MYCORRHIZAL SYMBIOSIS ALTERS ROOT H⁺ EFFLUXES AND ROOT SYSTEM ARCHITECTURE OF TRIFOLIATE ORANGE SEEDLINGS UNDER SALT STRESS

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ABSTRACT

H⁺ fluxes are important for exchange of nutrients and regulation of cytosolic pH and cell wall plasticity. Root system architecture (RSA) is a deciding factor in water- and nutrient-uptaked efficiency of a plant. Four-month-old trifoliate orange (Poncirus trifoliata L. Raf.) seedlings inoculated with or without an arbuscular mycorrhizal fungus (Funneliformis mosseae) were conducted to analyze root H⁺ effluxes and RSA under the conditions of 100 mM NaCl, 100 mM KCl, and 100 mM NaNO₃. Although salt stress partly inhibited growth performance (plant height, stem diameter, and leaf number) and RSA variables (length, projected area, volume, and average diameter), mycorrhizal inoculation generally significantly increased these growth performance and RSA traits. There was a more acid environment in rhizosphere of mycorrhizal seedlings than in non-mycorrhizal seedlings, based on bromocresol purple dyeing. The root H⁺ efflux rates were significantly increased by the salt treatments. On the other hand, F. mosseae significantly increased the root H⁺ efflux rates by 14.3%, 31.7%, 10.3%, and 16.7% under the NaCl, KCl, NaNO₃ and non-salt conditions, respectively. It is concluded that mycorrhizal symbiosis increased root H⁺ effluxes and improved RSA of trifoliate orange seedlings under salt stress, which might enhance salt tolerance of the host plant.

Key words: Arbuscular mycorrhizal fungi; Citrus; H⁺ efflux rates; Root system; Salt stress.

INTRODUCTION

Soil salinity, a common abiotic stress, can limit agricultural production all over the world and cause osmotic stress, ion (Na⁺) toxicity and nutrient imbalances (Sairam and Tyagi, 2004). Higher plants also induce many salt tolerance responses to cope with salt induced damage, such as enzymatic and non-enzymatic antioxidant systems, accumulation of compatible solutes, an extensive root system, high selectivity of K⁺ over Na⁺, expression of a number of salt-responsive genes, etc. (Din et al., 2008; Hameed and Ashraf, 2008; Li et al., 2010; Sairam and Tyagi, 2004; Wu and Zou, 2009).

Arbuscular mycorrhizal fungi (AMF) of the Phylum Glomeromycota, soil-inhabiting obligate biotrophic fungi, can form the mutualistic symbiosis with the host plant (Bainard et al., 2011). AMF benefit the host plant by increasing uptake of immobile nutrient elements due to the extraradical mycelium (Gosling et al., 2006). In return, the fungi must receive photosynthetic carbon from the host plant. Most evidence has shown that inoculation with AMF could enhance salt tolerance by improving plant nutrient uptake and ion balance, altering root plasticity, accumulation of compatible solutes, enhancing antioxidant systems, and facilitating water uptake (Echeverria et al., 2008; Nasim, 2010; Wu et al., 2010a, b). H⁺ fluxes present a direct evidence of ion exchange coupling with H⁺ (Sun et al., 2009). A significant correlation between increased P uptake by mycorrhizal and H⁺ efflux levels from hyphae was found in soybean seedlings (Ding et al., 2012). RNA gel blot analysis showed the expression of LHA1 and LHA4 genes encoding H⁺-ATPase in cortical cells containing arbuscles of Lycopersicon esculentum colonized by Glomus intraradices (Rosewarne et al., 2007). However, the mechanisms about the salt tolerance conferred by AMF have not yet been elucidated at H⁺ effluxes.

Root system architecture (RSA), the spatial configuration of a root system in soils, can be used to define the shape and structure of a root system (de Dorlodot et al., 2007). RSA is influenced by both genetic and environmental resources (Spanos et al., 2008). In general, salt stress limits RSA, thus reducing water- and nutrient-uptaked efficiency of plants (Galvan-Ampudia and Testerink, 2011). A small quantity of experiments had shown that inoculation with AMF might modify RSA of the host plant under Phytophthora fragariae stressed or elevated CO₂ concentration conditions (Norman et al., 1996; Gavito et al., 2001). Until now, it is unclear whether AMF affect RSA of citrus plants under salt stress conditions.

