Arbuscular Mycorrhizal Alleviated Ion Toxicity, Oxidative Damage and Enhanced Osmotic Adjustment in Tomato Subjected to NaCl Stress

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Abstract: This study investigated several aspects related to salt tolerance in arbuscular mycorrhizal (AM) tomato. Non-AM and AM tomato plants were grown in pots with organic soil and watered with different level of NaCl solution (0, 0.5 and 1%). Concentration of Na⁺ and Cl⁻ in shoots or roots, solute accumulation, MDA content, O₂⁻ generation rate and other parameters were determined for both treatments under continuous salt stress. A significantly positive impact of AM fungi on plant growth was observed. Leaves and roots accumulated more soluble sugar and showed higher leaf water potential (Ψ), soluble protein in AM symbiosis. Proline was also higher in AM roots, while the opposite was observed in leaves. MDA content, O₂⁻ generation rate increased in both AM and non-AM seedlings under salt stress, especially in non-AM seeding. In addition, AM colonization significantly decreased Na⁺ concentration in roots and shoots, but reduction of Cl⁻ concentration was indistinctive. As a consequence, we suggest that the improve salt tolerance of AM tomato is related to lower Na⁺ toxicity in shoots and roots, higher accumulation of soluble sugar, protein and proline under salinity. These could result in a greater osmotic adjustment in salinity.

Abbreviations: AM, arbuscular mycorrhiza; AMF, arbuscular mycorrhizal fungi; EC, electrical conductivity; ECs, electrical conductivity of substrate; MDA, malondialdehyde; O₂⁻, oxygen radical; TBA, thiobarbituric acid; Ψ, water potential.

Key words: Arbuscular mycorrhizal fungi · Ion toxicity · Osmotic adjustment · Salt stress · Tomato

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are associated with the roots of approximately 90% of terrestrial plant species [1]. These fungi establish symbioses with roots, contribute to improve water use [2,3], nutrient uptake and increase tolerance to biotic and abiotic stresses [2,4].

Salinity toxicity is a worldwide agricultural and eco-environmental problem. Generally, salinity inhibits plant growth and reduced its productivity. It is reported that AMF inoculation can improve the biomass of host plants, such as onion [5], pepper [6], guayule [7] and enhance water uptake into cucumber plants under salt stress[8]. However, the mechanism that AMF enhances salt resistance and water uptake remains unclear.

The effect of AMF on the salt resistance of tomato has been studied by Duke et al. [9] and Al-Karaki [10]. It has been shown that AM colonization enhanced water and nutrient uptake. Al-Karaki et al. [11] reported that mycorrhizal tomato presented greater tolerance to salt stress which could be due to a more efficient nutrient uptake by mycorrhizal plants, especially for phosphorus uptake. To investigate whether the enhanced tolerance was due to greater organic solutes accumulation in AM tomato is very essential under continuous salt stress. So, the physiological mechanism of enhanced salt tolerance of AM tomato still needs further research.

Given that Tomato is a major crop and organic soil culture is a trend in China or in the world, whereas organic soil also faces with salinity, especially in some semi-arid regions, where saline waters are frequently used for irrigation. So the AMF efficiency in organic soil culture may be more promising. The study aimed at evaluating the effects of Glomus mosseae on growth,

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oxidative damage to cell membrane, ion accumulation of tomato in organic soil under salt stress and elucidating the osmotic mechanism of enhanced salt tolerance by AM inoculation.

MATERIALS AND METHODS

Plant Material and Experimental Conditions: Seeds of tomato, *Lycopersicum esculentum* cv Zhongzhia No 91, were obtained from Institute of Vegetables and Flowers, CAAS, Beijing, PR China. Seeds were sterilized by immersing in 70% alcohol for 5 min, rinsed four times with distilled water and kept for germination on wet filter paper in Petri dishes at 28 degrees centigrade. After three days, seeds were planted into polystyrene trays. At the same time, a half of the pots (AM plants) were inoculated with 10 g *Glomus mosseae* (provided by Hungarian Institute of Soil Research) per pot. Non-AMF plants received the same weight of autoclaved inoculum. The inoculum was placed adjacent to each seeding root. 30-day-old seedlings of uniform size, were transplanted to 13 x 13 cm plastic pots containing 0.88 kg organized soil mixture (organic manure, soil and straw is 1:2:1). The soil mix was collected from greenhouse of Institute of Vegetables and Flowers and sterilized (160 degrees centigrade, 4 h). Soil properties were pH 7.26, 11.1% organic matter, 0.15% available phosphorus, 451 mg kg⁻¹ available nitrogen, 518 mg kg⁻¹ available potassium. The experimental pots were placed in greenhouse with natural light at 28/20 degrees centigrade (day/night) from September to December. The photon flux density ranged from 600 to 1200 mol m⁻² s⁻¹, relative humidity was between 65 and 95%.

