

Influence of arbuscular mycorrhiza on organic solutes in maize leaves under salt stress

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Abstract A pot experiment was conducted to examine the effect of the arbuscular mycorrhizal (AM) fungus, *Glomus mosseae*, on plant biomass and organic solute accumulation in maize leaves. Maize plants were grown in sand and soil mixture with three NaCl levels (0, 0.5, and 1.0 g kg⁻¹ dry substrate) for 55 days, after 15 days of establishment under non-saline conditions. At all salinity levels, mycorrhizal plants had higher biomass and higher accumulation of organic solutes in leaves, which were dominated by soluble sugars, reducing sugars, soluble protein, and organic acids in both mycorrhizal and non-mycorrhizal plants. The relative abundance of free amino acids and proline in total organic solutes was lower in mycorrhizal than in non-mycorrhizal plants, while that of reducing sugars was higher. In addition, the AM symbiosis raised the concentrations of soluble sugars, reducing sugars, soluble protein, total organic acids, oxalic acid, fumaric acid, acetic acid, malic acid, and citric acid and decreased the concentrations of total free amino acids, proline, formic acid, and succinic acid in maize leaves. In mycorrhizal plants, the dominant organic acid was oxalic acid, while in non-mycorrhizal plants, the dominant organic acid was succinic acid. All the results presented here indicate that the accumulation of organic solutes in leaves is a specific physiological response of maize plants to the AM symbiosis, which could mitigate the negative impact of soil salinity on plant productivity.

Keywords Arbuscular mycorrhiza · Organic solutes · Salt stress · *Zea mays*

Introduction

Salt-affected soils occupy approximately 7% of the global land surface (Ruiz-Lozano et al. 1996). In China alone, salinity affects about 3,630.5×10⁴ hm² land (Shi 1991). In general, salinity limits plant growth and productivity, especially in arid and semiarid regions (Apse et al. 1999). Arbuscular mycorrhizal (AM) fungi widely occur in saline environments (Sengupta and Chaudhuri 1990). Many researchers reported that AM fungi could enhance the ability of plants to cope with salt stress (Yano-Melo et al. 2003; Rabie 2005; Jahromi et al. 2008) in improving mineral nutrient absorption (Cantrell and Linderman 2001; Asghari et al. 2005), maintaining ion balance (Zandavalli et al. 2004; Giri et al. 2007), protecting enzyme activities (Giri and Mukerji 2004; Rabie and Almadini 2005), and facilitating water uptake (Berta et al. 1990; Ruiz-Lozano and Azcón 1995).

One of the most important responses of glycophytes to salinity is the accumulation of osmotically active organic solutes called osmolytes as a result of alterations in intermediary and secondary metabolism of nitrogen and carbon (Greenway and Munns 1980; Hasegawa et al. 2000; Hoque et al. 2007). This response is an important component of salinity tolerance in plants (Neocleous and Vasilakakis 2007). Organic solutes, such as proline, free amino acids, soluble protein, and sugars, maintain the osmotic balance and protect enzymes in presence of high cytoplasmic electrolyte concentrations (Greenway and Munns 1980; Hajlaoui et al. 2010). The regulation of organic acid metabolism also plays a key role in plant

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tolerance to saline conditions (Guo et al. 2010). The accumulation of organic acids may serve as counter ions to cations. They prevent toxic chloride accumulation in cells, contribute to cytosolic pH regulation (Yang et al. 2007; Hatzig et al. 2010), and are important osmolytes in plant vacuoles (Guo et al. 2010). Under salt stress, Francoise et al. (1991) found increased citric acid concentration in alfalfa roots, and Szalai and Janda (2009) found increased salicylic acid synthesis in the leaves and roots of young maize plants. It has been reported that AM fungi could modify the accumulation of soluble sugars in *Trifolium alexandrinum* (Khaled et al. 2003), soluble protein in *T. alexandrinum* (Khaled et al. 2003), *Glycine max* (Abdel-Fattah 2001), *Vigna radiata* (Rabie 2005), *Vicia faba* (Rabie and Almadini 2005), and *Lotus glaber* (Sannazzaro et al. 2006), and proline in *Lactuca sativa* (Ruiz-Lozano et al. 1996; Jahromi et al. 2008) and *T. alexandrinum* (Khaled et al. 2003).