The objective of the present study is to evaluate the RSA and the root H⁺ effluxes of trifoliate orange (Poncirus trifoliata L. Raf.) inoculated with or without AMF under salt stress.
MATERIALS AND METHODS

Experimental design: The experiment was arrayed in a completely randomized factorial design with two factors. The first factor included two levels of mycorrhizal inoculation: Funneliformis mosseae and non-AMF. The second factor consisted of four levels of salt-stress environments viz. NaCl (Na⁺ and Cl⁻), KCl (Cl⁻), NaNO₃ (Na⁺), and non-salinity. There were eight treatments in four replications per treatment, resulting in a total of 32 pots (three seedlings per pot).

Plant and fungal materials: Seeds of trifoliate orange were surface-sterilized with 70% of ethanol for 10 min, rinsed with distilled water, and sown into a plastic pot (17.5 cm upper mouth diameter × 13 cm bottom mouth diameter ×16.5 cm height) previously filled with 2.8 kg of autoclaved (121°C, 0.11 MPa, 2 h) mixture of soil, vermiculite and sphagnum (5/1/1, v/v/v), whose physical-chemical characteristics are pH 6.3, 9.8 g/kg organic matter, and 17.71 mg/kg available phosphorus.

The mycorrhizal fungus used here was F. mosseae (T.H. Nicol. & Gerd.) C. Walker & A. Schüßler [No.: BGC XJ02]. The mycorrhizal inoculums were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, and consisted of the infected root segments of Sorghum vulgare, spores, extraradical hyphae and growth substrates of soil and river sand (1/3, v/v). For AMF treatment, 15 g (30 spores/g) of mycorrhizal inoculums was placed below the seeds during sowing. The non-AMF pots received an equal amount of autoclaved (121°C, 0.11 MPa, 2 h) inoculums together with a 2 mL aliquot of a filtrate of mycorrhizal inoculums.

All the AMF and non-AMF seedlings were grown in the plastic greenhouse without any heated equipments from March 27 to July 11, 2010. During the experiment, the photo flux density ranged from 576 to 869 μmol/m²/s, day/night temperature was 18–35/14–30°C, and relative air humidity varied from 70% to 95%.

Salt stress: Two months after the mycorrhizal inoculation, the mycorrhizal and non-mycorrhizal plants were subject to salt stress with 100 mM NaCl, 100 mM KCl, and 100 mM NaNO₃. The salt treatments were gradual, beginning with 25 mM and then increasing in 25 mM per day until reaching the desired 100 mM at a 4-d-period to avoid osmotic shock. Generally, a 300 mL of the salt treatments was supplied into the designed pot enough for excess solution flowed out of the bottom of the pot. The non-salinity pots also received an equal amount of distilled water. The treated plants were watered every five days to keep the salt effects.

RSA and plant growth determinations: After 45 days of the salt treatments, all the plants were harvested and divided into shoots and roots. Plant height, stem diameter, and leaf number per plant were directly determined before plant harvest. The root system per plant was scanned by the Epson Perfection V700 Photo Dual Lens System (J221A, Indonesia) and analyzed by the professional WinRHIZO software in 2007 (Regent Instruments Inc., Quebec, Canada). The variables of RSA, including length, projected area, surface area, volume and length were automatically obtained.

Qualitative analysis of root H⁺ effluxes: Qualitative analysis of H⁺ effluxes of the intact root systems was obtained by the method of Peng et al. (2007) with slight modification. Simply, the fresh root systems were placed upon the indicator culture medium at 25°C for 5 h in darkness, which consisted in 0.75% agar, 0.006% bromocresol purple, 1 mM CaSO₄ and 2.5 mM K₂SO₄. The pH was adjusted to 5.6–6.0. The color change of the culture medium was observed and photographed.

