Alleviation of salt stress in citrus seedlings inoculated with mycorrhiza: changes in leaf antioxidant defense systems

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ABSTRACT

Citrus is a salt-sensitive plant. In the present study, the salt stress ameliorating the effect of arbuscular mycorrhizal fungi through antioxidant defense systems was reported. Three-month-old trifoliate orange (*Poncirus trifoliata*) seedlings colonized by *Glomus mosseae* or *G. versiforme* were irrigated with 0 and 100 mmol NaCl solutions. After 49 days of salinity, mycorrhizal structures were obviously restrained by salt stress. Mycorrhizal inoculation especially *G. mosseae* significantly alleviated the growth reduction of salinity. There were notably lower malondialdehyde and hydrogen peroxide contents in the leaves of mycorrhizal seedlings than in non-mycorrhizal ones. Mycorrhizal seedlings recorded notably greater activity of catalase and contents of ascorbate, soluble protein and glutathione under salinity or non-salinity conditions. The seedlings colonized by *G. mosseae* showed significantly higher antioxidant defense systems response to salinity than by *G. versiforme*. Our data demonstrate that mycorrhizal (especially *G. mosseae*) citrus seedlings exhibited greater efficient antioxidant defense systems, which provide better protection against salt damage.

Keywords: antioxidants; arbuscular mycorrhiza; reactive oxygen species; salinity; trifoliate orange

Soil salinization is an increasing problem of many irrigated, arid and semi-arid areas of the world, and approx. 20% of irrigated agricultural land is adversely affected by salinity (Misra et al. 2006). The deleterious effects of salinity may involve physiological drought, nutritional imbalance, toxicity of excessive Na^+ and Cl^- ions towards the cell, and a combination of these factors (Das et al. 1992, Misra et al. 2006, Evelin et al. 2009). These deleterious effects create secondary stresses like oxidative stress due to generation of reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and superoxide anions (O_2^-) in plants (Tunc-Ozdemir et al. 2009). These cytotoxic ROS can destroy normal metabolism through oxidative damage of lipids, proteins and nucleic acids. Plants possess an enzymatic antioxidant defense system like superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) and a non-enzymatic antioxidant defense system like ascorbate (ASC) and glutathione (GSH), to protect cells from oxidative stress induced by ROS. The efficiency of the antioxidant defense systems is correlated with tolerance to salt stress (Tunc-Ozdemir et al. 2009). So, an understanding of the enzymatic and non-enzymatic antioxidant defense systems is critical for salt tolerance of plants.

Arbuscular mycorrhizal fungi (AMF) from Glomeromycota form a symbiotic association by colonizing roots of many land plants (Parniske 2008). The association improves the supply of water and nutrients to the host plant and in return, up to 20% of plant-fixed carbon is transferred to the fungus (Parniske 2008). Arbuscular mycorrhizal (AM) colonization is reported to promote plant growth and salinity tolerance (Evelin et al. 2009). A potted experiment showed that SOD and POD

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activities in roots of tomato colonized by *Glomus mosseae* were significantly higher than either the non-AM plants under salinity or control conditions (He et al. 2007). Ghorbanli et al. (2004) reported the enhanced activities of SOD, POD and ascorbate-peroxidase in mycorrhizal soybean plants under salt stress.

Citrus is a highly salt-sensitive plant. Little information is available on the effects of AMF on ROS metabolism in salt-stressed citrus plants. The objective of the present study was therefore to evaluate whether AM symbiosis helps citrus plants to alleviate salt stress by enhancing antioxidant defense systems.

MATERIALS AND METHODS

Plant culture and salt induction. Seeds of trifoliate orange (*Poncirus trifoliata* L. Raf.) were surface sterilized for 5 min in 70% alcohol solution and germinated on moistened filter papers in the dark at 25°C. Seven-day-old seedlings were transferred to plastic pots (18 cm in depth and 20 cm in mouth diameter) containing 3.4 kg of autoclaved (121°C, 0.11 MPa, 2 h) soil mixture (soil/vermiculite/sphagnum, 5/2/1, v/v/v), which received 15 g *G. mosseae*, *G. versiforme* or autoclaved inoculum as the non-AMF control, respectively. Mycorrhizal inocula, consisting of spore, soil, hyphae and infected root fragment, were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences.