Maize (*Zea mays*) is a very common crop in saline soils in China. Salt stress can inhibit the growth of maize, but this inhibition can be mitigated by the AM symbiosis (Feng et al. 2000, 2002; Sheng et al. 2008) through the modification of root morphology, and improvement of root activity and photosynthetic capacity (Sheng et al. 2008, 2009). Little is known, however, about the effect of the AM symbiosis on the regulation of organic solute levels in maize plants under saline conditions. Two studies reported an increase in maize shoot and root soluble sugar content (Feng et al. 2002), and a decrease in maize leaf proline content (Feng et al. 2000). In this study, we tested the effect of the AM symbiosis on the accumulation of organic solutes in maize leaves at different levels of soil salinity. We measured the concentrations of proline, free amino acids, reducing sugars, soluble sugars, soluble protein, and organic acids and interpreted modifications with relation to possible mechanisms involved in the mitigation of salt stress by the AM symbiosis in maize.

Materials and methods

Plant and soil

The soil (Eum-Orthic Anthrosols) used in this study was collected from the top layer (0–20 cm) of a field where maize (in summer) and wheat (in winter) were grown, in Yangling City, Shaanxi Province, China. The soil (pH 7.6, soil/water ratio of 1:2.5 w/v) contained 20 g kg⁻¹ organic matter, 37 mg kg⁻¹ available nitrogen, 12 mg kg⁻¹ available phosphorus, and 207 mg kg⁻¹ available potassium, measured as described by Bao (2000). The soil was ground, sieved through 2 mm, and mixed with fine sand

(sand/soil, 1:2 v/v). The mixture was autoclaved at 121°C for 2 h.

Seeds of maize cultivar Shandan 16, which is sensitive to salinity, were surface-sterilized with 0.1% HgCl₂ for 3 min, rinsed five times with sterile distilled water, and allowed to germinate on moist sterile filter paper at 28°C. Five pre-germinated seeds were sown in 3-l pot containing 2 kg of the sand/soil mixture. Seedlings were thinned to two per pot 10 days after sowing. Plants were supplemented once a week with 50 ml of a nutrient solution containing 6 mM KNO₃, 1 mM NH₄H₂PO₄, 2.6 mM MgSO₄, 8 mM Ca(NO₃)₂, 10 μM H₃BO₃, 1.6 μM MnSO₄, 1 μM ZnSO₄, 0.5 μM CuSO₄, 50 μM (NH₄)₆Mo₂O₄, and 20 μM Fe-EDTA. The solution pH was adjusted to 6.5±0.3 (Li and Feng 2001).

AM inoculum

Glomus mosseae (Nicolson & Gerdemann) was isolated from the rhizosphere of *Agropyron cristatum* in a saline soil of the Inner Mongolia Autonomous Region, China, and multiplied in pot cultures using *Z. mays* as a host. The mycorrhizal inoculum consisted of soil containing spores (306–420 per 100 g dry soil), mycelium, and root fragments with a colonization level of 94%. Each pot received 30 g of inoculum or 30 g of sterilized inoculum plus 10 ml mycorrhizal fungi-free filtrate (1 μm) of inoculum washing as the non-mycorrhizal treatment. Live and sterile inocula were placed in a layer 3 cm below the maize seeds prior to sowing.

