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# EFFECTS OF *RHIZOGLOMUS INTRARADICES* ON PLANT GROWTH AND ROOT ENDOGENOUS HORMONES OF TRIFOLIATE ORANGE UNDER SALT STRESS

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## ABSTRACT

Soil salinity has negative effects on crop growth and production, while it is not clear whether mycorrhizal symbiosis mitigates the negative effect on citrus plants under salt stress. This study was to investigate the effects of an arbuscular mycorrhizal fungus, *Rhizoglomus intraradices* on plant growth, root morphology, leaf relative water content (RWC), and root endogenous hormone levels of trifoliate orange (*Poncirus trifoliata*) seedlings exposed to 0 and 150 mmol/L NaCl for 4 weeks. The 150 mmol/L NaCl treatment significantly inhibited root mycorrhizal colonization by 38%. Salt stress significantly reduced plant growth, root morphology, and RWC, while mycorrhizal seedlings represented greater growth performance (height, stem diameter, leaf number, and total plant biomass), root morphological traits (length, area, volume, diameter, and lateral root number) and RWC under non-salt and salt stress. Mycorrhizal fungal inoculation significantly increased root abscisic acid (ABA), indole-3-acetic acid (IAA), and methyl jasmonate (MeJA) levels by 27%, 16%, and 11% under non-salt and by 17%, 20%, and 14% under salt stress. Mycorrhizal treatment did not alter root gibberellins and zeatin riboside levels under non-salt or salt stress, whereas increased root brassinosteroids levels by 24% only under non-salt stress. It suggests that *R. intraradices* had positive effects on enhancing salt tolerance of trifoliate orange, which is involved in greater plant growth, root development, and root ABA, IAA, and MeJA.

**Key words:** arbuscular mycorrhizal fungi; citrus; phytohormone; salt stress.

## INTRODUCTION

Salt stress, one of the important abiotic stresses in plants, considerably declines crop growth and yields. The most important factor in response to salt damage is phytohormones, although there are varieties of other factors (Mahmud *et al.*, 2016). The mechanism of phytohormones in regulating the salt responses can be divided into two groups, namely, positive-related hormones (e.g., cytokinins (CKs), brassinosteroids (BRs), auxin, and gibberellins (GAs)) and stressed hormones (e.g., abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA)) (Kosová *et al.*, 2012).

Arbuscular mycorrhiza (AM) is a symbiotic association formed with arbuscular mycorrhizal fungi (AMF) and 80% of terrestrial plant roots. Studies in the past indicated that AMF improved plant growth and development, as well as conferred the vegetation restoration (Carvalho *et al.*, 2004). Although salt stress may restrain the development of AMF, mycorrhizal symbiosis still considerably promoted plant growth under salt stress (Al-Karaki, 2000). The stimulated effects of mycorrhiza can be explained by relevant mechanisms, including improved nutrition of host plants, higher $K^+/Na^+$ ratio, better osmotic adjustment, enhancement of antioxidant protected systems, and molecular changes (Evelin *et al.*, 2009; Wu *et al.*, 2013). Even so, phytohormones as the stressed signals are not known under mycorrhization, especially in salt stress.

Citrus, as an important economic crop, is widely cultivated around the world. Citrus plants are one of salt-sensitive horticultural crops. In this background, the purpose of the present study was conducted to evaluate AMF effects on plant growth, root morphology, and root hormone levels of trifoliate orange (*Poncirustrifoliata* L. Raf., a citrus rootstock) under salt stress.

## MATERIALS AND METHODS

**Plant set-up:** The 5-leaf-old trifoliate orange seedlings without mycorrhization were transplanted into 1.9–L pots containing autoclaved (0.11 MPa, 121°c, 1.5 h) substrates (soil : sand = 4 : 1, v/v).

An AM fungus *Rhizoglomus intraradices* (N.C. Schenck& G.S. Sm.) C. Walker & A. Schüßler was used in our study. When the seedlings were transplanted, 1400 spores of this AM fungal strain were inoculated in a pot as the mycorrhizal treatment. The non-mycorrhizal group also received the same amounts of autoclaved (0.11 MPa, 121°c, 1 h) inoculums.

