

Sodium Chloride Stress Induced Changes in Leaf Osmotic Adjustment of Trifoliolate Orange (*Poncirus trifoliata*) Seedlings Inoculated with Mycorrhizal Fungi

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Abstract

Citrus plants are sensitive to salinity, and thus employing new approaches to alleviate salt damage are necessary. The present study evaluated the effect of two arbuscular mycorrhizal fungi (AMF), *Glomus mosseae* and *G. versiforme*, on leaf osmotic adjustment of trifoliolate orange (*Poncirus trifoliata*) seedlings exposed to 100 mM NaCl. Salinity significantly inhibited mycorrhizal colonization, plant biomass and leaf relative water content, whereas the reduce of plant biomass was notably alleviated by the mycorrhizal colonization. Mycorrhizal seedlings exhibited significantly lower Na⁺ and Ca²⁺ concentrations, whilst also recorded higher K⁺ concentration and K⁺/Na⁺, Ca²⁺/Na⁺ and Mg²⁺/Na⁺ ratios at both salinity levels. Under salinity stress, mycorrhizal symbiosis markedly decreased sucrose concentrations of leaves and also increased glucose, fructose and proline concentrations of leaves. The results suggest that arbuscular mycorrhizas improved leaf osmotic adjustment responses of the seedlings to salt stress, thus enhancing salt tolerance of mycorrhizal plants.

Keywords: arbuscular mycorrhizal fungi, citrus, *Glomus mosseae*, osmotic adjustment, salt stress, trifoliolate orange

Introduction

Soil salinity is an important abiotic stress, which strongly affects crop productivity by way of accumulation of toxic Na⁺ and Cl⁻ ions and nutrient imbalance (Chinnusamy *et al.*, 2005; Sairam and Tyagi, 2004). Until now, >6% of the world's land and 30% of the world's irrigated areas have been adversely affected by soil salinity (Chaves *et al.*, 2009). Citrus, the second economically cultivated fruit tree in China, is relatively sensitive to salinity stress (Levy and Syvertsen, 2004). Soil salinity not only leads to the deleterious effects on citrus growth and fruit yield but also restricts citrus cultivation in south saline regions of China. Therefore, enhancing salt tolerance of citrus plants will probably be one of the important objectives.

The excess of salt is able to cause osmotic stress by way of the decrease of soil water potential, thus disturbing plant water relation (de Lacerda *et al.*, 2003). Osmotic adjustment, a net increase in intracellular inorganic and/or organic solutes, can maintain turgor and reduce the deleterious effects of salt stress on plants (Hajlaoui *et al.*, 2010; de Lacerda *et al.*, 2003). These intracellular solutes include K⁺, Ca²⁺, Mg²⁺, proline, glycine betaine, free amino acids, sugars, polyamines, polyphenols, etc. The response of the solute concentrations to salinity is regarded as a fundamental aspect of salinity tolerance (Reed, 1989). Citrus plants can improve osmotic adjustment for altering salt

tolerance (Pérez-Pérez *et al.*, 2009). In *Atriplex nummularia*, roots and leaves displayed contrary osmotic adjustment mechanisms in response to NaCl stress: an efficient osmotic adjustment only in leaves but not roots (Silveira *et al.*, 2009).

Arbuscular mycorrhiza (AM), a mutualistic association between roots of plants and soil arbuscular mycorrhizal fungi (AMF), provides the hosts with essential nutrients and water from soils (Requena *et al.*, 2007). In return, ~20% of plant photosynthetic carbon is transported into the root endosymbiotic fungi to maintain their growth. It is well documented that AMF colonization can alleviate damage of host plants caused by soil salinization through osmotic adjustment (Evelin *et al.*, 2009). Compared with non-mycorrhizal corresponding plants, the NaCl-stressed maize (*Zea mays*) plants colonized by *Glomus mosseae* exhibited higher soluble sugars and electrolyte concentrations, suggesting the higher osmoregulation capacity of these plants (Feng *et al.*, 2002). A field experiment also showed that the reduction in Na uptake together with a concomitant increase in Mg absorption in *G. macrocarpum*-colonized *Sesbania aegyptiaca* and *S. grandiflora* plants may be important salt-alleviating mechanisms for plants grown in saline soil (Giri and Mukerji, 2004). However, there are two different effects of AMF on citrus plants. *G. mosseae* and *Paraglomus occultum* significantly increased the K⁺/Na⁺ ratio in the NaCl-stressed