The seedlings were acclimated for 90 days and then were subjected to salinity stress. The soil salinity was developed by 100 mmol NaCl solution, and the control (0 mmol NaCl) seedlings were irrigated with distilled water. The soil was salinized stepwise using 25 mmol NaCl per day to avoid osmotic shock before 100 mmol NaCl treatment. The 0 and 100 mmol NaCl solutions were irrigated after five days again to maintain the salt effects.

Experiment design. The experiment was laid out in a randomized complete block design. Experimental treatments consisted of factorial combinations of three mycorrhizal treatments (*Glomus mosseae*, *G. versiforme* and non-AMF) with two salinity supply conditions (0 and 100 mmol NaCl). Each of the six treatments was replicated four times, leading to a total of 24 pots. The seedlings were harvested 49 days after 0 and 100 mmol NaCl treatments.

Parameter measurement. Plant height, stem diameter and leaf number per plant were recorded before harvest. Leaf area was measured using the WinRHIZO software (Regent Instruments Inc., Quebec, Canada) after scanning with an Epson Expression/STD 4800 Scanner. The shoot and root systems were separated. Root pieces were taken from the middle part of the root systems after root morphology analysis. One-cm root pieces were cleared with 10% (w/v) KOH solution and stained with 0.05% (w/v) trypan blue in lactophenol (Phillips and Hayman 1970). The AM colonization was quantified according to the following formula (Wu et al. 2008):

$$\text{AM colonization (\%)} = \frac{\text{root length infected}}{\text{root length observed}} \times 100$$

The mycorrhizal structures such as entry points, vesicles and arbuscles were calculated from the infected roots at the time of microscopical observation and expressed number/cm root.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation according to the method of Sudhakar et al. (2001). H_2O_2 was determined by the method described by Harinasut et al. (2003).

Fresh leaves (0.5 g) were homogenized in 7 ml of 0.1M phosphate buffer, pH 7.8. Insoluble material was removed by centrifugation at 4200 g for 10 min, with the resulting supernatant used for the assays of SOD, CAT and soluble protein. SOD activity was measured using the method of Giannopolitis and Ries (1977). CAT activity was performed as described by Kar and Mishra (1976) with the slight modifications. Five milliliters of the assay mixture for the CAT activity comprised: 2.5 ml enzyme solution and 2.5 ml 0.1M H_2O_2. After incubation at 30°C for 10 min, the reaction was stopped by adding 2.5 ml of 10% H_2SO_4 and the residual H_2O_2 was titrated against 0.1M $KMnO_4$ until a purple color persisted for at least 15 s. The unit of CAT activity was expressed as milligrams of H_2O_2 decomposed per gram sample per min. Hereinto, extinction coefficient for 1 ml 0.1M $KMnO_4$ is 1.7 mg H_2O_2. Soluble protein was evaluated by the method of Bradford (1976) using bovine serum albumin as the standard.

Approximately 0.4 g of leaf tissues were homogenized by the addition of 7 ml 5% trichloroacetic acid in an ice bath. The homogenates were centrifuged at 15 000 g at 4°C for 15 min. The supernatant was collected for determination of ASC and GSH using the method previously described by Wu et al. (2006).

Statistical analysis. Data from quantitative parameters were analyzed using ANOVA (SAS,

Table 1. Growth characteristics of *G. mosseae*-colonized, *G. versiforme*-colonized and non-AMF trifoliate orange (*Poncirus trifoliata*) seedlings under NaCl stress

Salt treatment (mmol)	AMF	Plant height (cm)	Stem diameter (cm)	Leaf number (No./plant)	Leaf area (cm^2)
0	*G. versiforme*	18.6^{b}	0.221^{b}	18.1^{b}	17.6^{b}
	G. mosseae	23.0^{a}	0.246^{a}	21.8^{a}	19.4^{a}
	non-AMF	15.6^{c}	0.206^{c}	15.8^{c}	15.7^{c}
100	*G. versiforme*	16.9^{bc}	0.215^{bc}	16.1^{c}	14.3^{c}
	G. mosseae	22.1^{a}	0.225^{b}	19.9^{ab}	18.0^{ab}
	non-AMF	13.6^{d}	0.191^{d}	12.5^{d}	12.4^{d}

The same letter within each column indicates no significant difference among treatments ($P < 0.05$)

version 8.1). Fisher's Protected Least Significant Difference (*LSD*, $P < 0.05$) was used to compare the means.