Experimental design

The experiment was conducted in a greenhouse under a temperature of 22–30°C, 12–14 h daylight, and 70–75% relative humidity, between July and September 2006. Pots were arranged in a randomized complete block design. Treatments were factorial combinations of two factors: (1) inoculation, with *G. mosseae* or non-mycorrhizal control, and (2) salinity, with 0, 0.5, and 1.0 g NaCl kg⁻¹ dry soil mix. Ten pots were prepared for each treatment. In order to avoid an effect of salinity on fine plants and AM establishment, maize plants were grown for 15 days before application of the NaCl treatments, which was achieved by adding NaCl in irrigation water (0, 10, and 20 g l⁻¹). To avoid osmotic shock, NaCl was introduced gradually by successively adding 20 ml of the NaCl solutions in each pot every day for 5 days, starting at day 15 after sowing. In total, each pot received 100 ml of a designated saline solution. This brought the electrical conductivity (EC) of saturated soil extracts to 0.8, 1.9, and 3.6 dS m⁻¹ in the 0, 0.5, and 1.0 g kg⁻¹ NaCl treatments, respectively. Leaching was prevented by keeping soil water below field capacity at

all times. EC of soil extract was monitored using a conductivity meter (DDS-11A) and adjusted once a month. Plants were harvested 70 days after sowing. Five pots of each treatment were used to determine the biomass of maize plants. The other five pots were used to determine AM colonization rate and concentrations of soluble protein, soluble sugars, reducing sugars, proline, total free amino acids, and organic acids.

Measurement and analysis

The biomass of maize plants was determined after oven-drying their root and shoot at 70°C for 90 h according to the method described by Gao (2000).

To estimate AM colonization, roots were collected, washed gently with tap water, and dried with paper towels. A subsample of 0.5 g fresh roots was cleared 15 min in 10% KOH at 90°C, bleached in alkaline hydrogen peroxide for 20 min, acidified in 1% HCl, and stained in lactophenol blue (Phillips and Hayman 1970). Colonization was estimated using the gridline intercept method described by Giovannetti and Mosse (1980).

The reducing sugars, soluble sugars, total free amino acids, proline, and soluble protein of the second fully expanded leaf were measured according to the method described by Gao (2000).

Organic acids were measured on the second fully expanded leaf. A subsample of 1.5 g fresh leaf material was selected for organic acid measurement. Organic acids in maize leaves were extracted using the method described by Gao (2000) and measured using high performance liquid chromatography (Waters-510 equipped with a Variable UV/Vis detector model 481). A hypersil BDS column (C18, 4.6×250 mm, 10 μm) was used, and the mobile phase was a 0.5% diammonium phosphate solution (pH=2.5). The column was held at 30°C, the flow rate of the mobile phase was 1.0 ml min⁻¹, and the detection wavelength was 205 nm.

Statistical analysis

The data was subjected to analysis of variance. Treatment means were compared by Duncan's test at the 5% level (SAS version 8.0).

Results

AM fungal colonization

Non-inoculated plants had no AM colonization. AM fungal colonization of inoculated plants ranged from 99% to 79% root length.

Plant biomass

Maize plants produced more biomass when mycorrhizal, but plant productivity decreased with increasing salinity in all plants (Fig. 1).

Soluble sugars, reducing sugars, total free amino acids, proline, and soluble protein

Concentrations of soluble and reducing sugars were significantly higher in mycorrhizal than in non-mycorrhizal plants regardless of salinity level and decreased with increasing soil salinity (Fig. 2a, b). At all salinity levels, total free amino acids and proline concentrations were lower in mycorrhizal than in non-mycorrhizal plants, although the difference in total free amino acid concentration was not significant in the 0 g kg⁻¹ NaCl treatment. Concentrations of total free amino acids and proline increased with increasing salinity in non-mycorrhizal plants, but not in mycorrhizal plants (Fig. 2c, d). Regardless of salinity level, soluble protein concentration was higher in mycorrhizal than in non-mycorrhizal plants, although the difference between mycorrhizal and non-mycorrhizal plants was not significant (Fig. 2e).

Organic acids

At all salinity levels, the concentration of total organic acids was higher in mycorrhizal than in non-mycorrhizal plants, although the difference was not significant in the 1.0 g kg⁻¹ NaCl treatment. At 0, 0.5, and 1.0 g kg⁻¹ NaCl levels, the concentration of total organic acids in mycorrhizal plants increased by 31.4%, 24.8%, and 8.1%, respectively, compared with the corresponding controls (Fig. 3).