Mycorrhizal Fungus Inoculum: Mycorrhizal fungus inoculum, consisting of spores, soil, hyphae and infected clove (*Trifolium repens*) root fragment from a stock culture of *Glomus mosseae*, was provided by Hungarian Institute of Soil Research. The inoculated dosage was 10 g inoculum per pot containing approx. 720 spores.

Experimental Design: The experimental design consisted of six treatments crossing two mycorrhizal inoculation levels (non-AMF and *Glomus mosseae*) with three soil salt levels (NaCl solution: treated 0, 0.5, 1.5%). Pots were arranged in a completely randomized block design. Six replicates of each treatment were applied totaling 36 pots (two seedling per pot).

From the 45th days after AMF inoculation, plants (salt treatments) were irrigated every 1, 2 or 3 days with 0.5, 1% NaCl water solution. Salt-free treatments were irrigated with tap water (EC=0.8 mS cm⁻¹). Soil EC values reached 0.9, 4.2, 7.1 mS cm⁻¹, respectively in the 0, 0.5 and 1% treatments and then salt solution irrigation was stopped. Measurement of water potential, soluble sugar, soluble protein, proline, Na⁺, Cl⁻ and MDA were performed every 5 or 10 days and kept the electrical conductivity in the growth substrate(ECs)value until the 100 days after planting. ECs was regularly monitored with a Model LF539 Conductivity Meter (WTW, Weilheim, Germany). When leaching occurred, the leachate was collected and added back to soil to maintain salinity treatments near target levels.

Measured Parameters

Biomass Measurements: The shoot and root were separated at harvest (100 d after planting). The dry weight (DW) was measured after oven drying at 80°C for 2 days.

Water Potential Determination: The fifth leaves from the apices of these seedlings were used for leaf Ψ at 9:00 am. Leaf Ψ was measured by using a pressure chamber [12]. The leaf was immediately placed in chamber and pressured at the speed of 30-50 KPa/min and recorded pressure gauge reading when watched water film on incision.

Soluble Sugar, Soluble Protein and Proline Determination: Soluble sugar content was determined by the anthrone method [12] using sucrose as standard. 0.3 g of fresh samples was placed in a 15 mL tube with 10 mL distilled boil water, boiled at 100°C for 10 min and filtered into 25 mL volumetric flasks, then distilled water was added to scale. Reaction mixture contained 1 mL extracts, 4 mL mixed reagent (0.2 gram anthrone + 100 mL 98% H₂SO₄) and then the mixture was heated at 100°C for 10 min and absorbance read at 630 nm. Soluble protein was determined by coomassie brilliant blue G-250 method [12], using BSA as standard.

For proline determination, fresh samples extracted with 3% sulfosalicyclic acid were placed in a boiling water bath for 10 min and finally filtered through filter paper. Two milliliter of extract was added to 6 mL (final volume) assay media containing 2 mL ninhydrin solution and 2 mL acetic acid and boiled at 100°C for 30 min and then cooled. The formed product was extracted with 5 mL toluene after 1 h by shaking enough, absorbance of the above organic layer was measured at 520 nm [12].

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**Sodium, Chloride Analyses:** Sodium concentrations was measured by flame emission spectrometer after digestion in flasks with H2SO4-salicylic acid-H2O2 [13]. Determination of chloride was carried out by the method of Cheng [14].

The thiobarbituric acid (TBA) was adopted to measure lipid peroxidation in leaves and roots. This determines the concentration of Malonaldehyde (MDA) as an end product of lipid peroxidation. MDA was determined using the method given by Li [12].

