



Inoculation response of mycorrhizas on morphology and physiological behaviour of trifoliolate orange (*Poncirus trifoliata*) roots under salt stress

PENG WANG¹, A K SRIVASTAVA², YI-CAN ZHANG³ and QIANG-SHENG WU⁴

Institute of Citrus Research, Zhejiang Academy of Agricultural Sciences, Taizhou, China 318 026 Received:

22 April 2016; Accepted: 1 June 2016

ABSTRACT

Citrus is highly sensitive to salt stress, and little efforts have been successful microbiologically to mitigate such abiotic stress. In this background, trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] seedlings were inoculated with *Diversispora versiformis* and exposed to 100 mM NaCl treatment for 85 days under controlled conditions. The NaCl application, though, strongly inhibited root mycorrhizal colonization in seedlings, but mycorrhizal inoculation considerably increased the root projected area and number of second- and third-order lateral roots under 100 mM NaCl treatment. Mycorrhizal-inoculated seedlings showed significantly higher soluble protein concentration, ornithine decarboxylase, arginine decarboxylase, and superoxide dismutase activity in leaves and roots, irrespective of NaCl concentration. While mycorrhizal seedlings displayed significantly lower polyamine oxidase activity and diamine oxidase activity in leaves and roots, irrespective of NaCl concentration. These results, thus, suggested that mycorrhizal plants were physiologically activated through mycorrhizal inoculation to downplay the adverse effect of salt stress.

Key words: Citrus, Mycorrhiza, Polyamine, Salt stress, SOD

Salt stress is globally considered as one of the important abiotic stresses (Srivastava and Singh 2009), responsible for substantial reduction in crop performance (Srivastava and Singh 2008). It is estimated that 20% of total cultivated lands and 33% of irrigated agricultural lands are affected by high salinity, in addition to annual 10% increase in salinized areas (Shrivastava and Kumar 2015). But incidently, the range of adaptations and mitigation strategies for fruit crops is still very limited to deal with impact of salt stress as abiotic stress. Studies carried out in the past ably demonstrated that arbuscular mycorrhizal fungi (AMF) are effective bio-stimulant to promote plant growth and enhance the ability of crop to withstand salt tolerance (Wu *et al.* 2013, Abdel Latif and Miransari 2014). Plants exposed to salt stress induce osmotic stress through accumulation of osmoprotectants like amino acid, proline, betain, and sugar in plant cells to increase cell osmotic potential (Munns 1993). However, the most distinct

response of such reaction is the accumulation of carbohydrate osmoprotectant, due to its direct relationship with other physiological processes like photosynthesis, respiration, and translocation (Kerepesi and Galiba 2014, Wu *et al.* 2015). Studies (Al-Garni 2006, Evelin *et al.* 2009) showed an increasing accumulation in soluble sugar with the increasing concentration of NaCl concentration.

During the process of photosynthetic electron transfer, losing electrons combine with oxygen to form reactive oxygen species (ROS) like superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen (1O_2) (Dionisio-Sese and Tobita 1998, Zhang *et al.* 2015, Zou *et al.* 2015). These ROS species strongly damage lipid, protein and nucleic acid (Imlay 2003). There is a much complicated system to scavenge these ROS in cells involving both components of enzyme and non-enzymatic nature, collectively called antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and so on. SOD is used for not only sweeping away H_2O_2 , but effectively deal with salt tolerance (Panda and Khan 2004).

Polyamines (PAs), a small molecular weight cation present in eucaryotes and prokaryotes, mainly consist of putrescine (Put), spermine (Spm), and spermidine (Spd) (Kaur-Sawhney *et al.* 2003). PAs have the vital functionings on growth, morphogenesis, embryogenesis, organ development, leaf senescence, and biotic/abiotic stress (Kusano *et al.* 2008). PAs as redox homeostasis regulators are reported to accumulate under salt stress, and thus, assist the plant to maintain cellular ROS homeostasis (Saha

