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Salinity effects on carbohydrates, protein, and free amino acids in *Miscanthus sinensis*

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with 2 figures

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Summary

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Salinity is a major environmental stress that limits plant growth and development in arid and semi-arid regions. In order to better understand the salt-induced effects in *Miscanthus sinensis*, which has been identified as a good candidate for biomass production, seedlings of two accessions (salt-tolerant 'JM0119' and salt-sensitive 'JM0099') were subjected to 0 mM NaCl (control) or 250 mM NaCl (salt stress treatment) for two weeks. The contents of carbohydrates, soluble protein and total free amino acids in leaves were determined. Dramatically raised levels of soluble sugars were observed in the leaves of both accessions during salinity exposure. The starch content was slightly affected by salt stress. The total soluble protein content showed a significant reduction in leaves of JM0099 after long-time salt treatment, and the total content of free amino acids in leaves significantly increased in JM0119, while no remarkable changes were found for JM0099. Overall, accumulated soluble sugars and free amino acids provided osmotic adjustment and osmoprotection in *M. sinensis* leaves, and higher amount of sucrose, total free amino acids and soluble protein were associated with higher salt tolerance.

1. Introduction

Soil salinity as a major environmental stress factor adversely affects plant growth and biomass production by disturbing the physiological, biochemical, morphological, ecological, and genetic processes involved in photosynthesis, ion homeostasis, antioxidant phenomena, and osmolyte accumulation (Munns & Tester 2008, Acosta-Motos & al. 2017). To cope with the detrimental effects of salt stress, plants have evolved adaptation strategies that include osmotic regulation. Several compounds involved in the osmotic regulation process are well known: soluble carbohydrates such as glucose, sucrose, and fructose, as well as nitrogen compounds such as proteins and amino acids. Soluble sugars and amino acids are compatible solutes that possess osmoprotectant properties. They can greatly accumulate in the cytosol to balance the osmotic pressure which is created by ions (e.g. Na^+ and Cl^-) sequestered in the vacuole or in the intercellular apoplastic space (Tilbrook & Roy 2014, Singh & al. 2015). Changes in the salt-induced accumulation of these compatible solutes have been reported to prevent membrane injury, to stabilize proteins and enzymes, and to mitigate damaging risk caused by ROS (Hajlaoui & al. 2010, Hu & al. 2013, Akbari & al. 2018, Simões & al. 2019, de Oliveira & al. 2020). Furthermore, soluble sugars also act as signaling molecules involved in the plant sugar sensing and signaling network under stress, thus modifying gene expression and proteomic patterns governing photosynthetic metabolism (Chaves & al. 2009). Previous studies suggested that tolerant plants could use starch for different physiological processes to cope with the salinity challenge (Acosta-Motos & al. 2017) and that the inhibition of proteins metabolism in salt stress was associated with the alteration of N assimilation and protein catabolism (Ashraf & al. 2018).

Miscanthus sinensis Anderss., a C_4 perennial lignocellulosic grass, has been identified as a good candidate for biomass production. To meet the challenge of developing second-generation energy crops capable of growing on marginal land, the physiological and biochemical responses of *M. sinensis* to salinity were studied recently (Sun & al. 2014, Chen

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& al. 2017). However, the salinity effects on the compounds involved in osmotic regulation process in *M. sinensis* are still unclear. In the present study, we investigated the contents of carbohydrates, total soluble protein, and total free amino acids to NaCl stress in the leaves of JM0119 (salt-tolerant) and JM0099 (salt-sensitive), two *M. sinensis* accessions with contrasting salt tolerance determined in our previous greenhouse study (Sun & al. 2014). These salt-induced variations could provide insights into the salt-tolerance mechanisms of *M. sinensis*.

2. Materials and methods

Seeds of JM0119 (salt-tolerant) and JM0099 (salt-sensitive) were surface-sterilized with 70 % ethanol for 30 s and with 1 % (v/v) sodium hypochlorite (NaClO) for 10 min, then rinsed three times with distilled water. Twenty-five seeds of each accession were sown in a 2-l pot filled with mixed peat moss (PRO-MIX PGX; Grower's Nursery Supply, Inc.; Salem, OR). The pots were placed in a growth chamber at 12 h photoperiod with 500 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF), 70 % humidity and 28/22 °C day/night regime, and watered with distilled water daily. Seedlings were thinned to ten per pot at the five-leaf stage then irrigated with salt-free 1/2 Hoagland solution until the seedlings had eight fully expanded leaves. Each pot was treated daily either with 200 ml 1/2 Hoagland solution containing 0 mM NaCl (control) or with 250 mM NaCl (distributed over a three-day period at a rate of 83 mM per day in order to avoid osmotic shock). Samples taken from leaves of four seedlings were harvested just before the treatment (day 0) and 1, 3, 7, and 14 days after salt application. Leaf samples were immediately immersed in liquid nitrogen upon harvest and stored at –80 °C.