After two months of AMF inoculation, salt treatments (0 and 150 mmol/L NaCl) were applied. In order to avoid the salt shock, 150 mmol/L NaCl solutions were gradually increasing with a 50 mmol/L NaCl gradient in three days. Subsequently, a 150 mL designed NaCl solution was weekly watered into each pot. Such salt treatments were maintained for four weeks until the plants were harvested. These seedlings were grown in a glasshouse of Yangtze University campus between March 18 and July 18, 2015.

**Experimental design:** The experiment contained four treatments in a completely randomized arrangement with two factors: inoculation with or without *R. intraradices* (+AMF; -AMF), and salt treatments with 0 and 150 mmol/L NaCl solution (non-salt stress; salt stress). Each treatment replicates five times, with three seedlings per pot.

**Determinations of variables:** The whole seedlings were determined for total fresh weight at harvest, and then divided into the roots and the shoots. The number of lateral roots at different orders was calculated. Root morphological traits that include total length, projected area, surface area, volume, and average diameter were determined by an EPSON Flatbed Scanner (V700, Seiko Epson Corp, Japan) and a WinRHIZO 2007d (Regent Instruments Incorporated, Quebec, Canada).

Mycorrhizal structure was stained by the protocol described by Phillips and Hayman (1970), in terms of 0.05% (w/v) trypan blue. Root AMF colonization was expressed as the percentage of colonized root lengths versus total root lengths.

Leaf relative water content (RWC) was carried out as per Bajji and Kinet (2001).

Root endogenous hormones, including BRs, GAs, ABA, indole acetic acid (IAA), zeatin riboside (ZR), and methyl jasmonate (MeJA), were extracted by the protocol of Chen *et al*. (2009) and were determined with the Enzyme-Linked Immunosorbent Assay (ELISA).

**Statistical analysis:** The experimental data were statistically analyzed according to the variance (ANOVA) of SAS software (8.1v), and the significant difference between the treatments was compared as per the Duncan's Multiple Range (DMR) test at 0.05 levels.

## RESULTS AND DISCUSSION

**Mycorrhizal colonization in roots:** Our study showed no colonization by AMF found in the roots of the non-AMF seedlings, regardless of NaCl levels used. Root colonization of AMF seedlings was 59.26% and 36.86% under 0 and 150 mmol/L NaCl, respectively (Table 1). Moreover, NaCl treatment considerably decreased root mycorrhizal colonization by 38%, as compared with no-NaCl treatment. The result is in line with earlier studies (Zou *et al*., 2013; Wang *et al*., 2016). The inhibition of both spore germination and hyphal growth may explain such reduction of root colonization under salt stress.

**RWC:** The present study showed that salt stress significantly reduced leaf RWC, no matter whether AM or non-AM seedlings (Fig. 1). Mycorrhizal treatment significantly increased leaf RWC by 9% under non-salt stress and 17% under salt stress, respectively. This result is similar to the findings of Wu *et al*. (2015) in *Populus cathayana* plants colonized by *Rhizoglomus irregularis*. Possibly, mycorrhizal extraradial hyphae directly take part in water absorption of host plants (Wu *et al*., 2015). Earlier studies showed that water could be taken up from soil for the hydrophilic of hyphae (Allen, 2007) and move through the AMF for the flowing of apoplastic water in the roots (Barzana *et al*., 2012). As reported by Zhang *et al*. (2018), mycorrhizal hyphal water absorbed rate varied from 0.13 to 1.97 mg $H_2O$/h/mm. The water contribution of mycorrhizas is relatively important for host plants exposed to soil water deficient status, relative to soil ample water status.