Oxalic, fumaric, acetic, malic, citric, formic, lactic, and succinic acids were found in all plants, but the concentrations of these organic acids differed with plant mycorrhizal status. Differences were most striking for oxalic acid and succinic acid, especially in the 1.0 g kg⁻¹ NaCl

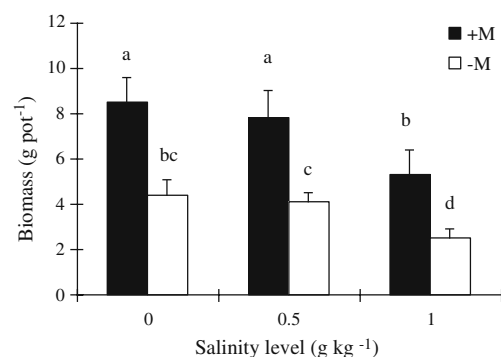
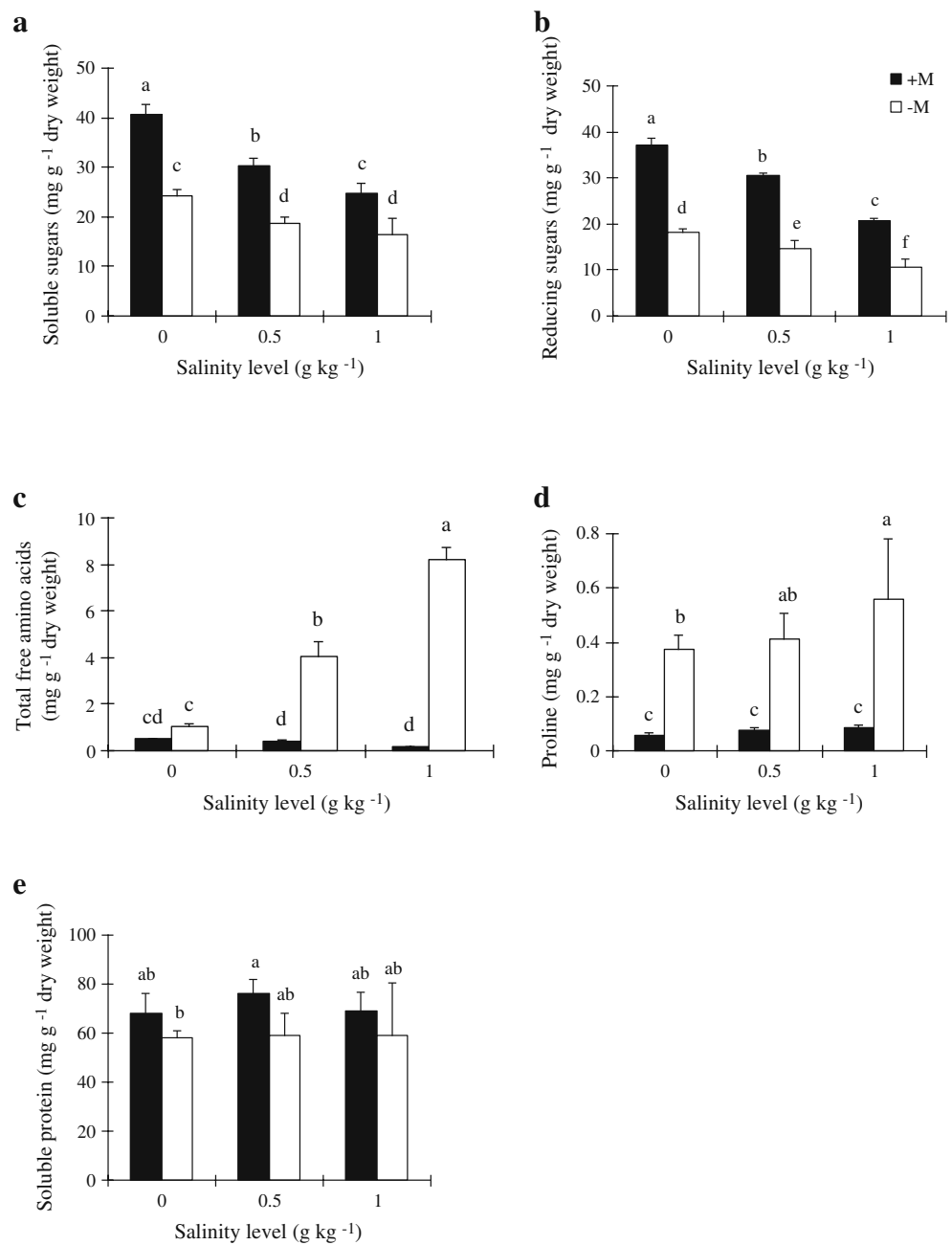