RESULT AND DISCUSSION

Changes in plant growth of citrus plants as affected by NaCl salinity are shown in Table 1. The present study confirmed the reduction in plant height, stem diameter, leaf number and leaf area at high salinity level. Oxidative stress was shown to be one of the major causes of damage to plants under salinity (Das et al. 1992, Misra et al. 2006, Tunc-Ozdemir et al. 2009). Our study showed that soil salinity significantly induced the accumulation of ROS like H_2O_2 (Figure 1) in AM and non-AM trifoliate orange seedlings. Excess H_2O_2 could induce the toxic effects on cellular membranes (Das et al. 1992), thus rising the degree of membrane damage (MDA) (Figure 2). The growth characteristics of trifoliate orange seedlings, irrespective of salinity, were improved significantly with AM inoculation, especially with *G. mosseae* (Table 1), implying that mycorrhizal symbiosis can alleviate the deleterious effects of salt stress.

Salinity, not only affects the host plant but also the development of AMF. Root colonization, vesicle, and entry point of *G. versiforme*- and *G. mosseae*-inoculated seedlings were markedly suppressed by salinity in this study (Table 2). Salinity significantly decreased arbuscule number of *G. mosseae*-inoculated seedlings, but not that of *G. versiforme*-inoculated seedlings. Interactions between salinity and AMF were significant for both AM colonization and entry point, but not for vesicle and arbuscule number. This is in conformity with earlier reports on the effect of salinity on

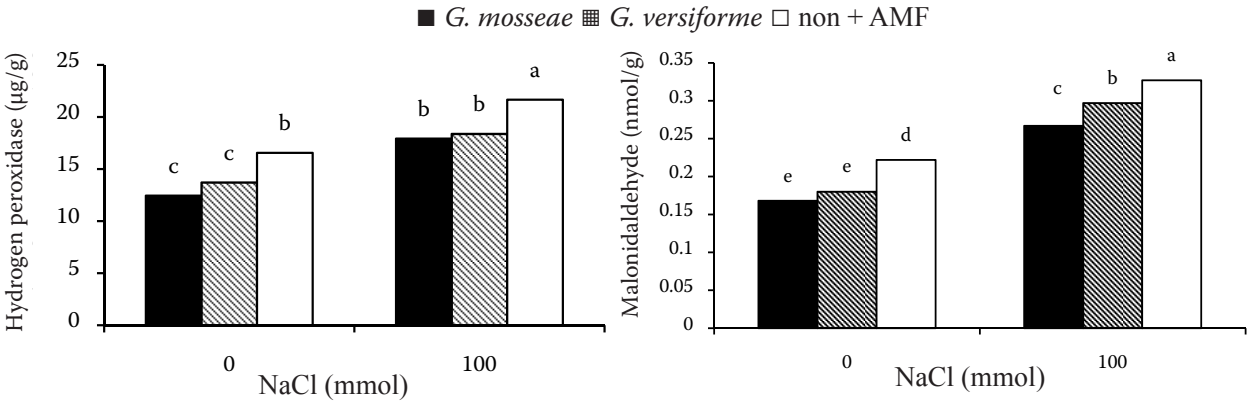

Figure 1. Hydrogen peroxide contents in leaves of *G. mosseae*-colonized, *G. versiforme*-colonized and non-AMF trifoliate orange (*Poncirus trifoliata*) seedlings under NaCl stress. Means followed by the same letter indicate no significant difference among treatments at 5% level

Figure 2. Malondialdehyde contents in leaves of *G. mosseae*-colonized, *G. versiforme*-colonized and non-AMF trifoliate orange (*Poncirus trifoliata*) seedlings under NaCl stress. Means followed by the same letter indicate no significant difference among treatments at 5% level

Table 2. Root AM colonization, vesicle, arbuscule and entry point of *G. mosseae*-colonized, *G. versiforme*-colonized trifoliate orange (*Poncirus trifoliata*) seedlings under NaCl stress

Salt treatment (mmol)	AMF	AM colonization (%)	Vesicle	Arbuscule	Entry point
			(No./cm root)		
0	*G. versiforme*	65.3^{c}	2.8^{b}	1.9^{bc}	2.6^{b}
	G. mosseae	75.7^{a}	3.6^{a}	2.6^{a}	3.2^{a}
	non-AMF	0^{e}	0^{d}	0^{d}	0^{d}
100	*G. versiforme*	59.5^{d}	2.1^{c}	1.7^{c}	2.1^{c}
	G. mosseae	67.8^{b}	2.9^{b}	2.1^{b}	2.7^{b}
	non-AMF	0^{e}	0^{d}	0^{d}	0^{d}

The same letter within each column indicates no significant difference among treatments ($P < 0.05$)

AMF interaction with host plant (Murkute et al. 2006, Sheng et al. 2008). The suppressed growth and colonization of arbuscular mycorrhiza under salinity may be attributed to the reduced hyphal extension of AMF.