¹Assistant Research Fellow (e mail: peter_wang81@163.com), Institute of Citrus Research, Zhejiang Academy of Agricultural Sciences, Taizhou 318026, China, ²Principal Scientist (e mail: aksrivas2007@gmail.com), Soil Science, ICAR–Central Citrus Research Institute, Nagpur 440 033, Maharashtra, India; ³Master Student (1842017832@qq.com), College of Horticulture and Gardening, Yangtze University, Jingzhou, Hubei 434025, China, ⁴Professor (e mail: wuqiangsh@163.com), Department of Chemistry, Faculty of Science, University of Hradec Kralove, Hradec Kralove, Czech Republic

et al. 2015). Put is originated from the decarboxylation of arginine and ornithine by arginine or ornithine decarboxylase (ADC or ODC) and transformed into Spd and Spm (Hussain *et al.* 2011). The level of polyamine is, hence, related with PA synthesis and catabolism. PAs are degraded by oxidation reaction, catalyzed by diamine oxidase (DAO) for Put degradation and polyamine oxidase (PAO) for Spd and Spm degradation (Couée *et al.* 2004). Inoculation with AMF is reported to increase leaf Spd and root Put concentrations in *Citrus tangerina* seedlings coupled with, improved root morphology as influenced by higher activity of root Put synthetases (ADC and ODC) (Wu *et al.* 2012).

The central theme of our study was to investigate the response of AMF-inoculated trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] to salt stress considering changes in root morphology, and plant's defense profile governed by the activities of ADC, ODC, PAO, DAO, and SOD.

MATERIALS AND METHODS

The experimental plant material was trifoliolate orange a widespread used citrus rootstock for world's premier mandarin cultivar like Satsuma mandarin (*Citrus reticulata* Blanco). Seeds of trifoliolate orange were germinated under controlled conditions using autoclaved (0.11 Mpa, 121°C, 2h) sands at 26/20°C day/night temperature and 80% relative air humidity conditions. Three 4-leaf-old trifoliolate orange seedlings without mycorrhizas were transplanted into the plastic pot, having size dimension of 19 cm upper diameter, 13 cm bottom diameter and 17 cm height. The autoclaved (0.11 Mpa, 121°C, 2 h) soils were supplied into each pot, mixed with 800 spores of *Diversispora versiformis*. This soil was collected from a citrus orchard in the Yangtze University and contained pH 6.2, organic matter 9.8 g/kg, available N 114.2 mg/kg, Olsen-P 15.7 mg/kg, and available K 20.8 mg/kg. The non-AMF treatment used the same quantity of autoclaved (0.11 Mpa, 121°C, 2h) mycorrhizal inocula. The AM- and non-AM seedlings were acclimatized for 94 days under controlled conditions, characterized by 721–967 $\mu\text{mol}/\text{m}^2/\text{s}$ photo flux density, 25/19°C average day/night temperature, and 75–95% relative air humidity. Subsequently, the salt treatment of 0 and 100 mM NaCl treatments were initiated. The 100 mM NaCl treatment was regulated by gradually increasing the concentration of NaCl from 25mM to 100 mM NaCl within four days time with irrigation at weekly interval so that seedlings are gradually exposed to salt stress.

The experiment was designed with two factors, in a completely randomized factorial arrangement involving inoculation with or without *D. versiformis*, and the other was NaCl treatment at 0 mM and 100 mM concentration, thus having, a total of four treatments, with each treatment replicated three times.

The seedlings were harvested following 85 days of NaCl treatments. Total plant biomass was weighed and recorded. The root system of each plant was scanned with the Epson Perfection V700 Photo Dual Lens System and

analyzed by a WinRHIZO professional software for root projected area and average diameter. The number of lateral roots in different orders was counted, and the length of taproot was measured by vernier caliper. Root AM colonization was determined by Phillips and Hayman (1970) based on trypan blue staining.

Soluble sugar content was determined using anthrone colorimetry using sucrose as a standard (Zou *et al.* 2013). Activity of superoxide dismutase (SOD) was assayed according to procedure as suggested by Giannopolitis and Ries (1977). One unit of SOD was expressed as the amount of enzyme that inhibited 50% nitro blue tetrazolium by light. ADC and ODC activity were determined by the protocol described by Wu *et al.* (2012). One unit of ADC and ODC activity was defined as the absorbance increased 1.0 at 254 nm during 1 min. DAO and PAO activity in leaf and root were assayed according to Wu *et al.* (2012). Absorbance increased 1.0 at 470 nm during 1 min was defined as one unit of DAO or PAO activity.