Fig. 1 Biomass of maize plants inoculated (+M) or not (-M) with *G. mosseae* and grown at three NaCl levels. Means (\pm SD) labeled with different letters are significantly different ($p < 0.05$, $n = 5$) by Duncan's test

Fig. 2 Concentrations of soluble sugars (a), reducing sugars (b), total free amino acids (c), proline (d), and soluble protein (e) in leaves of maize plants inoculated (+M) or not (-M) with *G. mosseae* and grown at three NaCl levels. Means (\pm SD) labeled with different letters are significantly different ($p < 0.05$, $n = 5$) by Duncan's test



treatment. The dominant organic acid in mycorrhizal plants was oxalic acid, while in non-mycorrhizal plants, succinic acid was dominant.

At all salinity levels, the concentrations of oxalic, fumaric, acetic, malic, and citric acids were higher in mycorrhizal than in non-mycorrhizal plants, although the difference in concentrations of malic and citric acids was not significant in the 0.5 and 1.0 g kg⁻¹ NaCl treatment. There were no significant differences in lactic acid concentration between mycorrhizal and non-mycorrhizal plants. At 0 and 0.5 g kg⁻¹ NaCl levels, no significant effect of inoculation on the concentrations of formic and succinic acids was recorded, while at 1.0 g kg⁻¹ NaCl level,

non-mycorrhizal plants had higher concentrations of both organic acids (Fig. 4).

Organic solute concentrations and relative abundance

The amount of total organic solutes was higher in mycorrhizal plants regardless of salinity level (Fig. 5). The AM symbiosis reduced the relative abundance of free amino acids and proline in total organic solutes, increased that of reducing sugars, but did not significantly affect the balance of soluble protein, organic acids, and soluble sugars (Fig. 6). The relative abundance of soluble protein was high in the organic solutes of all plants, while proline only accounted for 0.03–0.05% of

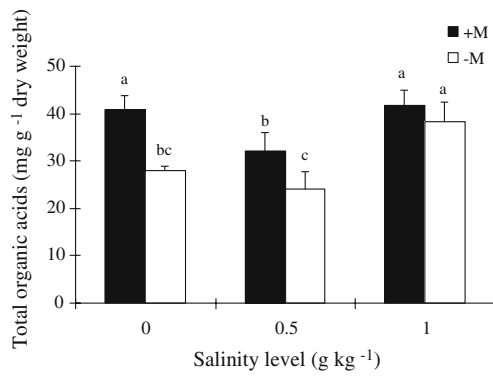


Fig. 3 Concentration of total organic acids in leaves of maize plants inoculated (+M) or not (-M) with *G. mosseae* and grown at three NaCl levels. Means (\pm SD) labeled with *different letters* are significantly different ($p < 0.05$, $n = 5$) by Duncan's test

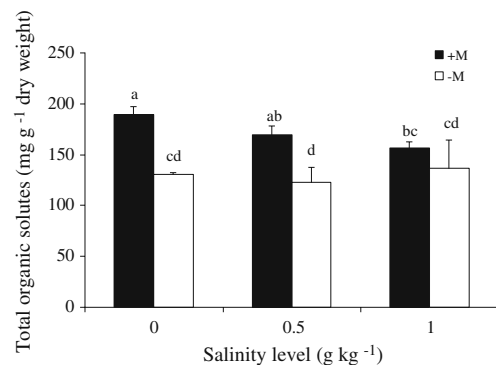


Fig. 5 The amount of total organic solutes in leaves of maize plants inoculated (+M) or not (-M) with *G. mosseae* and grown at three NaCl levels. Means (\pm SD) labeled with *different letters* are significantly different ($p < 0.05$, $n = 5$) by Duncan's test