**Plant growth performance:** Salt stress significantly inhibited plant growth performance, including stem diameter, height, leaf number and total biomass than non-salt stress, irrespective of AMF or non-AMF treatments (Table 1). Inoculation with *R. intraradices* conferred significantly greater plant height, leaf number, stem diameter, and total plant biomass by 64.6%, 50.0%,29.3%, and 256.3% under non-salt stress and by 109.9%, 44.4%,32.5%, and 219.7% under salt stress. Similar results were also observed in strawberry and banana under mycorrhization conditions (Fan *et al*., 2001; Yano-Melo *et al*., 2003). The growth improvement under mycorrhization is due to improved nutrient absorption and root modification (Trouvelot *et al*., 2015).

**Root morphology:** The present study indicated that 150 mM NaCl treatment had the inhibited effects on total root length, root projected area, root surface area, root diameter, root volume and lateral root number (Table 2). As compared with non-AM plants, AM plants exhibited considerably higher root total length, root projected area, root surface area, root e diameter, volume and number of 1$^{st}$ and 2$^{nd}$ order lateral roots by12.7%, 7.1%, 4.8%, 2.6%, 87.5%, 30.2%, and 117.8% under non-salt stress and by 16.4%, 5.0%, 5.5%, 3.9%, 63.6%, 46.9%, and 67.1% under salt stress, respectively. This result is in accordance with previous studies in maize and citrus under AMF inoculation conditions (Sheng *et al*., 2009; Wu *et al*., 2010). Better root morphology of AM plants under salt stress means the enhanced absorptive capacity and the viability of root system in soils (Yi *et al*., 20007), which benefits the growth and surviving of the host plant (Wu *et al*., 2010).

**Changes in root endogenous hormone levels**

**BRs:** BRs, a kind of steroid hormones, are necessary for plant growth and development, and can tolerate environmental stresses by inducing antioxidant activities

(Bajguz and Piotrowska-Niczyporuk, 2014). In this study, salt treatment did not significantly alter root BR concentration in mycorrhizal and non-mycorrhizal trifoliate orange seedlings, while mycorrhizal inoculated treatment significantly increased root BR levels only in non-salt-stress-treated plants (Fig. 2). Perhaps in salt stress, the regulation of AMF to other phytohormones is sufficient for plants to resist salt stress.

**GAs:** GAs are an essential for many plants in response to abiotic stress and also take part in plant growth and development (Colebrook *et al.*, 2014). In our study, root GAs concentration of AM and non-AM plants was significantly increased by salt stress, relative to non-salt stress (Fig. 2), indicating that root GAs have a strong response to salt stress in trifoliate orange. However, AMF inoculation had not significant effects on root GAs of trifoliate orange seedlings, irrespective of non-salt and salt stress. Studies in the past showed that GAs had an adverse impact on the colonization of AMF in host plants (Foo *et al.*, 2013).

**ABA:** ABA is an ubiquitous plant hormone as the signal molecule to respond environmental stresses, including salt stress, pathogen invasion, and drought stress, as well as plant development processes (Stec *et al.*, 2016). Our study showed a strong increase in root ABA levels of AM and non-AM seedlings by salt stress (Fig. 2). Similarly, AMF inoculation resulted in a significant increase in root ABA concentration by 27% and 17% under non-salt and salt stress condition. This is similar to a previous study in *Lycium barbarum* (Liu *et al.*, 2016a). Salt stress can lead to dehydration of plant tissues, while the increase in ABA is an important signal in plant tissue dehydration (Zörb *et al.*, 2013). It is generally believed that under salt stress, ABA content in plants is generally increased, which thereby induces stomatal closure to maintain water balance in plants, decrease photosynthesis, promote arbuscule formation, and induce the synthesis of related stressed proteins, so as to improve stress resistance (Martin-Rodriguez *et al.*, 2011). As a result, mycorrhiza-modulated ABA increase may be a responsive mechanism of AM-enhancing salt tolerance in host plants.