Citrus tangerine seedlings (Wu *et al.*, 2010b). Mycorrhizal colonization also reduced the proline concentrations of Carrizo citrange, irrespective of salinity level (Duke *et al.*, 1986). However, Graham and Syvertsen (1989) observed the increase of Cl⁻ concentrations in leaves and roots of salinized sweet orange and sour orange. Murkute *et al.* (2006) reported no significantly physiological effects on salt-stressed citrus rootstocks by AMF inoculation.

The objective of the present study was to evaluate the effects of two *Glomus* species on inorganic (Na⁺, K⁺, Ca²⁺ and Mg²⁺) and organic (proline and sugars) solutes of citrus seedlings exposed to salt stress.

Materials and methods

Experimental design

The experiment was laid out in a completely randomized block design with two factors in four replications. The first factor was mycorrhization in three levels, including inoculation with *G. mosseae*, *G. versiforme* and non-AMF. The second factor was salinization in two levels, including 0 and 100 mM NaCl concentrations, which are based on the results conducted by Pérez-Tornero *et al.* (2009) in *C. macrophylla* grown in 0~150 mM NaCl and Ferreira and Lima-Costa (2008) in *Citrus* cv. 'Carvalho' tangor grown in 100 mM NaCl. Each treatment had three replicates for a total of 18 pots.

Plant culture

The trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] seedlings germinated at 25°C for 7 days were transferred into the plastic pots (18 cm in depth and 20 cm in mouth diameter) containing 3.4 kg of autoclaved soil, vermiculite, and sphagnum mixture (5/2/1, v/v/v). Fifteen gram mycorrhizal inocula of *G. mosseae* and *G. versiforme* containing fungal spores, infected maize roots, and growth substrate was inoculated into the pots before transplanting. The non-AMF control received the same autoclaved inoculum. The inoculum was supplied by the Institute of

Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences.

The salt treatments began at 90 days of acclimation. Half of AMF and non-AMF seedlings was randomly used as the non-salt treatment, and the other as salt-stressed treatment. The salt-stressed treatment was applied by 100 mM NaCl solution stepwisely using 25 mM NaCl per day to avoid osmotic shock, whilst the non-salt treatment received 0 mM NaCl solution as the control. The plants were randomly placed in a plastic greenhouse (Jingzhou, China) without any temperature equipments from March to August 2009.

Parameter measurements

The experiment was ended after 7 weeks of salt treatments, and the shoots and roots were separated. The AM structures were stained with trypan blue method described by Phillips and Hayman (1970), and the AM colonization was quantified by Wu *et al.* (2008).

Leaf relative water content (RWC) was determined by the method of Wu and Xia (2006). The proline concentration was determined according to the method of Troll and Lindsley (1955). The sucrose, glucose and fructose concentrations were carried out following Wu *et al.* (2010a).

The K⁺, Na⁺, Mg²⁺ and Ca²⁺ concentrations were determined directly using an Atomic Absorption Spectrometer (AI 1200, Aurora Instruments Limited, Canada).

Statistical analysis

The data were analyzed by two-factor ANOVA with SAS v 8.1. Fisher's protected least significant difference (LSD, $P < 0.05$) was used to compare the significant differences between treatments and interactions.

Results

The present research did not observe mycorrhizal colonization in un-inoculated seedlings growing under 0 and 100 mM NaCl conditions (Tab. 1). Mycorrhizal coloniza-

Tab. 1. Arbuscular mycorrhizal colonization and biomass of mycorrhizal and non-mycorrhizal trifoliolate orange (*Poncirus trifoliata*) seedlings under salt stress

NaCl (mM)	Mycorrhizal status	AM colonization (%)	Dry weight (g plant ⁻¹)		
			Shoot	Root	Plant
0	<i>G. versiforme</i>	65.31c	0.37ab	0.12b	0.49b
	<i>G. mosseae</i>	75.69a	0.42a	0.17a	0.59a
	Non-AMF	0e	0.31cd	0.09c	0.40c
100	<i>G. versiforme</i>	59.52d	0.33bc	0.09c	0.42c
	<i>G. mosseae</i>	67.76b	0.38a	0.11bc	0.49b
	Non-AMF	0e	0.26d	0.07d	0.33d
ANOVA					
Salt stress		**	*	**	**
AMF		**	**	**	**
Interaction		**	NS	*	NS