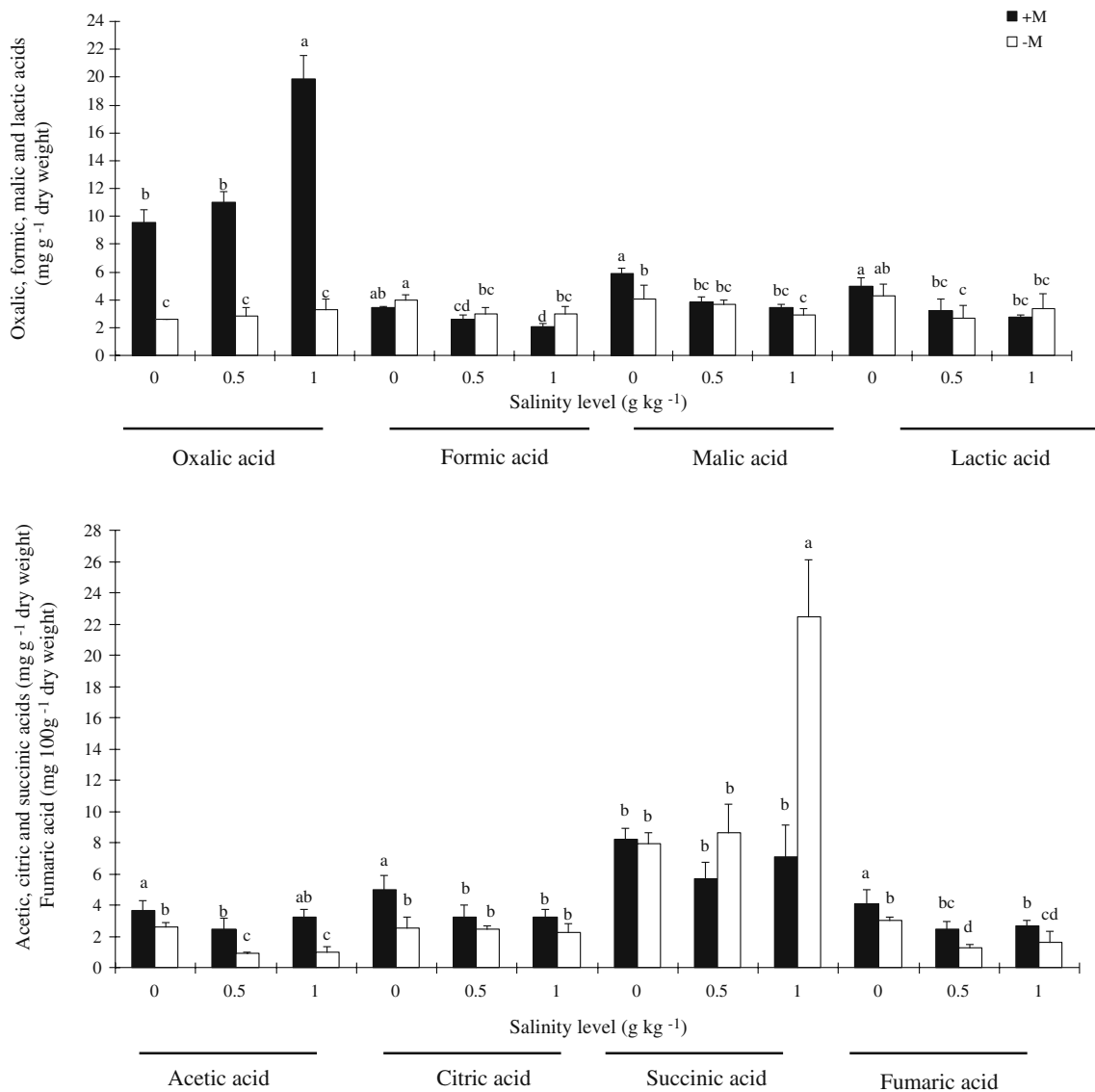
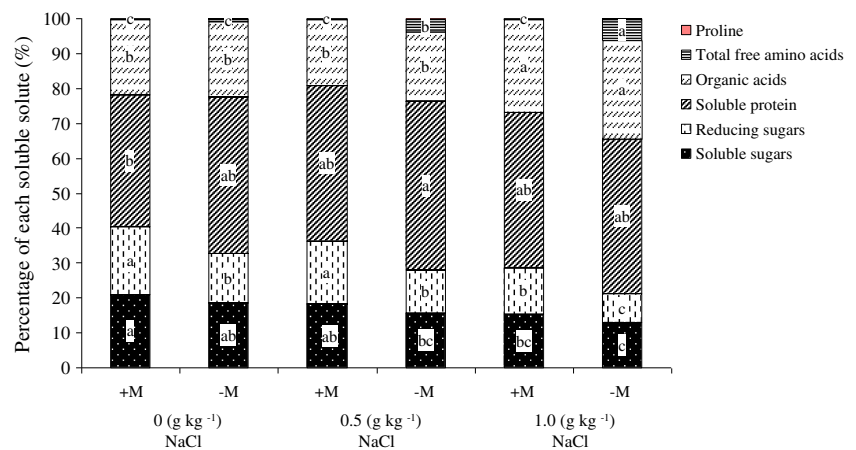