**IAA:** IAA plays an important role in regulating plant growth under adverse stresses (Iqbal and Ashraf, 2007). The study conducted by Kaya *et al.* (2013) showed that exogenous IAA application significantly enhanced the tolerance of salt stress in maize. Our study showed that there was a significant decrease in root IAA levels of AM and non-AM trifoliate orange seedlings under the salt stress versus the non-salt stress (Fig. 2). At the same time, mycorrhizal treatment notably increased root IAA concentration by 16% and 20% under non-salt and salt stress conditions, respectively. The result is similar to the findings of Liu *et al*. (2016b). Furthermore, IAA is closely related to the growth and development of plant roots (Liu *et al.*, 2018). Therefore, the mycorrhizal effect on IAA is effectively associated with AMF-induced growth improvement, root modification and salt tolerance.

**ZR:** ZR is an important form of cytokinin (CTK) transported in the xylem. ZR has been reported to have the ability to enhance plant salt tolerance and temperature stress (Javid *et al.*, 2011). In this study, root ZR concentration was drastically increased by the salt stress by 19% and 24% in AM and non-AM seedlings, respectively (Fig. 2). At the same time, the inoculation with AMF did not significantly increase root ZR concentration under non-salt and salt stress, respectively. Studies have shown that ABA and CTK antagonize each other (Peleg and Blumwald, 2001). Mycorrhizae promoted a significant increase in ABA in our study, which antagonized CTK changes under mycorrhization. Future studies need to analyze the relationship of ABA and CTK in plants under mycorrhization conditions.

**MeJA:** MeJA, a kind of jasmonates (JAs), is involved in seed germination, root growth, fruit ripening and senescence, and plant development (Zhu *et al.*, 2006). In this study, compared with non-salt stress treatment, salt stress significantly reduced root MeJA levels in non-AM seedlings, other than in AM seedlings (Fig. 2). In addition, mycorrhizal inoculation conferred a significant increase in root MeJA concentrations by 11% and 14% under non-salt and salt stress conditions, respectively. Augé (2011) also reported high levels of JAs to enhance the salt tolerance in the host plant. It seems that AM-responded MeJA increase is a potential role of AM plants maintaining better root growth.

**Table 1. Effect of *Rhizoglomus intraradices* inoculation on plant growth performance of trifoliate orange seedlings under non-salt (0 mM) and salt stress (150 mM) conditions.**

| Salt status | AMF status | Root AMF colonization (%) | Plant height (cm) | Stem diameter (mm) | Leaf number (#/plant) | Total plant biomass (g FW/plant) |
|---|---|---|---|---|---|---|
| Non-salt | -AMF | 0.00±0.00c | 25.54±0.99c | 2.83±0.03c | 20±1c | 0.96±0.08c |
| stress | +AMF | 59.26±3.88a | 42.01±3.47a | 3.66±0.23a | 30±2a | 3.42±0.27a |
| Salt stress | -AMF | 0.00±0.00c | 15.53±4.04d | 2.40±0.12d | 18±2c | 0.71±0.46d |
| | +AMF | 36.86±2.72b | 32.59±3.60b | 3.18±0.11b | 26±2b | 2.27±0.42b |

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

**Table 2. Effect of *Rhizoglomus intraradices* inoculation on root morphology of trifoliate orange seedlings under non-salt (0 mM) and salt stress (150 mM) conditions.**

| Salt status | AMF status | Total length (cm) | Projected area ($cm^2$) | Surface area ($cm^2$) | Average diameter (mm) | Volume ($cm^3$) | Number of lateral root | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 1st | 2nd |
| Non-salt stress | -AMF | 236±2b | 12.6±0.4b | 18.8±0.5bc | 5.09±0.04c | 0.64±0.06c | 43±2b | 90±5c |
| | +AMF | 266±9a | 13.5±0.3a | 19.7±0.4a | 5.22±0.09b | 1.20±0.06a | 56±2a | 196±9a |
| Salt stress | -AMF | 219±3c | 12.1±0.2c | 18. 2±0.4c | 5.17±0.04bc | 0.55±0.02d | 32±5c | 76±3d |
| | +AMF | 255±12a | 12.7±0.2b | 19.2±0.4ab | 5.37±0.08a | 0.90±0.06b | 47±1b | 127±6b |