Liquid-nitrogen-frozen leaf samples (c. 30 mg) were extracted with 300 µl 80 % ethanol (v/v) (10 mM Hepes-KOH, pH 7.4) at 80 °C for 15 min and centrifuged for 2 min at room temperature (Leakey & al. 2006). A 50-µl aliquot of supernatant was used for each determination of glucose, fructose and sucrose using a continuous enzymatic substrate assay (Maness 2010). A 16-µl aliquot of the ethanol-extracted supernatant was used for the determination of the total free amino acid content using a fluorescamine assay (Bantan-Polak & al. 2001) with a fluorescence spectrophotometer (F-2500, Hitachi, Ltd.; Tokyo, Japan). Histidine was used as a standard. Pellets of the ethanol extraction were suspended in l ml 0.1 M NaOH by heating to 95 °C for 30 min. The soluble protein content was assayed with a commercial kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific Inc., Rockford, USA) with bovine serum albumin as a standard. The remainder of the NaOH solution was then acidified to pH 4.9 with an HCl/sodium acetate solution and mixed well for the starch assay. A 50-µl aliquot of the suspension was incubated with 10 µl α-amylase after diluting with 250 µl distilled water at 95 °C for 5 minutes then digested with 10 µl 300 U ml^{-1} amyloglucosidase for 15 min at 60 °C. The starch content was calculated with the measured glucose content.

All data was analyzed by Statistical Product and Service Solutions (SPSS Statistics, Version 20; IBM Corp.; New York, USA). Differences in the physiological traits among accessions and salt treatments were tested by analysis of variance. The least significant difference ($LSD_{0.05}$) test was used to separate treatment means.

3. Results and discussion

Striking salt-induced increases ($P < 0.05$) of the contents of soluble sugars including glucose, fructose, and sucrose were observed from day 3 or later in both accessions (Fig. 1A–D). Plants of JM0099 showed relative higher level of the glucose content under control conditions compared with those of JM0119, and varied in a single peak curve, with a peak value 149 % greater than the control on day 6, during the time course of the salt treatment (Fig. 1A). However, the content of fructose in the plants of JM0119 was much greater under control conditions relative to that in JM0099, and increased dramatically on day 3, 123 % greater than the control, followed by a valley curve (Fig. 1B). The contents of sucrose and soluble sugars (Glu+Fru+Suc) were greater in the salt-treated plants of JM0099 than those in JM0119 on day 10, then became lower on day 17 (Fig. 1C and 1D). On day 17, relative to the control, contents of glucose, fructose, sucrose, and total soluble sugars (Glu+Fru+Suc) increased by 125 %, 54 %, 97 % and 96 %, respectively, for JM0119, and 67 %, 116 %, 80 % and 81 %, respectively, for JM0099. Although the content of starch showed a decline under salt stress from day 10 in both accessions, no significance was observed (Fig. 1E).

Non-structural carbohydrates regulate growth and development of plants, and are considered to be involved in the adaptation strategy to salt stress. Hexoses such as glucose and fructose provide carbon skeletons and energy for normal functioning of cellular metabolism and act as important signal molecules in source-sink regulation, while other sugars, namely disaccharides such as sucrose and sugars with longer chains, have storage and transport functions (Singh & al. 2015, Hütsch & al. 2016). The present study determined that the salt-tolerant accession, in contrast to the sensitive accession, had