Quantitative analysis of root H⁺ effluxes: The root H⁺ efflux rates were measured following the method described by Peng et al. (2007) with slight modification. The fresh root systems from each treatment were weighted as the fresh weight and then gently put on the culture medium at 25°C for 5 h in darkness containing 0.006% bromocresol purple, 1 mM CaSO₄ and 2.5 mM K₂SO₄. Subsequently, the pH value was recorded by an acidimeter (PHS-3C, Leici, Shanghai Youke Instrument. Co., Ltd., China). The root H⁺ efflux rates were expressed as μmol H⁺ efflux per g root fresh weight at one hour.

Statistical analysis: All data were subjected to analysis of variance (ANOVA) with SAS software (version 8.1). Fisher’s protected the least significant differences (LSD, P<0.05) were used to compare the treatment means.

RESULTS AND DISCUSSION

Plant growth performance: Salt stress is known to restrict plant growth. In the present study, only NaCl treatment significantly inhibited plant height and leaf number of the non-AMF seedlings, compared with the non-salinity + non-AMF seedlings (Table 1). In general, KCl and NaNO₃ treatments represented better growth performance of mycorrhizal and non-mycorrhizal seedlings than NaCl treatment. On the other hand, the seedlings inoculated with F. mosseae exhibited significantly higher plant height, stem diameter and leaf number per plant than the non-AMF seedlings, irrespectively of salt treatments. The result is in harmony with the findings of Porras-Soriano et al. (2009), who observed that F. mosseae, G. intraradices or G. claroideum significantly increased stem diameter, number of shoots, and shoot length in olive plants growing under saline or non-saline conditions. The growth improvement caused by AMF colonization might
be related to increase of P uptake and alteration of RSA (Wu et al., 2011b).

**Root system architecture (RSA):** RSA can be influenced by numerous environmental factors such as soil salinity, availability and distribution of water and nutrients, soil microorganisms, etc. (Wang et al., 2009). In the present study, compared to the non-salinity control, NaCl treatment generally decreased RSA variables such as root length, root projected area, root surface area, and root volume, and KCl and NaNO₃ treatments partly decreased the RSA variables. On the other hand, mycorrhizal seedlings showed higher root length, root projected area, root surface area and root volume, and lower root average diameter than non-mycorrhizal seedlings, regardless of salt stressed or not. The result is consonant with that in inoculation with *F. mosseae, G. versiforme* and *Paraglomus occultum* significantly altered the root traits of trifoliate orange seedlings (Wu et al., 2011a). Greater root traits of mycorrhizal trifoliate orange with large surface area and smaller root diameter would help the host plant to uptake more water and nutrients (Ashraf et al., 2005). As a result, the root alteration caused by AMF means that the mycorrhizal seedlings were more salt-tolerant than non-mycorrhizal seedlings.

**Root H⁺ effluxes:** Generally, bromocresol purple exhibits yellow color under acid conditions and purple color under alkaline conditions, respectively. The response of bromocresol purple to changes in pH of the medium has been used to compare H⁺ effluxes (Jackson and Crofts, 1969). In the present study, both mycorrhizal inoculations and salt treatments obviously induced different colors of the culture medium (Fig. 1). In general, mycorrhizal roots exhibited more yellow color than non-mycorrhizal roots, irrespectively of salt treatments (Fig. 1). The result suggests that mycorrhizal inoculation could induce the roots releasing more H⁺ into rhizosphere of trifoliate orange. On the other hand, salt treatments resulted in increasing H⁺ effluxes into rhizosphere, and the rank was NaCl > KCl > NaNO₃ > non-salinity (Fig. 1).