Note: Same letter within each column indicates no significant difference among treatments ($P < 0.05$). NS, no significant. *, $P < 0.05$. **, $P < 0.01$

tion of inoculated seedlings ranged from 59.52 to 75.69%, and salt stress significantly decreased mycorrhizal colonization. *G. mosseae*-colonization was markedly higher than *G. versiforme*-colonization, regardless of the salt levels.

Analysis of dry biomass showed that all the dry weights decreased with the increasing NaCl concentrations (Tab. 1). There were significant differences between mycorrhizal and non-mycorrhizal seedlings. Generally, the inoculated seedlings under salt stress conditions recorded significantly higher shoot, root and plant dry weights compared to the un-inoculated ones. Moreover, the significant differences were more obvious in *G. mosseae*-colonized seedlings.

Leaf RWC reduced with the increasing salinity, and the different significances were among mycorrhizal seedlings but not non-mycorrhizal seedlings (Fig. 1). Under the non-salinity stress conditions, the two AMF significantly increased leaf RWC compared to non-AMF treatment. Moreover, *G. mosseae*-inoculation recorded notably higher effects on leaf RWC than *G. versiforme*-inoculation. Under salinity stress conditions, *G. mosseae* but not *G. versiforme* markedly increased leaf RWC as compared to non-AMF seedlings.

For ionic concentrations, soil salinity reduced K^+ concentration but increased Na^+ and Ca^{2+} concentrations in leaves of both AMF and non-AMF seedlings compared to non-salinity stress conditions (Tab. 2). Na^+ concentration was always lower in AMF than in non-AMF seedlings, and *G. mosseae*-colonized seedlings exhibited lower Na^+ concentration than *G. versiforme*-colonized seedlings under 100 mM NaCl but not 0 mM NaCl conditions. K^+ concentration was notably enhanced by AMF inoculation, whilst the significant differences were not observed the mycorrhizal seedlings. In leaves, AMF plants had lower Ca^{2+} concentration than non-AMF plants regardless of soil salinization, and the significant differences were not observed between the mycorrhizal seedlings. Mycorrhizal seedlings under non-salinity stress recorded lower Mg^{2+} concentrations of leaves as compared to non-mycorrhizal seedlings; under salinity stress, only *G. versiforme*-colonized seedlings maintained higher Mg^{2+} concentrations of leaves.

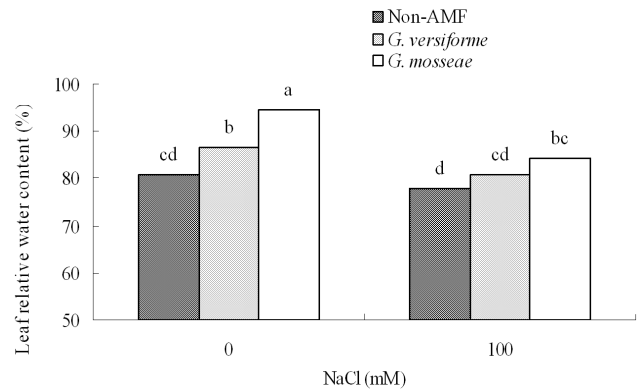


Fig. 1. Effect of salinity and mycorrhizal inoculation on leaf relative water content of trifoliolate orange (*Poncirus trifoliata*) seedlings

For ionic balance, salt stress markedly reduced the ratio of K^+/Na^+ , Ca^{2+}/Na^+ or Mg^{2+}/Na^+ (Tab. 2). The AMF seedlings had higher K^+/Na^+ , Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratios than the non-AMF control seedlings at both salinity levels. Herein, under the non-salinity stress, compared to *G. versiforme*-colonized seedlings, *G. mosseae*-colonized seedlings recorded significantly higher K^+/Na^+ and Mg^{2+}/Na^+ ratios. Similar response was observed in the ratio of Ca^{2+}/Na^+ between *G. versiforme* and *G. mosseae*-colonized seedlings.