Fig. 4 Concentrations of oxalic, formic, malic, lactic, fumaric, acetic, citric, and succinic acids in leaves of maize plants inoculated (+M) or not (-M) with *G. mosseae* and grown at three NaCl levels. Means

(\pm SD) labeled with *different letters* are significantly different ($p < 0.05$, $n = 5$) by Duncan's test

Fig. 6 Relative abundance of organic solutes in leaves of maize plants inoculated (+M) or not (-M) with *G. mosseae* and grown at three NaCl levels. Means (\pm SD) labeled with different letters are significantly different ($p < 0.05$, $n = 5$) by Duncan's test



solutes in mycorrhizal plants and for 0.27–0.33% in non-mycorrhizal plants. Soluble sugars, reducing sugars, soluble protein, and organic acids made up over 99% and 93% of the organic solutes of mycorrhizal plants and non-mycorrhizal plants, respectively (Fig. 6). Consequently, soluble sugars, reducing sugars, soluble protein, and organic acids were the dominant organic solutes in both mycorrhizal and non-mycorrhizal plants.

Discussion

Free amino acids are important osmolytes contributing to osmotic adjustment in plants (Hajlaoui et al. 2010). With increasing external salt concentration, free amino acids accumulate in the leaves and roots of maize (Abd-El Baki et al. 2000; Neto et al. 2009; Hajlaoui et al. 2010). We also observed the increase of free amino acid levels in maize leaves under salt stress, but to a lesser extent in AM plants. Among free amino acids, proline is a contributor to osmotic adjustment in salt-stressed maize plants (Hajlaoui et al. 2010). Reports on the effect of AM symbiosis on proline accumulation are somewhat contradictory. Some studies have shown an increase in proline accumulation in mycorrhizal plants subjected to salt stress (Khaled et al. 2003; Sharifi et al. 2007). Enhanced proline accumulation in plant cells can increase plant osmotic potentials (Hajlaoui et al. 2010) and abscissic acid level (Ober and Sharp 1994), thereby improving the tolerance of mycorrhizal plants to salinity. On the contrary, some studies have shown a reduction of proline levels in AM plants under salt stress (Duke et al. 1986; Ruiz-Lozano et al. 1996; Jahromi et al. 2008), as we have found in this study. This and the low relative abundance of proline in total organic solutes suggest that proline contributes little to osmotic balance and salt tolerance in mycorrhizal plants. The lower accumulation of proline in AM plants appears to indicate a lower level of salt stress in mycorrhizal plants.