Data (means \pm SD, $n=3$) were subjected to analysis of variance (ANOVA) using SAS (SAS Institute Inc., Cary, NC, USA). Fisher's Protected Least Significant Difference was used at the 5% level to compare the significant differences between different treatments.

RESULTS AND DISCUSSION

Root changes

AMF inoculation responded significantly when compared with two conditions, salt stress against no salt stress. No AMF-colonization was observed in the non-AMF seedlings. AMF-seedlings under non-salt registered 77% roots colonized by AMF compared to 57% colonization under salt stress. Exposure of salt stress significantly inhibited the root mycorrhizal colonization, as compared with non-salt stress, indicating the negative effect of salt stress on root colonization and some reversal upon *D. versiformis*. Similar results were earlier observed in our previous studies (Zou *et al.* 2013). AMF-inoculation did not alter other root properties such as root average diameter, taproot length, and number of first-order lateral root, irrespective of salt or non-salt stress treatment. However, root projected area and number of second- and third-order lateral roots were significantly higher in AMF-seedlings compared to non-AMF seedlings, regardless of salt stress or not. Better development of root morphology in mycorrhizal seedlings aided the host plant to absorb more water and nutrients from the inoculated soil to negate the adverse effect of salts (Asghari *et al.* 2005). The AMF-induced alteration in root morphology is linked to endogenous PA metabolism (Wu *et al.* 2012), but without being used as common symbiosis signaling (Gutjahr *et al.* 2009).

Changes in soluble sugar accumulation

AMF-treatment significantly increased soluble sugar concentration by 408% and 398% in leaf and 85% and 71%

Table 1 Effect of *Diversispora versiformis* (AMF) on root morphological traits of trifoliolate orange seedlings under non-salt (0 mM NaCl) and salt stress (100 mM NaCl) conditions

Salt treatment	AMF	Root projected area (cm ²)	Root average diameter (mm)	Taproot length (cm)	Number of lateral roots		
					First-order	Second-order	Third-order
Non-salt stress	+AMF	20.4 ± 0.4a	0.50 ± 0.02a	26.5 ± 0.50b	46 ± 1b	164 ± 1b	13 ± 1a
	-AMF	14.0 ± 0.9b	0.46 ± 0.03a	26.6 ± 0.47b	51 ± 1b	117 ± 1d	8 ± 1b
Salt stress	+AMF	22.6 ± 1.0a	0.46 ± 0.01a	30.7 ± 0.77ab	60 ± 0a	197 ± 1a	13 ± 1a
	-AMF	16.7 ± 0.8b	0.48 ± 0.02a	33.6 ± 0.69a	60 ± 1a	137 ± 10c	5 ± 1c
<i>Significance</i>							
Salt treatment		*	NS	*	**	**	*
AMF		**	NS	NS	NS	**	**
Interaction		NS	NS	NS	NS	NS	**

Note: The different letters followed within the same column indicate the significant differences at $P < 0.05$.

in root under non-salt and salt stress conditions, respectively (Fig 1). These results are in consistent with the earlier results obtained by Feng *et al.* (2002) in maize and Zou *et al.* (2013) in trifoliolate orange. Inoculation with AMF promoted photosynthetic ability of the host plant through elevation in soluble sugar production (Wu *et al.* 2015) since soluble sugar is known as osmolyte facilitating host plant for osmotic adjustment in cytosol of root cells under salt stress condition (Nemati *et al.* 2011). Greater accumulation of soluble sugar in AMF-seedlings, hence, provide better osmotic adjustment capacity to the host plant to cope with salt stress, since AMF-inoculation and root development are highly dependent of total soluble sugar concentration (Zou *et al.* 2013).