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

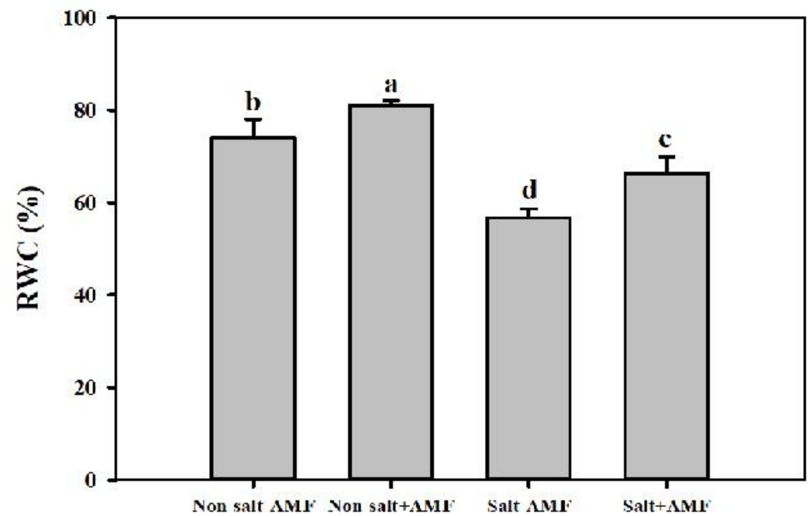


**Fig. 1. Effect of *Rhizoglomus intraradices* inoculation on leaf relative water content (RWC) of trifoliate orange seedlings under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. Data showed significant ($P < 0.05$) differences with different letters above the bars.**

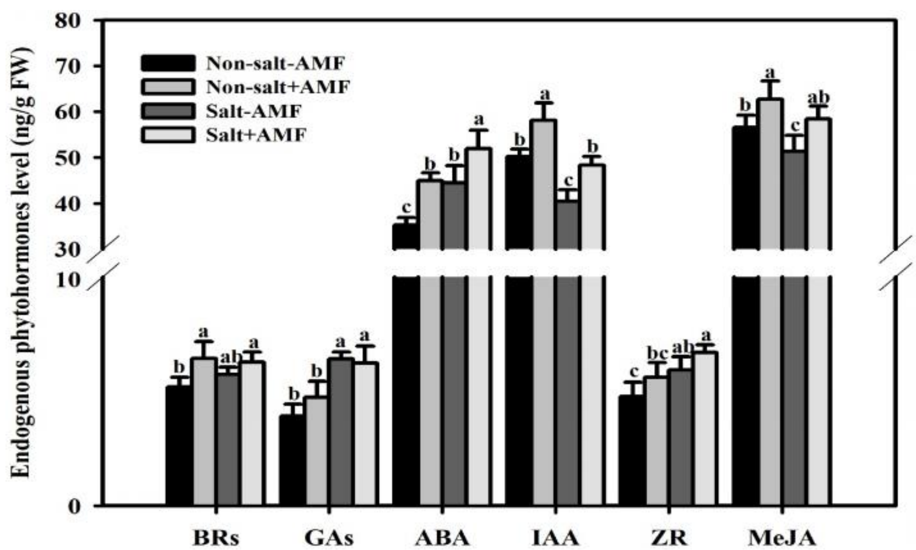


**Fig. 2. Effect of *Rhizoglomus intraradices* inoculation on concentrations of root endogenous brassinosteroids (BRs), gibberellins (GAs), abscisic acid (ABA), indoleacetic acid (IAA), zeatin riboside (ZR), and methyl jasmonate (MeJA) of trifoliate orange seedlings under non-salt (0 mM) and salt stress (150 mM) conditions. Data showed significant ($P < 0.05$) difference with different letter above the bars.**

**Conclusions:** Four-week 150 mM NaCl treatment significantly reduced plant growth and root development of trifoliate orange seedlings. Inoculation with *R. intraradices* significantly improved plant growth performance and root morphology and also maintained greater leaf water status under salt stress. AM plants possessed significantly higher root IAA, ABA, and MeJA concentrations than non-AM plants under salt stress, which is important for the enhancement of salt tolerance in host plants.

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