Oxygen radical generation rate was measured by the method of Li [12]. 0.5 g of fresh leaf sample was homogenised in an ice bath in 1.5 mL 0.2 M sodium borate buffer (pH 8.8). Homogenate was centrifuged at 4000 rpm for 20 min at 4 degrees centigrade. The supernatant was used as extract for O2- generation rate assayed. The reaction mixture contained 0.5 mL extract, 0.5 mL 50 mM sodium borate buffer (pH 7.8), 1 mL 1 mM Hydroxylamine hydrochloride was incubated at 25 centigrade for 1 h and then 17 mM 1-naphthylamine was added. After shaking enough, the mixture was incubated at 25 degrees Celsius for 20 min and absorbance at 530 nm was measured. The absorbance value should be changed to NO2- concentration according to NO2- standard curve and O2- generation rate was obtained by [NO2-] multiplying 2. O2- generation rate was caculated by noting reaction time and protein content in sample and expressed as nmol min-1 mg-1. Protein was determined by Bradford [15].

**Statistical Analysis:** Data was analyzed and compared by the Duncan's multiple new range test (P<0.05 or P<0.01).

**RESULTS**

**Biomass:** NaCl stress significantly reduced plant growth (Table 1). However, AM seedlings under salt and saltless conditions were significantly taller and stem diameter, shoot and root dry weight increase were significantly larger than corresponding non-AM seedlings.

**Water Relation Parameters:** Leaf \( \Psi \) in AM and non-AM seedlings decreased during the salt stress period (Fig. 1). Whatever the salt concentration was, AM plants displayed higher leaf \( \Psi \) than the corresponding non-AM plants. At 45 days, leaf \( \Psi \) of 0.5% and 1% treatments in AM plants were 5.6 and 8.0% higher than those of non-AM plants respectively.

**Organic Osmotic Adjustment**

**Soluble Sugar:** AM seedlings had higher soluble sugar in leaves and roots than corresponding non-AM seedlings during the experimental stage. (Fig. 2). There was very distinct difference between AM and non-AM seedling in leaves under 0.5% and 1% salt stress at 10 days (Fig. 2a, \( P<0.01 \)). In AM roots, very distinct difference was detected from non-AM seedlings at 20 and 40 days under 1% salt stress (Fig. 2b, \( P<0.01 \)). In addition, a decrease in leaves from 40 days after salt stress was observed, while a constant increase occurred in roots.

**Soluble Protein:** Treatments with NaCl could effectively induce protein. A larger value on this parameter was recorded in AM plants in the experiment period (Fig. 3). From 0 to 40 days after beginning of salt stress, AM and non-AM plants increased 24.36 and 20.64 mg·g\(^{-1}\) at 0.5% level, 27.06 and 24.10 mg·g\(^{-1}\) at 1% level respectively. Consequently, AM plants accumulated more soluble protein than that of non-AM plants under salt stress in our experiment.

**Proline:** Proline content of leaves was lower in AM seedlings regardless of salt treatments (Fig. 4a), while the opposite was observed in roots (Fig. 4b). Significant difference between AM and non-AM seedlings was observed. Plants under 1% salt stress accumulated more proline than that of 0.5% salt stress during the experimental time. After 10 days salt stress, leaf and root proline were increased while at 40 days were decreased.

Table 1: Effect of AMF on the seedlings growth in tomato under salt stress

<table>
<thead>
<tr>
<th>NaCl (%)</th>
<th>AMF</th>
<th>Non-AMF</th>
<th>AMF</th>
<th>Non-AMF</th>
<th>AMF</th>
<th>Non-AMF</th>
<th>AMF</th>
<th>Non-AMF</th>
<th>AMF</th>
<th>Non-AMF</th>
<th>AMF</th>
<th>Non-AMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height</td>
<td>Stem diameter</td>
<td>Dry weight</td>
<td></td>
<td>Height</td>
<td>Stem diameter</td>
<td>Dry weight</td>
<td></td>
<td>Height</td>
<td>Stem diameter</td>
<td>Dry weight</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>AMF</td>
<td>4.80 aA</td>
<td>0.115 aA</td>
<td>6.26 aA</td>
<td>1.37 aA</td>
<td>9.90 aA</td>
<td>0.096 aB</td>
<td>5.99 bB</td>
<td>1.10 bB</td>
<td>9.90 aA</td>
<td>0.096 aB</td>
<td>5.99 bB</td>
</tr>
<tr>
<td>0.5%</td>
<td>AMF</td>
<td>2.38 cC</td>
<td>0.061 bB</td>
<td>5.64 cC</td>
<td>0.86 cC</td>
<td>5.32 cE</td>
<td>0.050 cC</td>
<td>5.38 deDE</td>
<td>0.42 deE</td>
<td>5.38 deDE</td>
<td>0.018 dD</td>
<td>5.08 deE</td>
</tr>
<tr>
<td>1</td>
<td>AMF</td>
<td>2.20 cC</td>
<td>0.052 cC</td>
<td>5.38 deDE</td>
<td>0.42 deE</td>
<td>5.38 deDE</td>
<td>0.018 dD</td>
<td>5.08 deE</td>
<td>0.28 deE</td>
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</tr>
</tbody>
</table>