In the two fungal species, only *G. mosseae* significantly increased sucrose concentrations of leaves under non-salinity stress (Tab. 3). However, under salinity stress, the two AMF markedly decreased sucrose concentrations of leaves. The seedlings inoculated with AMF had higher concentrations of glucose and fructose when grown with 0 and 100 mM NaCl (Tab. 3). Though proline concentration increased with the increasing salinity, no significant differences were observed. Compared to the non-AMF control seedlings, proline concentration was increased by 36 and 43% in *G. versiforme* and *G. mosseae*-colonized seedlings under the non-salinity stress, respectively; Under the salinity stress, *G. versiforme* and *G. mosseae*-colonized seedlings by 45 and 67%, respectively.

Tab. 2. Effects of salinity and mycorrhizal inoculation on ionic concentrations and ionic balance in leaves of trifoliolate orange (*Poncirus trifoliata*) seedlings

NaCl (mM)	Mycorrhizal status	Ionic concentration (mg g ⁻¹)				Ionic balance		
		Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	K ⁺ /Na ⁺	Ca ²⁺ /Na ⁺	Mg ²⁺ /Na ⁺
0	<i>G. versiforme</i>	4.27e	41.05ab	18.77d	8.39c	9.65b	4.41a	1.97b
	<i>G. mosseae</i>	4.17e	43.46a	18.30d	9.20b	10.44a	4.39a	2.21a
	Non-AMF	6.91d	38.26cd	24.89c	10.82a	5.57c	3.61b	1.57c
100	<i>G. versiforme</i>	12.06b	40.46bc	25.98bc	9.84b	3.37d	2.15d	0.82d
	<i>G. mosseae</i>	10.97c	39.00bc	27.68ab	8.29c	3.56d	2.52c	0.76d
	Non-AMF	14.20a	35.87d	28.84a	8.31c	2.53e	2.03d	0.59e
ANOVA								
Salt stress		**	**	**	**	**	**	**
AMF		**	**	**	*	**	**	**
Interaction		NS	NS	*	**	**	*	**

Note: Same letter within each column indicates no significant difference among treatments ($P < 0.05$). NS, no significant. *, $P < 0.05$. **, $P < 0.01$

Tab. 3. Effect of salinity and mycorrhizal inoculation on organic solute concentrations in leaves of trifoliolate orange (*Poncirus trifoliata*) seedlings

NaCl (mM)	Mycorrhizal status	Sucrose (mg g ⁻¹)	Glucose (mg g ⁻¹)	Fructose (mg g ⁻¹)	Proline (mg g ⁻¹)
0	<i>G. versiforme</i>	8.28bc	36.37a	28.25bc	0.25b
	<i>G. mosseae</i>	9.27ab	41.83a	28.58bc	0.26ab
	Non-AMF	7.39cd	29.27b	24.22d	0.18c
100	<i>G. versiforme</i>	6.43d	38.01a	31.94a	0.27ab
	<i>G. mosseae</i>	7.54cd	35.82a	30.93ab	0.32a
	Non-AMF	10.63a	21.63c	25.89cd	0.19c
ANOVA					
Salt stress		NS	*	**	NS
AMF		*	**	**	**
Interaction		**	NS	NS	NS

Note: Same letter within each column indicates no significant difference among treatments ($P < 0.05$). NS, no significant. *, $P < 0.05$. **, $P < 0.01$

Discussion

The present study here indicated that soil salinity significantly reduced the AM colonization. This is in agreement with the previous results (Kumar *et al.*, 2010; Wu *et al.*, 2010b). The AM colonization was always significantly higher in *G. mosseae*-colonized than in *G. versiforme*-colonized seedlings at both salinity levels, suggesting that *G. mosseae* possesses a more efficient potential for enhancing salt tolerance of citrus seedlings than *G. versiforme*.

It is well known that soil salinity obviously inhibits plant growth (Taffouo *et al.*, 2010). The present results also indicated that, though the salt stress reduced the dry weights of the citrus seedlings, especially root and plant dry weights, the AMF seedlings exhibited significantly higher dry biomass as compared to non-AMF seedlings exposed to 0 and 100 mM NaCl, implying that AMF can improve plant growth of salt-stressed citrus plants. Moreover, the significant differences were more obvious in *G. mosseae*-colonized seedlings. The results are in consonant with previous reported on *C. tangerine* (Wu *et al.*, 2010b), *Jatropha curcas* (Kumar *et al.*, 2010), *Lactuca sativa* (Jahromi *et al.*, 2008) and *Zea mays* (Sheng *et al.*, 2008).

