treatments at 0.05 level by LSD.

Fig. 2 Effects of AMF inoculation on phosphorus content of *C. tangerine* leaves under W and S conditions

content in W seedlings by 45% and by 27% in S seedling. There was no significant difference for P content of leaves between AM S seedlings and non-AM W seedlings. No significant interaction between water stress and AMF was found for P content in leaves.

3.3 Effects of AMF on MDA, H_2O_2 and $O_2^{\cdot-}$ contents in *C. tangerine* leaves under W and S conditions

Water stress induced the accumulation of MDA, H_2O_2 and $O_2^{\cdot-}$ in both W and S seedlings (Table 1). Under W conditions, *G. versiforme* colonization notably reduced the MDA and H_2O_2 contents of leaves by 25% and 16%, respectively. Under S conditions, *G. versiforme* colonization notably reduced the MDA, H_2O_2 and $O_2^{\cdot-}$ contents in leaves by 21%, 16% and 31%, respectively. A significant water stress × AMF interaction was noted for $O_2^{\cdot-}$ content in leaves ($P < 0.05$).

3.4 Effect of AMF on the enzymatic antioxidant activities in *C. tangerine* leaves under W and S conditions

Water stress notably increased the SOD, CAT, APX and GR activities in the leaves of AM seedlings compared with W conditions (Table 2). The SOD, POD and APX activities significantly increased in the leaves of non-AM seedlings as a consequence of water stress. Under W conditions, *G. versiforme* infection markedly increased the SOD, POD, CAT and APX activities of leaves by 44%, 81%, 30% and 113%, respectively. Under S conditions, *G. versiforme* colonization greatly increased the SOD, POD, CAT, APX and GR activities of leaves by 12%, 24%, 28%, 48% and 83%,

Table 1 Effects of AMF inoculation on MDA, H_2O_2 and $O_2^{\cdot-}$ contents in *C. tangerine* leaves under W and S conditions

Item	Inoculation	MDA /(μmol·g^{-1} FW)	H_2O_2 /(μmol·g^{-1} FW)	$O_2^{\cdot-}$ /(μmol·g^{-1} FW)
W	AMF	7.59c	48.50c	0.539b
	Non-AMF	10.25b	57.25b	0.594b
S	AMF	10.58b	58.43b	0.567b
	Non-AMF	13.46a	69.42a	0.825a
Significance	S	**	**	**
	AMF	**	**	**
	S × AMF	ns	ns	*

Note: Different small letters mean significant differences within the same column at 0.05 level by LSD; W, S and AMF stand for well-watered, watere-stressed and arbuscular mycorrhiza fungi, respectively; * stands for $P < 0.05$, ** for $P < 0.01$, and ns for no significant difference.

Table 2 Effects of AMF inoculation on the activities of SOD, POD, CAT, APX and GR in *C. tangerine* leaves under W and S conditions

Item	Inoculation	SOD/ (U·g^{-1} FW)	POD/ (U·g^{-1} FW)	CAT/(U·g^{-1} FW)	APX/(U·g^{-1} FW)	GR/(U·g^{-1} FW)
W	AMF	596.42b	281.94ab	895.83b	796.88b	534.72b
	Non-AMF	424.55c	155.56c	687.50c	375.00c	453.93b
S	AMF	705.22a	301.39a	1062.50a	1223.96a	1235.51a
	Non-AMF	627.17b	243.06b	833.33bc	828.13b	673.61b
Significance	S	**	*	*	**	**
	AMF	**	**	**	**	*
	S × AMF	ns	*	ns	ns	*

Note: Different small letters mean significant differences within the same column at 0.05 level by LSD; W, S and AMF stand for well-watered, water-stressed and arbuscular mycorrhiza fungi, respectively; *, ** and ns stand for $P < 0.05$, $P < 0.01$ and no significant difference, respectively.

respectively. We also found a significant water stress × AMF interaction for POD or GR activities in leaves ($P < 0.05$).

3.5 Effect of AMF on the non-enzymatic antioxidant contents in *C. tangerine* leaves under W and S conditions

For AM seedlings, water stress reduced the contents of ASC, TASC, soluble protein, GSH, GSSG and TGSH in leaves (Table 3). However, for non-AM seedlings, water stress caused a significant decrease in the ASC, TASC, soluble protein and GSSG contents in leaves.

Under W conditions, *G. versiforme* colonization notably increased the ASC, DHA and TASC contents in leaves by 14%, 59% and 21%, respectively, compared with non-AM treatment (Table 3). Under S conditions, *G. versiforme* inoculation also greatly promoted the ASC and TASC contents in leaves by 37% and 33%. Arbuscular mycorrhizal symbiosis did not affect the ratio of ASC : DHA under S conditions but reduced it under W conditions.

Arbuscular mycorrhizal leaves had 11% higher soluble protein content in both W and S seedlings than non-AM leaves regardless of water treatment (Table 3).

The GSH and TGSH contents in the S leaves were similar to that in AM and non-AM seedlings, but the GSH and TGSH contents in the W leaves increased by 41% and by 37% in AM seedlings compared with non-AM seedlings (Table 3). Arbuscular mycorrhizal treatment did not affect the GSSG content and the ratio of GSH : GSSG in the leaves regardless of soil water status. A significant ($P < 0.05$) water stress × AMF interaction was noted for GSH or TGSH contents in leaves.