Note: Data was analyzed by Duncan's multiple new range test and the different capital and small letters indicate significant differences at \( P<0.01 \) and \( P<0.05 \) level, respectively.
Fig. 1: Effect of AMF on leaf water potential of tomato under NaCl stress. Asterisk denotes that AM and non-AM treatments differed significantly, *P<0.05, **P<0.01. In contrast, no asterisk denotes no significant difference. Values are mean ±SE (n=6).

Fig. 2: Effect of AMF on soluble sugar content in leaf and root of tomato under NaCl stress. Rest legend is same as in Fig. 1.

Fig. 3: Effect of AMF on soluble protein level in leaf of tomato under NaCl stress. Rest legend is same as in Fig. 1.

Fig. 4: Effect of AMF on proline level in leaf and root of tomato under NaCl stress. Rest legend is same as in Fig. 1.

**ION TOXICITY:** Na⁺ and Cl⁻ concentration in shoot and root increased with salinity (Fig. 5). AM plants showed lower concentrations than non-AM plants. The Na⁺ and Cl⁻ concentrations were higher in roots than that in shoots. Na⁺ concentration in shoots and roots of non-AM seedlings was 1.11 and 1.22 times of AM plants respectively under saltless condition, 1.57 and 1.26 times under 0.5% salt stress, 1.07 and 1.06 times under...
Fig. 5: Effect of AMF on Cl (a-b) and Na⁺ (c-d) concentration in leaf and root of tomato under NaCl stress. Rest legend is same as in Fig. 1

Fig. 6: Effect of AMF on MDA content in leaf and root of tomato under NaCl stress. Rest legend is same as in Fig. 1
1% salt stress, respectively. AM colonization seemed to decrease Na⁺ under 0.5% salt stress \((p<0.05)\) (Fig. 5c-d) remarkably. The increase of Cl⁻ concentration was not obvious with salinity compared to Na⁺ (Fig. 5a-b). A insignificant differences in Cl⁻ content in shoots and roots was found between AM and non-AM seedlings under salt stress \((p<0.05)\).

**Degree of Cell Membrane Damage**

**MDA Content:** MDA of leaves and roots accumulated constantly under saltless or salt condition (Fig. 6). MDA content in leaves was higher than that in roots. AM colonization reduced MDA accumulation significantly in contrast to non-AM plants at the same salt level \((p<0.05\) or \(p<0.01\) ).

**O₂⁻ Generation Rate:** O₂⁻ generation rate in AM plants was significantly lower than that in non-AM counterparts during experiment stage under salt or saltless condition (Fig. 7). At 5th day after salt stress, O₂⁻ generation rate increased suddenly in AM and non-AM plants. At 40 days, under 0.5 and 1% salt stress, O₂⁻ generation rate in non-AM plants was 2.7 and 3.1 times of AM plants, respectively.

**DISCUSSION**

**AM Inoculation Alleviated Cell Membrane Damage under Salt Stress:** In our experiment, salinity had a negative effect on plant growth and AM colonization increased tomato growth under different salt level stress (Tab 1). Similar result were reported by Ruiz-Lozano *et al.* [2] and AL-Karaki *et al.* [11].

The lowest MDA content and O₂⁻ generation rate in AM tomato was also detected (Fig. 6). The lowestest O₂⁻ generation rate reflects the lowest reactive oxygen level.

The lowest MDA displays higher anti-oxidative ability which reflecting lower injury or higher salt tolerance. Our results confirmed that the AM colonization indeed increased tomato salt resistance by alleviating oxidative damage to cell membrane. The reasons for reducing the cell membrane injury may be due to changing antioxidative defense system or other physiological processes, such as osmotic adjustment by AM colonization.

**Osmotic Adjustment Improved Salt Tolerance in AM tomato:** Osmotic adjustment is considered to be an important component of salt tolerance mechanisms in higher plants. Under salt stress conditions, higher plants accumulate some small molecules including organic solutes (soluble sugar, soluble protein and amino acids etc) and inorganic ions (K⁺, Ca⁺⁺ etc) to make higher osmotic adjustment and maintain a favorable gradient for water flow from soil into roots[16,17].