We found higher soluble protein concentrations in mycorrhizal than in non-mycorrhizal plants under salt stress. Similar results were reported in *L. glaber* (Sannazzaro et al. 2006), *V. radiata* (Rabie 2005), *V. faba* (Rabie and Almadini 2005), *T. alexandrinum* (Khaled et al. 2003), and *G. max* (Abdel-Fattah 2001). Normally, the steady-state levels of soluble protein in plant cytoplasm depend on the rates of protein degradation and synthesis (Guo et al. 1999). Thus, AM-mediated increase in soluble protein has been attributed to AM-mediated activation of certain plant genes (Ouziad et al. 2006; Jahromi et al. 2008). However, the observation of reduced levels of total free amino acids concurrent with higher levels of soluble protein, in our study, suggests that the AM-mediated increase in soluble protein concentration is rather due to reduced protein degradation in AM maize.

In our study, sugar (soluble sugars and reducing sugars) accumulation in maize leaves decreased when salinity increased, but at the same NaCl level, the AM symbiosis favored sugar accumulation. Similar results were observed in the shoots of *V. radiata* (Rabie 2005) and in roots and shoots of maize (Feng et al. 2002). The high levels of sugars in mycorrhizal plants may be the result of an increase in photosynthetic capacity (Sheng et al. 2008; Wu et al. 2009). The accumulation of sugars induced by the AM symbiosis is a positive response to salt stress since sugars can prevent structural changes in soluble protein, maintain the osmotic equilibrium in plant cells, and protect membrane integrity (Abd-El Baki et al. 2000).

Zhang et al. (2003) reported that AM fungal colonization could change the concentrations of organic acids in root exudates, thus causing a decrease in soil pH, soil electrical conductivity, organic carbon, and an increase in soil availability of N, P, and K (Hoffland et al. 1992; Dinkelaker et al. 1997; Usha et al. 2004). With regard to the influence of AM fungi on organic acid accumulation in plant tissues under salt stress, our data firstly revealed that AM symbiosis increases the accumulation of organic acids in

maize leaves. It is well known that organic acids, as metabolically active solutes, play a role in osmotic adjustment (Guo et al. 2010), in the balance of cation excess (Hatzig et al. 2010), and in pH homeostasis (Hasegawa et al. 2000; López-Bucio et al. 2000; Hatzig et al. 2010). Besides, high amounts of organic acids, especially malic acid, can enhance sugar synthesis through the C₄ pathway since it plays an anaplerotic role in delivering CO₂ to the Calvin cycle (Chollet et al. 1996). Thus, it appears that AM-mediated accumulation of organic acids is involved in the mitigation of deleterious effects of salt stress in maize plants.

In addition, our data also indicated that AM symbiosis changes the dominant organic acid in maize leaves, elevated concentrations of oxalic, fumaric, acetic, malic, and citric acids, decreased formic and succinic acid concentrations, but did not significantly affect lactic acid concentration. This suggests that the effect of AM symbiosis on organic acids differed according to the organic acid. Increases in the concentrations of oxalic, fumaric, acetic, malic, and citric acids could compensate for a reduction in formic and succinic acid levels in mycorrhizal maize and result in an overall increase in organic acid concentration in the plant. As we know, the AM symbiosis plays a key role in protecting enzyme activity under salt stress (Giri and Mukerji 2004; Rabie and Almadini 2005). This, together with the fact that the metabolic regulation of organic acids under saline conditions involves enzymes participating in basal metabolic pathways—such as the tricarboxylic acid cycle, glyoxylate cycle, or glycolysis—suggests that the observed changes in organic acid synthesis result from the influence of the AM symbiosis on enzyme activities in basal plant metabolic pathways. In order to clarify the mechanisms of AM-mediated changes in concentrations of organic acids, further investigations should be conducted to define the effect of AM symbiosis on enzyme activities regulating organic acid synthesis and utilization.

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