4 Discussion

In the present work, *G. versiforme* inoculation affected the enzymatic antioxidant activities in *C. tangerine* leaves under W and S conditions. Arbuscular mycorrhizal symbiosis markedly increased the SOD, CAT and APX activities in the leaves under the above water conditions, and AM inoculation only increased the GR activities in leaves exposed to water stress. This agrees with previous reports obtained from the shoots of three shrub species inoculated with *G. claroideum* in a degrade semi-arid soils (Alguacil et al., 2003), from the shoots and roots of *Lactuca sativa* colonized by *G. mosseae* or *G. deserticola* grown under W or S conditions (Ruiz-Lozano et al., 1996), from the shoots and roots of *Glycine max* inoculated with *G. intraradices* subjected to W or S (Porcel and Ruiz-Lozano, 2004), and from the roots of *Phaseolus vulgaris* inoculated with *G. clarum* or *G. intraradices* under low and high P concentration conditions (Lambais et al., 2003). In addition, our study further confirmed that the activity of POD and the contents of ASC, TASC and soluble protein in *C. tangerine* leaves were higher in AM seedlings than in non-AM seedlings exposed to W or S. Similarly, the contents of GSH and TGSH in leaves were increased by AM colonization under W conditions. A positive correlativity occurs between AM colonization and content of GSH ($r = 0.858\,8$, $P < 0.05$), and a negative correlativity exists between AM colonization and activity of SOD ($r = -0.857\,5$, $P < 0.05$). Thus, the increment of the antioxidant enzyme activity of *C. tangerine* and the contents of ASC and GSH by AM inoculation made AM *C. tangerine* in favorable place enhance drought resistance. There were similar contents of MDA, H_2O_2 and $O_2^{\cdot-}$ between AM *C. tangerine* leaves and non-AM leaves under S conditions. This reasonably explained why AM symbiosis improved water relations of plants, which alleviates the negative effects by water stress.

As expected, AM seedlings kept the higher concentration of P whether water stressed or not. The result was in accordance with the finding by Nelsen and Safir (1982), who reported that the higher growth of the stressed, AM onion plants (*Allium cepa*) could be attributed to improved P nutrition when compared with that of the stressed, non-AM plants, despite the presence of lower levels of soil P available to the AM plants. Owing to the direct absorption of P by external hyphae, the increment of P concentration might regulate indirectly reactive oxygen metabolism of AM plants, thus enhancing plant drought resistance. Therefore, the improved reactive oxygen metabolism of AM plants might be indirectly ascribed to AM colonization.

In the present study, AM colonization markedly reduced the content of H_2O_2 in *C. tangerine* leaves regardless of soil

Table 3 Effects of AMF inoculation on the contents of ascorbate, soluble protein and glutathione in *C. tangerine* leaves under W and S conditions

Item	Inoculation	ASC /(μmol·g^{-1} FW)	DHA /(μmol·g^{-1} FW)	TASC /(μmol·g^{-1} FW)	ASC: DHA	Soluble protein /(mg·g^{-1} FW)	GSH /(μmol·g^{-1} FW)	GSSG /(μmol·g^{-1} FW)	TGSH /(μmol·g^{-1} FW)	GSH: GSSG
W	AMF	5.53a	1.46a	6.99a	3.93b	44.31a	3.29a	0.55a	3.83a	6.07a
	Non-AMF	4.85b	0.92b	5.78b	5.39a	39.79b	2.33b	0.51a	2.85b	4.57a
S	AMF	4.50b	1.76a	6.26b	2.62bc	39.90b	2.47b	0.39b	2.86b	6.47a
	Non-AMF	3.29c	1.43a	4.72c	2.31c	35.69c	2.36b	0.39b	2.75b	5.90a
Significance	S	**	*	**	**	**	*	**	**	ns
	AMF	**	*	**	ns	**	**	ns	**	ns
	S × AMF	ns	ns	ns	ns	ns	*	ns	*	ns

Note: Different small letters mean significant difference within the same column at 0.05 level by LSD; W, S and AMF stand for well-watered, water-stressed and arbuscular mycorrhiza fungi, respectively. *, ** and ns stand for $P < 0.05$, $P < 0.01$ and no significant difference, respectively.

water status. H_2O_2 is moderately reactive and long-lived in a molecule so that it can diffuse to some distances from its production site. Thus, H_2O_2 is regarded as a diffusible signal for the induction protection system (Levine et al., 1994). A diaminobenizidine staining technique showed that, H_2O_2 accumulated in clumped and less branched arbuscules but was not observed in hyphal tips, appressoria and vesicles (Salzer et al., 1999). The accumulation of H_2O_2 by arbuscules reduced the production of ROS in order to alleviate ROS damage.

Superoxide dismutase can catalyse the dismutation of $O_2^{\cdot-}$ to H_2O_2. *G*. *versiforme* colonization may increase the activity of SOD in *C*. *tangerine* leaves. When *Trappe-Trifolium pratense* and *G*. *mosseae* were associated, AM roots showed two new isozymes: Mn-SOD II and mycCu, Zn-SOD, which have relative molecular masses of 37,800 and 33,300, respectively (Palma et al., 1993). Mn-SODII considerably increased its level of transcript accumulation in mycorrhizal *Lactuca sativa* under S conditions (over 50% in *G*. *mosseae*-colonized plants and over 138% in *G*. *intraradices*-colonized plants relative to non-AM ones) (Ruiz-Lozano et al., 2001). Arbuscular mycorrhizal symbiosis down-regulated the expression pattern of SOD genes under W conditions. The cDNAs that encoded SODs could bring different regulation to AM symbiosis. It suggests that the effect of AMF on SOD in plants may be related to new SOD isozymes due to AM inoculation or to the regulation of cDNAs that encoded SODs. Further studies on the molecular level of SOD in plant-AMF association are under way.

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