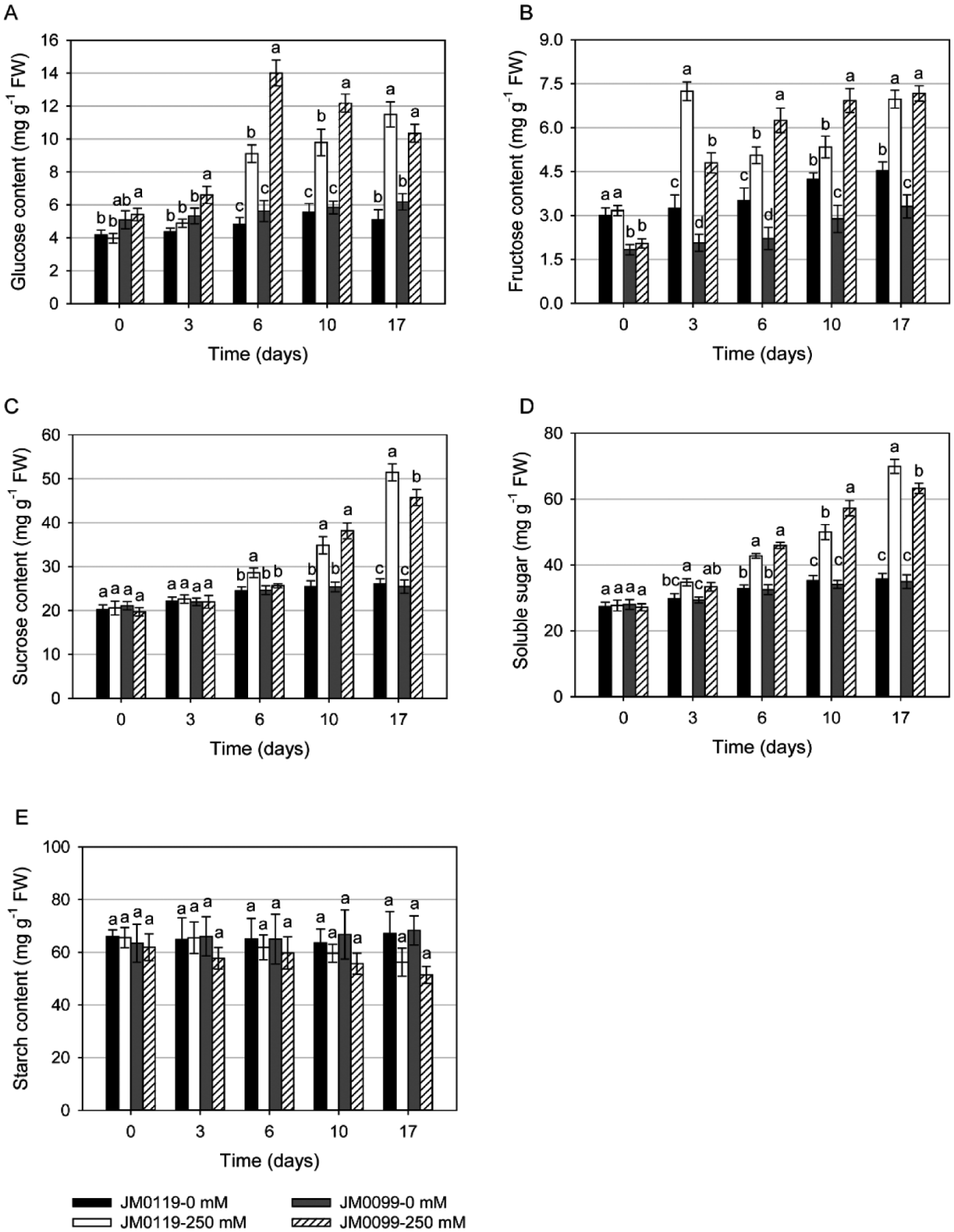

Fig. 1. Time-course changes in contents of (A) glucose (Glu), (B) fructose (Fru), (C) sucrose (Suc), (D) total soluble sugar (Glu+Fru+Suc), and (E) starch in the absence or presence (250 mM) of NaCl for two *Miscanthus sinensis* accessions. Values are means ± SE of four replicates. Different letters indicate significant differences within each sampling date ($LSD_{0.05}$ test).