Changes in SOD activity

SOD is the part of the antioxidant defense system for scavenging over ROS accumulation. SOD activity was significantly higher in AMF-seedling (15.77% and 104.48% in leaf and root, respectively) than non-AMF seedlings (10.67% and 17.65% in leaf and root, respectively) exposed

to non-salt and salt stress: 15.77 (Fig 2). Our earlier results also observed similar findings (Wu *et al.* 2010) in trifoliolate orange. Hence, greater SOD activity in AMF-seedlings is anticipated to enhance the salt tolerance ability of trifoliolate orange.

Changes in PA metabolic enzyme activities

Put synthesis originates from ADC pathway or ODC pathway (Liu *et al.* 2015). In this study, salt stress markedly increased the leaf as well as root ODC activity in non-AMF seedlings coupled with salt stress, but not in AMF seedlings, with non-salt stress (Fig 3). Inoculation with AMF showed two entirely different types of responses on leaf ODC activity; an increase under non-salt stress condition and a decrease under salt stress condition. Mycorrhizal inoculation did not alter root ODC activity under salt stress but significantly increased root ODC activity under non-salt stress. These results imply that AMF inoculation increased leaf and root ADC and ODC activity under non-salt stress condition and leaf and root ADC activity under salt stress, possibly resulting in

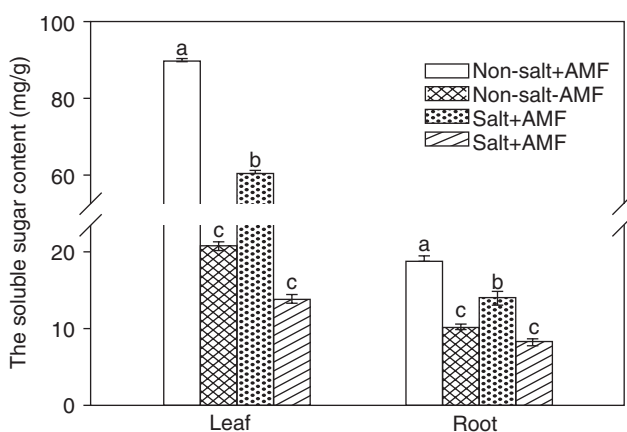


Fig 1 Effect of AMF-inoculation on soluble sugar concentration in leaf and root of trifoliolate orange seedlings under non-salt (0 mM NaCl) and salt stress (100 mM NaCl) conditions. Data showed significant difference ($P < 0.05$) with different letter above the bars.

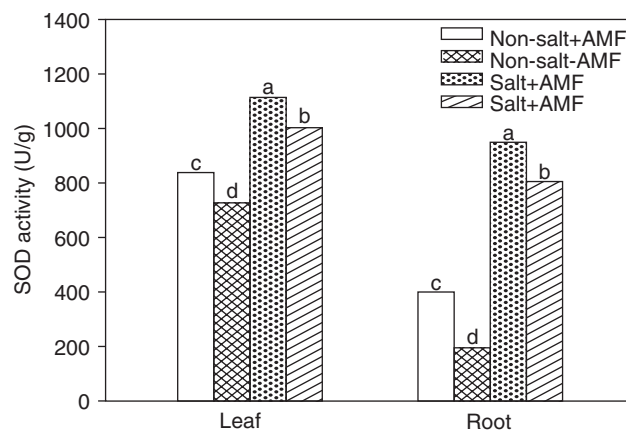


Fig 2 Effect of AMF-inoculation on SOD activity in leaf and root of trifoliolate orange seedlings under non-salt (0 mM NaCl) and salt stress (100 mM NaCl) conditions. Data showed significant difference ($P < 0.05$) with different letters above the bars.