Proline as a compatible osmoprotectant solute can protect cytosolic enzymes and cellular organelles, thus maintaining normal osmotic conditions (Jiménez-Bremont *et al.*, 2006; Khedr *et al.*, 2003). The proline concentrations here were notably higher in AMF than in non-AMF seedlings at both salinity levels, suggesting that exogenous AMF trigger internal proline accumulation to alleviate salt damage. The proline accumulation of the AMF seedlings may be due to the result that mycorrhizal seedlings recorded higher activity of a proline synthase, Δ^1 -pyrroline-5-carboxylate synthetase (Kavi Kishor *et al.*, 1995). The present result is in agreement with the previous observation by Garg and Manchanda (2009), who reported that the quantum of an increase in synthesis and accumulation of proline was higher in mycorrhizal than in non-mycorrhizal *Cajanus cajan* plants subjected to salt stress. However, in another experiment, AMF trifoliolate

orange seedlings accumulated less proline concentration than non-AMF seedlings exposed to drought stress (Wu *et al.*, 2007). These incompatible results suggest that the variation of the proline accumulation in the AMF trifoliolate orange seedlings is dependent on stressed types.

Glucose, fructose and sucrose are preferred as the regulated substances responsible for osmotic adjustment in tissue water osmotic stress (Shatnawi *et al.*, 2006). In the present work, the inoculated seedlings with AMF had higher concentrations of glucose and fructose when grown with 0 and 100 mM NaCl. Under non-salinity stress, exogenous AMF increased sucrose concentrations of leaves, whereas under salinity stress these AMF markedly decreased sucrose concentrations. As the obligate symbionts, AMF must obtain a mount of their carbon (mainly hexose) from the hosts (Bago *et al.*, 2003). Therefore, lower levels of sucrose in the salt-stressed seedling and higher levels of fructose and glucose in the present study imply that host sucroses transform more into hexose to support the symbiotic development and maintain normal water relations (Wu *et al.*, 2010a). The phenomenon is more obvious under salinity stress. Compared to non-mycorrhization, the changed tendency of sucrose concentration due to mycorrhization was increased under non-salinity stress but decreased under salinity stress, adequately indicating that under salinity stress, mycorrhizal plants need more hexose to sustain the benefit effects on salt tolerance.

Osmotic adjustment involves not only organic solutes but also inorganic ions. The present study here observed that salt stress induced Na⁺ and Ca²⁺ accumulation but decreased K⁺ accumulation in both the AMF and non-AMF seedlings, which are in agreement with Al-Karaki (2000). On the other hand, AMF seedlings exhibited lower levels of Na⁺ and Ca²⁺ but higher concentrations of K⁺, which would help the mycorrhizal seedlings to reduce cellular Na⁺ accumulation to a toxic level, thus protecting host plants against salt damage. For the ratios of K⁺/Na⁺, Ca²⁺/Na⁺ and Mg²⁺/Na⁺, the inoculated seedlings had higher the values than the non-AMF control seedlings at both

salinity levels, which are essential for normal cellular functions of mycorrhizal plants. The similar increases in K^+/Na^+ ratio of mycorrhizal *Acacia nilotica*, Ca^{2+}/Na^+ ratio of mycorrhizal *C. tangerine* and Mg^{2+}/Na^+ ratio of both mycorrhizal *Sesbania aegyptiaca* and *S. grandiflora* have also been reported previously (Giri and Mukerji, 2004; Giri *et al.*, 2007; Wu and Zou, 2009).

Conclusions

From these results, it concluded that the salt stress inhibited trifoliolate orange biomass, but the inhibition was alleviated by the exogenous AMF inoculation. Mycorrhizal colonization obviously improved the osmotic adjustment of the trifoliolate orange seedlings at both salinity levels based on higher levels of organic solutes (sucrose, glucose, fructose and proline), higher K^+ concentration, higher ratios of K^+/Na^+ , Ca^{2+}/Na^+ and Mg^{2+}/Na^+ , and lower Na^+ level, thus alleviating partly salt damage.

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