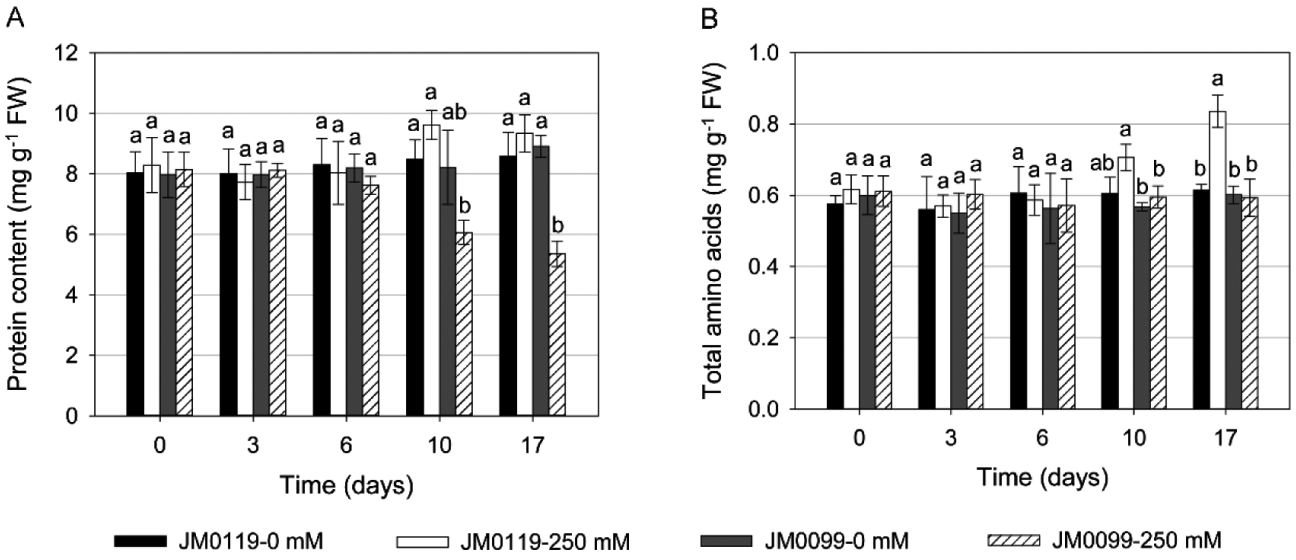

Fig. 2. Time-course changes in the content of total soluble protein (A) and total free amino acid (B) in the absence or presence (250 mM) of NaCl for two *Miscanthus sinensis* accessions. Values are means ± SE of four replicates. Different letters indicate significant differences within each sampling date ($LSD_{0.05}$ test).

significantly higher fructose content under control conditions and showed earlier increases in fructose and sucrose in response to salinity. These findings suggested that the salt-tolerant plants had constitutive adaptation and rapid osmoprotection to osmotic stress. Interestingly, the contents of hexoses (i.e., glucose and fructose) in salt-treated sensitive plants were significantly higher than those in the tolerant ones, in accordance with the results reported in ryegrass (Hu & al. 2013) and sugarcane (Simões & al. 2019), accompanied with increases of sucrose in both accessions. Thus, the salt-induced accumulation of these hexoses may be due to the reduced sink carbon demand caused by the limited growth rate, particularly for the sensitive accessions (Hajlaoui & al. 2010, Hu & al. 2013, Tang & al. 2013). Previous studies in pistachio (Akbari & al. 2018), sesame (Zhang & al. 2019), and sorghum (de Oliveira & al. 2020) reported that salinity-tolerant plants could maintain higher concentrations of soluble sugars than the sensitive ones. Consistent with these results, the salt-tolerant accession in the present study accumulated a greater amount of sucrose than the sensitive accession after 17-day salt treatment. This higher accumulation of sucrose under salt stress might enhance the plant's ability to avoid tissue death and maintain growth and development under long-time salt stress by acting as osmolytes in elevating osmotic adjustment, protecting biomolecules, and balancing gathered ions in vacuoles (Hatzig & al. 2010, Tang & al. 2013). The slight decrease of starch content observed in both accessions could be dependent upon both triose-phosphate utilization and glucose generation (Paul & Foyer 2001), and indicated that the elevated levels of sucrose may mainly result from sucrose synthesis instead of hydrolyzation of carbohydrates such as starch.

Trifling increases in the total soluble protein content in the salt-treated plants were found in JM0119 from day 10, paralleling with a significant ($P < 0.001$) reduction of protein content on day 17 in JM0099 (Fig. 2A). The content of total soluble protein declined 40 % relative to the control in JM0099 after 17-day salinity exposure. The content of total free amino acids was changeless in the plant leaves of JM0099, while a significant ($P = 0.001$) increase relative to the control was observed in JM0119 leaves on day 17 (Fig. 2B).

Under salt stress, plants are reported to accumulate low-molecular-weight organic solutes such as free amino acids to reduce water loss and achieve long-term osmotic adjustment (Khalafallah & al. 2013). Consistent with previous findings in sesame (Zhang & al. 2019) and sugar beet (Geng & al. 2019), the tolerant plants in our study showed superior osmotic accommodation to the build-up of ion concentrations, according to the accumulated total free amino acids content observed in the tolerant accession. The stable protein content observed in the salt-tolerant plants implied that the salt-tolerant accession showed higher resistance to salt-induced protein degradation caused by accumulated ionic toxicity. Our results supported the notion that plants maintaining enhanced levels of free amino acids were capable of relieving inhibitory effects of ions on enzymes, enhancing thermal stability of proteins, and limiting dissociation of enzyme com-

plexes during protein metabolism under salt stress (Khalafallah & al. 2013, Rahman & al. 2017). The steady state of the total free amino acids content in the sensitive accession may be due to a balance between the rate at which they were released during protein degradation and the rate at which they were removed by efflux from the leaf (Hajlaoui & al. 2010).

In conclusion, the two *M. sinensis* accessions in this study, JM0099 and JM0119, varied in tolerance during salinity exposure. Higher amounts of sucrose, total free amino acids and soluble protein were essential for the osmoregulation and associated with the higher salt tolerance.

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