Although the qualitative analysis of root H⁺ effluxes could clarify the mycorrhizal and salt effects, these root weights were obviously different among the treatments. Therefore, the quantitative analysis of root H⁺ efflux rates would reflect the effects of AMF and salt treatments on H⁺ effluxes by rule and line, based on the fresh root weights and incubated times. This experiment showed that root H⁺ efflux rates ranged from 0.35–040 µmol/g/h under the NaCl conditions, from 0.41–0.54 µmol/g/h under the KCl conditions, from 0.29–0.32 µmol/g/h under the NaNO₃ conditions, and from 0.24–0.28 µmol/g/h under the non-salinity conditions (Fig. 2). The root H⁺ efflux rates were significantly increased by the salt treatments and ranked the salt treatments in order of KCl > NaCl > NaNO₃ > non-salinity, regardless of the seedlings inoculated with AMF or non-AMF (Fig. 2). The result suggests that CI might have precedence over both NaCl and Na⁺ in root H⁺ effluxes. The result is consistent with the findings of Sun et al. (2009), who observed a strong H⁺ efflux in the root apex of sos1 mutants (Arabidopsis) given the NaCl shock.

On the other hand, inoculation with *F. mosseae* significantly increased the root H⁺ efflux rates by 14.3%, 31.7%, 10.3%, and 16.7% under the NaCl, KCl, NaNO₃ and non-salinity conditions, respectively (Fig. 2). The result suggests that under salinity and non-salinity conditions, mycorrhizal symbiosis can induce the root H⁺ effluxes more into the rhizosphere of the host plant, leading to an increased capacity of the mycorrhizal roots to acidify the rhizosphere. The result is in agreement with the findings of Ramos et al. (2009), who observed a high vanadate-sensitive H⁺ efflux in maize roots inoculated with *G. clarum* during the establishment phase of AM interaction. Interestingly, those increments were found at the elongation zone and root hairs but not at the apex and mature zones. Bago et al. (1998) also found high acidic pH values around extraradical hyphae and spores. There is increasing evidence that a H⁺-ATPase transport protein was localized in plant membrane around arbuscule hyphae (Gianinazzi-Pearson et al., 1991, 2000), and

<table>
<thead>
<tr>
<th>Salt treatment</th>
<th>AMF</th>
<th>Plant height (cm)</th>
<th>Stem diameter (cm)</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl +AMF</td>
<td>17.5±1.2b</td>
<td>0.260±0.008ab</td>
<td>14.5±0.9bc</td>
<td></td>
</tr>
<tr>
<td>NaCl −AMF</td>
<td>11.0±1.1d</td>
<td>0.235±0.012d</td>
<td>10.8±0.2e</td>
<td></td>
</tr>
<tr>
<td>KCl +AMF</td>
<td>19.7±0.6a</td>
<td>0.271±0.007a</td>
<td>15.5±0.2ab</td>
<td></td>
</tr>
<tr>
<td>KCl −AMF</td>
<td>15.1±0.8c</td>
<td>0.259±0.011ab</td>
<td>13.7±0.5c</td>
<td></td>
</tr>
<tr>
<td>NaNO₃ +AMF</td>
<td>19.8±2.0a</td>
<td>0.267±0.009a</td>
<td>15.5±0.6ab</td>
<td></td>
</tr>
<tr>
<td>NaNO₃ −AMF</td>
<td>14.5±1.1c</td>
<td>0.253±0.004bc</td>
<td>13.5±0.4cd</td>
<td></td>
</tr>
<tr>
<td>Non-salinity +AMF</td>
<td>18.0±1.3ab</td>
<td>0.270±0.004a</td>
<td>16.4±0.7a</td>
<td></td>
</tr>
<tr>
<td>Non-salinity −AMF</td>
<td>13.4±1.1c</td>
<td>0.244±0.008cd</td>
<td>12.5±0.8d</td>
<td></td>
</tr>
</tbody>
</table>

Note: Means±SD (n=4) followed by the same letter within a column are not significantly different among treatments at P<0.05.
Table 2. Effect of *Funneliformis mosseae* and salt stress on RSA of trifoliate orange (*Poncirus trifoliata*) seedlings