Of the various organic osmotica, soluble sugar contribute up to 50% of the total osmotic potential in glyophytes subject to saline conditions[18]. Their major functions are osmoprotection, osmotic adjustment, carbon storage and radical scavenging.

Under continuous salt stress, our results showed that the enhanced salt tolerance was closely related to the increase soluble sugar accumulation in leaves and roots of AM plants. The greatest sugar accumulation in AM plant may be due to (1) the ability of plants to cope with salt stress, (2) the sink effect of the mycorrhizal fungus demanding sugars from shoot tissues, (3) the fact that AM colonization enhanced photosynthesis and therefore allowed higher allocation of sugars from leaves to roots. The results of Fig. 2 also showed a sugar content was decreased after 20 days under salt stress, this may be due to the limiting photosynthesis with increasing salt damage.
to plant, however, carbohydrates was still needed for AM growth. So, the accumulation of soluble sugar in roots could have provided the roots with an osmotic mechanism to maintain a favourable Ψ gradient for water entrance into the roots. This result was similar to the study on AM maize under salt stress [19].

Proline is an important organic compound which is involved in the osmotic adjustment [16,20]. Proline concentration has indeed been shown to be higher in many salt tolerant plants. However, the role of proline in osmoregulation and salt tolerance generally has been questioned. Tal et al. [21] reported that more proline accumulated in salt sensitive species of tomato than tolerant wild relatives. A similar negative relationship between proline accumulation and salt tolerance in tomato was observed by Aziz et al. [22]. Similarly, the proline measured in this study accumulated less in leaves of AM plants than in non-AM ones, but higher in roots. The data in our experiment showed that the enhanced osmotic adjustment in roots may alleviate the shoot damage indirectly and thus needed less proline to protect the shoot. So, the proline accumulation in root was important for the enhanced salt tolerance of AM tomato.

Proteins that accumulated in plants under saline conditions may provide a storage form of nitrogen that could be re-utilized when stress was over and may play a role in osmotic adjustment [23]. A higher content of soluble proteins had been observed in salt tolerance than in salt sensitive cultivars of barley [24], sunflower [25]. The study indicated that AM colonization increased the soluble protein accumulation of AM seedlings throughout the period of stress. The increase in protein was induced by AMF infection or by salt stress, which was important for AM plants salt tolerance to be elucidated by studying gene express. In according to previous studies [23,24,25], our results suggested that the increase in protein was contributed to the enhanced tolerance of AM plants.

In short, these increased organic solutes enhanced osmotic adjustment in AM plants and significantly improved water potential (Fig. 1), which caused higher stress adaptation compared to non-AM plants.

**Lower Na⁺ Accumulation Enhanced Salt Tolerance in AM Tomato:** One of the harmful effects of salinity on plant growth involves the excessive accumulation of Na⁺ and Cl⁻ [25,26]. More Na⁺ accumulation resulted in ionic imbalance, specific ion effects and nutrient deficiency symptoms in plants. Copeman [27] reported AM plant reduced Cl⁻ concentration under salt stress. Our results showed that Na⁺ and Cl⁻ concentration, especially Na⁺ was significantly lower in AM tomato compared to non-AM plants, but we did not find significant difference in Cl⁻ concentrations. So, AM colonization helped to sustain ion balance and lower Na⁺ accumulation which played a key role in improving salt tolerance of AM tomato. Accordingly, non-AM plant injured seriously by Na⁺ toxicity. But how the AM symbiosis reduced the two ion was still unclear. The main potential mechanisms is that the enhanced plant growth diluted the ion concentration indirectly [28]. In present study, the analyzing expression of vacuolar Na⁺/H⁺ antiporter genes in dependence on salt and mycorrhizal colonization was particular interest because overexpression of Na⁺/H⁺ antiporters in plants renders them more tolerant to salt stress [29]. According to our results, the future study on expression of Na⁺/H⁺ antiporters may be important to explain the mechanism of enhanced salt tolerance in AM tomato.

**ACKNOWLEDGEMENTS**

This research was supported by cooperation project between China and Hungary (CHIV-31/2004), Office of Education key project of Sichuan Province (072A064) and Shuanghui project of Sichuan Agricultural University. The authors express sincere thanks to Dr Tunde Takacs for providing the AMF inoculums.

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