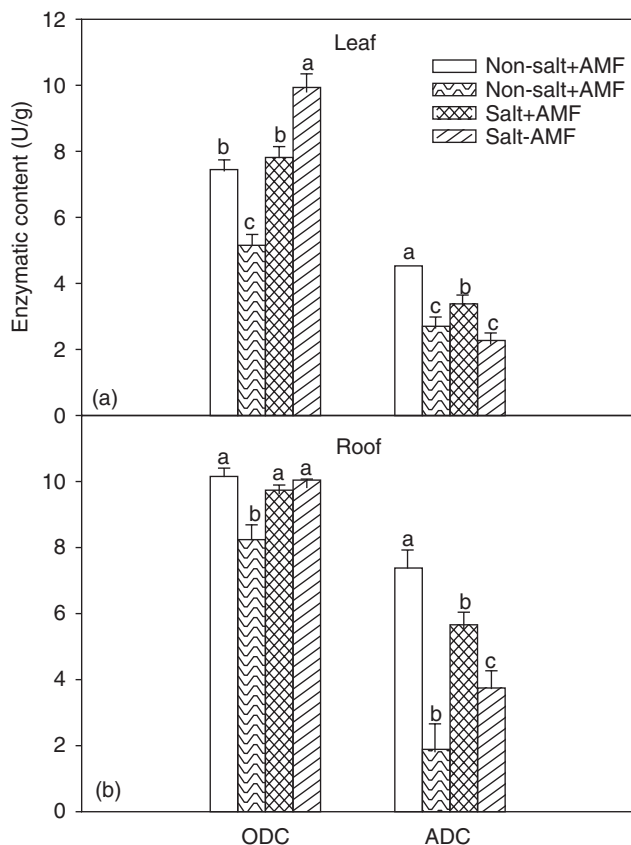


Fig 3 Effect of AMF-inoculation on ODC and ADC activity in leaf and root of trifoliata orange seedlings under non-salt (0 mM NaCl) and salt stress (100 mM NaCl) conditions. Data showed significant difference ($P < 0.05$) with different letters above the bars.

elevated Put accumulation. Wu *et al.* (2012) showed that AMF colonization was associated with accelerated accumulation of Put evident from an increase in ADC and ODC activity in *Citrus tangerina* plants. Hence, ADC pathway of Put was more sensitive to mycorrhization than ODC pathway of Put under salt stress conditions. However, Franceschetti *et al.* (2004) reported that ODC was the most active enzyme in *Nicotiana tabacum* overexpressing *Datura stramonium* plants. It appears that AMs mainly stimulated ODC pathway to synthesize Put in trifoliata orange under salt stress conditions.

Mycorrhizal inoculation was also associated with distinct changes in PAO and DAO activity in leaf and root (Fig 4). In leaf, AM-seedlings displayed considerably lower DAO and PAO activity under salt stress and non-salt stress conditions. In root, mycorrhizal inoculation did not alter DAO activity under salt stress condition but increased DAO activity under non-salt stress and decreased PAO activity under non-salt and salt stress conditions. In plants, Put is degraded by DAO, while Spd and Spm are degraded by PAO into H_2O_2 (Couée *et al.* 2004). AM-plants showed a greater SOD activity for H_2O_2 . Therefore, AM-trifoliata orange plants, lower Put, Spd, and Spm degradation could be the strong possibility under salt stress conditions. Hussain *et al.* (2011) observed sub-optimal growth by Put