<table>
<thead>
<tr>
<th>Salt treatment</th>
<th>AMF</th>
<th>Average diameter (cm)</th>
<th>Length (cm)</th>
<th>Projected area (cm²)</th>
<th>Surface area (cm²)</th>
<th>Volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl +AMF</td>
<td>0.448±0.007c</td>
<td>298±4bc</td>
<td>13.4±0.4c</td>
<td>41.9±1.2c</td>
<td>0.470±0.023c</td>
<td></td>
</tr>
<tr>
<td>NaCl-AMF</td>
<td>0.573±0.016a</td>
<td>192±10f</td>
<td>10.7±0.5d</td>
<td>33.7±1.5d</td>
<td>0.474±0.027c</td>
<td></td>
</tr>
<tr>
<td>KCl +AMF</td>
<td>0.486±0.023cd</td>
<td>301±13b</td>
<td>14.7±1.0ab</td>
<td>46.1±3.0ab</td>
<td>0.560±0.061ab</td>
<td></td>
</tr>
<tr>
<td>KCl-AMF</td>
<td>0.563±0.020a</td>
<td>231±9e</td>
<td>12.9±0.5c</td>
<td>40.5±1.4c</td>
<td>0.566±0.031ab</td>
<td></td>
</tr>
<tr>
<td>NaNO₃ +AMF</td>
<td>0.508±0.023bc</td>
<td>285±6cd</td>
<td>14.5±0.8b</td>
<td>45.4±2.4b</td>
<td>0.578±0.055a</td>
<td></td>
</tr>
<tr>
<td>NaNO₃-AMF</td>
<td>0.527±0.015b</td>
<td>239±12e</td>
<td>12.5±0.3c</td>
<td>39.4±1.0c</td>
<td>0.518±0.012bc</td>
<td></td>
</tr>
<tr>
<td>Non-salinity +AMF</td>
<td>0.460±0.015d</td>
<td>336±9a</td>
<td>15.5±0.9a</td>
<td>48.7±3.0a</td>
<td>0.564±0.056ab</td>
<td></td>
</tr>
<tr>
<td>Non-salinity-AMF</td>
<td>0.480±0.025d</td>
<td>273±6d</td>
<td>13.1±0.3c</td>
<td>41.0±1.0c</td>
<td>0.492±0.033c</td>
<td></td>
</tr>
</tbody>
</table>

Note: Means±SD (n=4) followed by the same letter within a column are not significantly different among treatments at P<0.05.

*LHA1* and *LHA4* were expressed in cortical cells containing arbuscules (Rosewarne *et al.*, 2007), suggesting that arbuscules of mycorrhizal symbiosis might take part in the H⁺ efflux. However, in the present work, it was unclear whether the mycorrhizal fungus induced the expression of the H⁺-ATPase gene under the salt-treated conditions. The acidic rhizosphere caused by mycorrhizal symbiosis is important to secondary active transporter of organic and inorganic nutrients, turgor regulation, and in the regulation of cell wall plasticity, as suggested in “Acid-Growth Theory” (Ramos *et al.*, 2008). Therefore, the mycorrhizal inducement might help the host plant to enhance the salt tolerance.

![Fig. 1](image1.png) **Fig. 1** Color changes of the culture medium containing different treated root systems of trifoliate orange (*Poncirus trifoliata*) seedlings at 25°C for 5 h in darkness. In the figure, the treatments of the upper row were in turn NaCl+AMF, KCl+AMF, NaNO₃+AMF, and non-salt+AMF from left to right; those of the lower row NaCl–AMF, KCl–AMF, NaNO₃–AMF, and non-salt–AMF.

![Fig. 2](image2.png) **Fig. 2** Effect of *Funneliformis mosseae* and salt stress on root H⁺ efflux rates of trifoliate orange (*Poncirus trifoliata*) seedlings. Data (means ± SE, n=4) followed by the same letter above the bars are not significantly different among treatments at P<0.05.

**Conclusions:** Based on the quantitative and qualitative analysis of root H⁺ effluxes, the salt treatments and inoculation with *F. mosseae* could significantly induce more H⁺ effluxes into rhizosphere, resulting in a more acidic rhizosphere environment of trifoliate orange seedlings. Generally, higher H⁺ levels were found in mycorrhizal seedlings grown in KCl conditions. On the other hand, mycorrhizal seedlings recorded better RSA variables, which would benefit the host plant to enhance salt tolerance.

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