Our study showed that the leaf H_2O_2 content was significantly lower in *G. versiforme*- or *G. mosseae*-inoculated seedlings compared to that in non-AMF trifoliate orange seedlings regardless of salinity, implying lower accumulation of ROS in AM seedlings and lower membrane damage (Figure 1). The present work also observed that AM inoculation notably decreased the MDA contents of the seedlings exposed to salt stress (Figure 2). This agrees with the previous reports obtained from the roots of trifoliate orange seedlings inoculated with *G. versiforme* exposed to drought stress (Wu et al. 2006), and from the roots of tomato colonized by *G. mosseae* under different NaCl stress (He et al. 2007). Lower H_2O_2 level due to mycorrhization may also function as a molecular signal in plant cells, triggering tolerance against various abiotic stresses (De Azevedo Neto et al. 2005).

SOD and CAT are important for plants to tolerate the salinity, because SOD catalyses the dismutation of O_2^- to H_2O_2, and CAT dissociates H_2O_2 to oxygen and water. A strong correlation between the enzymatic antioxidant defense system and salt tolerance in many plants are reported (Das et al. 1992, Misra et al. 2006, Tunc-Ozdemir et al. 2009). The present study clearly showed that SOD activity was largely induced by *G. mosseae* but not *G. versiforme* under salt or saltless conditions, and AMF infection significantly increased the CAT activity of the citrus leaves under salt stress (Figure 3). The results are similar in part to the results obtained by Ghorbanli et al. (2004) and He et al. (2007), who reported greater SOD activity of salt-stressed soybean inoculated with *G. etunicatum* or tomato inoculated with *G. mosseae*. However, several studies suggested that AM symbiosis did not affect CAT activity of salt-

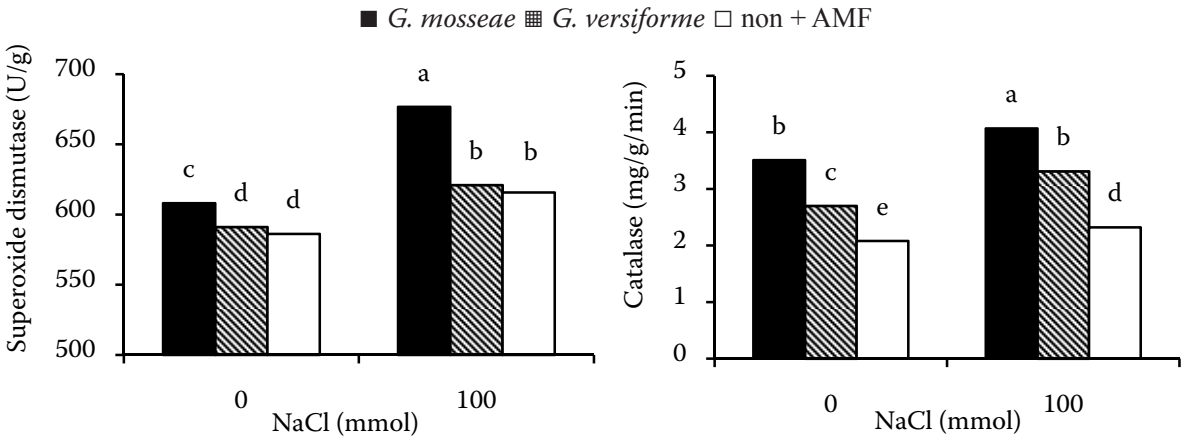

Figure 3. Superoxide dismutase and catalase activities in leaves of *G. mosseae*-colonized, *G. versiforme*-colonized and non-AMF trifoliate orange (*Poncirus trifoliata*) seedlings under NaCl stress. Means followed by the same letter indicate no significant difference among treatments at 5% level