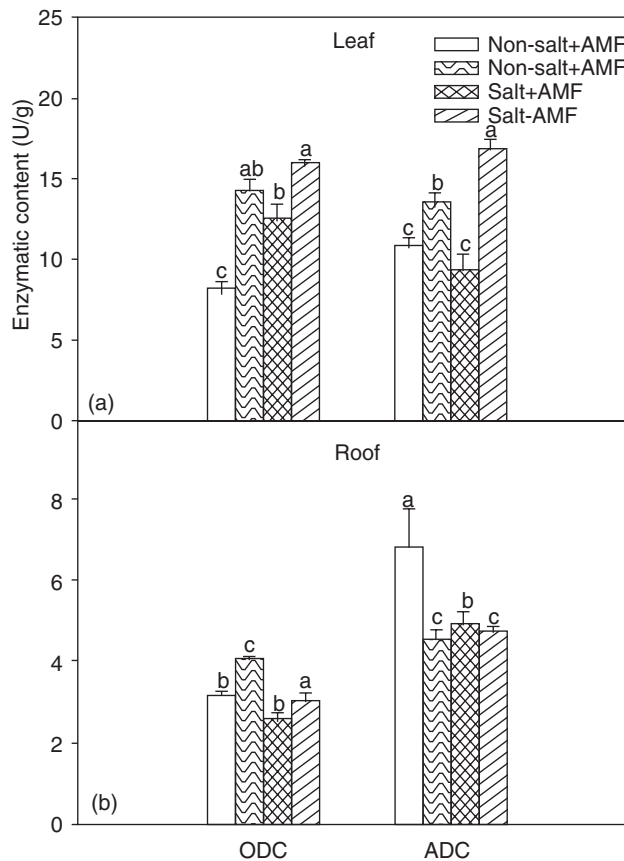


Fig 4 Effect of AMF-inoculation on PAO and DAO activity in leaf and root of trifoliata orange seedlings under non-salt (0 mM NaCl) and salt stress (100 mM NaCl) conditions. Data showed significant difference ($P < 0.05$) with different letters above the bars.

and eliminate ROS (especially H_2O_2) by Spd and Spm.

Trifoliata orange seedlings inoculated with AMF expressed considerably strong root morphological traits under salt stress, stimulating further the soluble protein accumulation coupled with enhanced activities of ADC and SOD, reduced PAO activity and ROS accumulation under salt stress, the necessary biochemical pre-conditions for enhancement of salt tolerance.

ACKNOWLEDGEMENT

This work was funded by the National Natural Science Foundation of China (No. 31301737), the General Program of Agricultural Science and Technology of Taizhou city (No. 121KY18), the Science and Technology Innovation Ability Promotion Project of Zhejiang Academy of Agricultural Sciences (No. 2013R27Y01E01), and the Young Talent Development Program of Zhejiang Academy of Agricultural Sciences.

REFERENCES

- Abdel Latef A A H and Miransari M. 2014. The role of arbuscular mycorrhizal fungi in alleviation of salt stress. (*In*) *Use of Microbes for the Alleviation of Soil Stresses* pp 23–38. Miransari M (Ed). Springer Science+Business Media, New York.