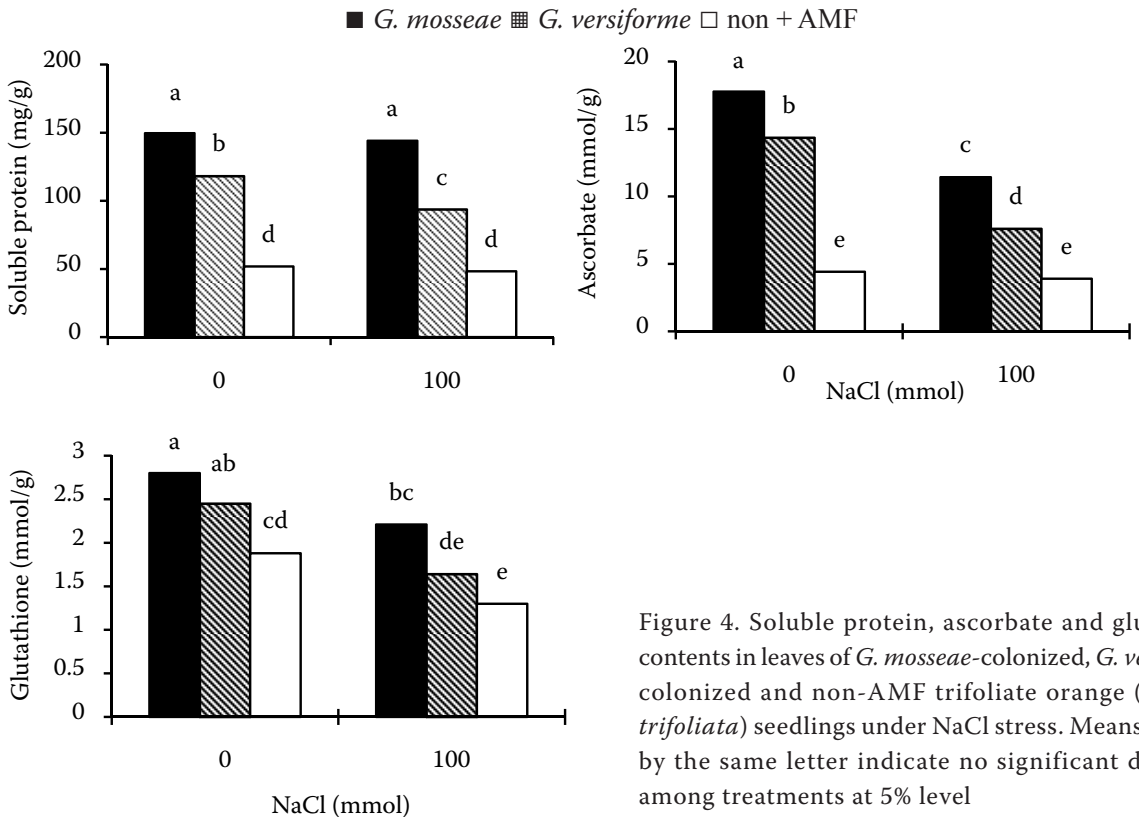

Figure 4. Soluble protein, ascorbate and glutathione contents in leaves of *G. mosseae*-colonized, *G. versiforme*-colonized and non-AMF trifoliate orange (*Poncirus trifoliata*) seedlings under NaCl stress. Means followed by the same letter indicate no significant difference among treatments at 5% level

stressed plants (Ghorbanli et al. 2004, He et al. 2007). The different behavior of CAT was perhaps due to different AMF species, plant species and phenology in these experiments. Since enzymatic antioxidant defense system is related to salt tolerance of plant, the greater SOD and CAT activities in the mycorrhizal seedlings might enhance salt tolerance of trifoliate orange seedlings.

ASC reacts non-enzymatically with ROS like O_2^-, H_2O_2 and OH. GSH, the major source of non-protein thiols in most plant cells, takes part in the control of H_2O_2 levels. The non-enzymatic antioxidants, soluble protein, GSH and ASC, are involved directly in the reduction of most ROS generated by stress (Das et al. 1992, Misra et al. 2006, Tunc-Ozdemir et al. 2009). The data from our experiment showed that although salinity significantly restricted the ASC and GSH contents, mycorrhizal inoculation notably elevated the soluble protein, ASC and GSH contents of the seedlings (Figure 4). These results agree with those of Wu et al. (2006) who reported an increase in soluble protein, ASC and GSH in drought-stressed trifoliate orange. Maintaining higher non-enzymatic antioxidant contents due to mycorrhization would help hosts to eliminate ROS generation, thus resulting in the H_2O_2 reduction in the present study.

In conclusion, these data suggested that although salinity significantly inhibited the symbiosis establishment of trifoliate orange seedlings, the inoculated seedlings were more tolerant to salt stress than un-inoculated ones, which can attribute to the higher capacity of the seedlings to control ROS formation and to activate enzymatic and non-enzymatic antioxidant defenses.

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