- Al-Garni S M S. 2006. Increasing NaCl-salt tolerance of a halophytic plant *Phragmites australis* by mycorrhizal symbiosis. *American-Eurasian Journal of Agricultural and Environment Science* **1**: 119–26.
- Asghari H R, Marschner P, Smith S E and Smith F A. 2005. Growth response of *Atriplex nummularia* to inoculation with arbuscular mycorrhizal fungi at different salinity levels. *Plant and Soil* **273**: 245–56.
- Couée I, Hummel I, Sulmon C, Gouesbet G and Amrani A E. 2004. Involvement of polyamines in root development. *Plant Cell, Tissue and Organ Culture* **76**: 1–10.
- Dionisio-Sese M L and Tobita S. 1998. Antioxidant responses of rice seedlings to salinity stress. *Plant Science* **135**: 1–9.
- Evelin H, Kapoor I R and Giri B. 2009. Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. *Annals of Botany* **104**: 1 263–80.
- Feng G, Zhang F S, Li X L, Tian C Y, Tang C and Rengel Z. 2002. Improved tolerance of maize plants to salt stress by arbuscular mycorrhiza is related to higher accumulation of soluble sugar in roots. *Mycorrhiza* **12**: 185–90.
- Franceschetti M, Fornalé S, Tassonia A, Zuccherelli K, Mayer M J and Bagni N. 2004. Effects of spermidine synthase overexpression on polyamine biosynthetic pathway in tobacco plants. *Journal of Plant Physiology* **161**: 989–1001.
- Giannopolitis C N and Ries S K. 1997. Superoxide dismutases: occurrence in higher plants. *Plant Physiology* **59**: 309–14.
- Gutjahr C, Casieri L and Paszkowski U. 2009. *Glomus intraradices* induces changes in root system architecture of rice independently of common symbiosis signaling. *New Phytologist* **182**: 829–37.
- Hussain S S, Ali M, Ahmad M and Siddique K H M. 2011. Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. *Biotechnology Advances* **29**: 300–11.
- Imlay J A. 2003. Pathways of oxidative damage. *Annual Review of Microbiology* **57**: 395–418.
- Kaur-Sawhney R, Tiburcio A F, Altabella T, Galston A W, Vegetal L F and Farmacia F. 2003. Polyamines in plants: an overview. *Journal of Cell and Molecular Biology* **2**: 1–12.
- Kerepesi I and Galiba G. 2014. Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. *Crop Science* **40**: 482–7.
- Kusano T, Berberich T, Tateda C and Takahashi Y. 2008. Polyamines: essential factors for growth and survival. *Planta* **228**: 367–81.
- Liu J H, Wang W, Wu H, Gong X and Moriguchi T. 2015. Polyamines function in stress tolerance: from synthesis to regulation. *Frontiers in Plant Science* **6**: 827.
- Munns R. 1993. Physiological process limiting plant growth in saline soils: some dogmas and hypotheses. *Plant Cell and Environment* **16**: 15–24.
- Nemati I, Moradi F, Gholizadeh S, Esmaeili M A and Bihamta M R. 2011. The effect of salinity stress on ions and soluble sugars distribution in leaves, leaf sheaths and roots of rice (*Oryza sativa* L.) seedlings. *Plant Soil and Environment* **57**: 26–33.
- Panda S K and Khan M H. 2004. Changes in growth and superoxide dismutase activity in *Hydrilla verticillata* L. under abiotic stress. *Brazilian Journal of Plant Physiology* **16**: 115–8.
- Phillips J M and Hayman D S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**: 158–61.
- Saha J, Brauer E K, Sengupta A, Popescu S C, Gupta K and Gupta B. 2015. Polyamines as redox homeostasis regulators during salt stress in plants. *Frontiers in Environmental Science* **3**: 21.
- Shrivastava P and Kumar R. 2015. Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi Journal of Biological Sciences* **22**: 123–31.
- Smith S E and Read D J. 2008. Mycorrhizal symbiosis. Academic Press, San Diego, CA, USA:
- Srivastava A K and Shyam Singh. 2008. Citrus nutrition research in India : Problems and prospects. *Indian Journal of Agricultural Sciences* **78**: 3–16.
- Srivastava A K and Shyam Singh. 2009. Citrus decline : Soil fertility and plant nutrition. *Journal of Plant Nutrition* **32**: 197–245.
- Wu Q S, Zou Y N and He X H. 2013. Mycorrhizal symbiosis enhances tolerance to NaCl stress through selective absorption but not selective transport of K⁺ over Na⁺ in trifoliate orange. *Scientia Horticulturae* **160**: 366–74.
- Wu Q S, Zou Y N, Liu W, Ye X F, Zai H F and Zhao L J. 2010. Alleviation of salt stress in citrus seedlings inoculated with mycorrhiza: change in leaf antioxidant defense system. *Plant Soil and Environment* **56**: 470–5.
- Wu Q S, He X H, Zou Y N, Liu C Y, Xiao J and Li Y. 2012. Arbuscular mycorrhizas alter root system architecture of *Citrus tangerine* through regulating metabolism of endogenous polyamines. *Plant Growth Regulation* **68**: 27–35.
- Wu Q S, Lou Y G and Li Y. 2015. Plant growth and tissue sucrose metabolism in the system of trifoliate orange and arbuscular mycorrhizal fungi. *Scientia Horticulturae* **181**: 189–93.
- Zhang F, Du P, Song C X and Wu Q S. 2015. Alleviation of mycorrhiza to magnesium deficiency in trifoliate orange: changes in physiological activity. *Emirates Journal of Food and Agriculture* **27**: 763–9.
- Zou Y N, Liang Y C and Wu Q S. 2013. Mycorrhizal and non-mycorrhizal responses to salt stress in trifoliate orange: plant growth, root architecture and soluble sugar accumulation. *International Journal of Agriculture and Biology* **15**: 565–9.
- Zou Y N, Huang Y M, Wu Q S and He X H. 2015. Mycorrhiza-induced lower oxidative burst is related with higher antioxidant enzyme activities, net H₂O₂ effluxes, and Ca²⁺ influxes in trifoliate orange roots under drought stress. *Mycorrhiza* **25**: 143–52.