MODULAÇÃO DO PROTEOMA DE FOLHAS DE SORGO SOB ESTRESSE SALINO PELA FONTE DE NITROGÊNIO.

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RESUMO: A salinidade é um fator abiótico que afeta a produtividade de várias culturas no mundo. Assim, a elucidação de mecanismos de tolerância ao estresse salino faz-se necessária. Estudos recentes mostraram que a nutrição com amônio (NH_4^+) ativa mecanismos essenciais para a aclimatação de plantas de sorgo ao estresse salino. Aqui se realizou uma investigação detalhada do proteoma de plantas de sorgo sob estresse salino usando fontes diferenciais de nitrogênio. Plantas de sorgo foram inicialmente cultivadas em soluções nutritivas modificadas para conter NH_4^+ ou nitrato (NO_3^-) a 5 mM, e depois sujeitas a um estresse salino de 75 mM de NaCl. A significância na diferença de abundância dos spots proteicos foi mensurada com o teste t de Student. O estudo proteômico revelou que 115 *spots* proteicos foram alterados pela salinidade. Sob estresse salino, plantas nutridas com NO_3^- mostraram regulação em 67 proteínas: 28 supermoduladas, 23 subreguladas, 6 reprimidas e 10 sintetizadas *de novo*. Em contraste, plantas cultivadas com NH_4^+ mostraram variação em 53 *spots*: 25 superreguladas, 10 submoduladas, 4 reprimidas e 14 sintetizadas *de novo*. Os dados sugerem que o melhor desempenho de plantas nutridas com NH_4^+ se relacionam especialmente a uma ativação de um metabolismo energético mais eficiente. Estes resultados fornecem pistas de como a modulação do proteoma pode levar a uma melhor aclimatação ao estresse salino.

PALAVRAS-CHAVE: Salinidade, perfil proteico, nutrição nitrogenada.

PROTEOME MODULATION OF SORGHUM LEAVES GROWN IN DIFFERENTIAL NITROGEN SOURCES UNDER SALT STRESS

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ABSTRACT: Salinity is a major abiotic factor that affects several crops yield around the world. Thus, the elucidation of mechanisms of salt stress tolerance is necessary. Recent studies have shown that ammonium (NH_4^+) nutrition activates essential mechanisms for the acclimation of sorghum plants to salt stress. Here, a detailed investigation of the proteome of sorghum plants under salt stress using differential nitrogen sources was performed. Plants were grown in nutrient solutions modified to contain 5 mM of either $NO3^-$ or $NH4^+$ and then subjected to 75 mM NaCl salt stress. The significance of abundance protein spot differences was assessed by Student's t-test. The proteomic study revealed that 115 protein spots were changed due to salinity. Under salt stress, NO_3^--fed plants exhibited regulation in 67 proteins: 28 upregulated, 23 downregulated, 6 repressed and 10 synthesized de novo. In contrast, NH_4^+-fed plants showed variation in 53 proteins: 25 upregulated, 10 downregulated, 4 repressed and 14 synthesized de novo. Data suggest the better performance of NH_4^+-fed plants is related especially to an activation of a more efficient energetic metabolism. These results provide hints of how proteome modulation can cause a better acclimation to salt stress.
KEYWORDS: Salinity, protein profile, nitrogen nutrition.

INTRODUCTION

Salinity in arable lands is an increasing problem and restricts plant development, resulting in loss of productivity. In Brazil, this issue is found all over the country, especially in the Northeastern Region, where approximately 25% of irrigated areas are salinized (Pedrotti *et al.*, 2015). Salinity induces a series of morphological, physiological and biochemical responses in plants due to changes in molecular processes, which widely vary among species and depend on the type, salt concentration, duration of exposition to the stress, as well as other factors (Shinozaky *et al.*, 2015).

Nitrogen is a crucial element for plant growth, and its main sources naturally absorbed by plants are nitrate (NO_3^-) and ammonium (NH_4^+). Nitrate is usually the main N source assimilated by plants; however, when there is low NO_3^- availability in the soil, ammonium is taken up by plant roots (Helali *et al.*, 2010). Although in many species NH_4^+ is toxic at high concentrations, recent studies have shown that some species display tolerance to this ion, such as sorghum, and this tolerance provides alleviation of the deleterious effects of abiotic stresses like salinity (Miranda *et al.*, 2016). NH_4^+ assimilation by plant cells is less energetically cost-effective than nitrate (Xu *et al.*, 2012), and several authors reported that salinity can inhibit

NO3- uptake through competition with Cl^- ions (Miranda et al., 2012) In addition, sorghum, NH4+ supply was able to overcome the salt deleterious damages by activating effective mechanisms of defense, including reduced Na^+ accumulation and better photosynthetic performance under salinity (Miranda *et al.*, 2016).

Proteomics has revealed to be a powerful tool in the study of gene expression. Combining 2-DE with mass spectrometry, it has been quite employed to investigate stress-induced alterations in crops of economic importance. Swami et al (2011) studying proteomic changes in sorghum plants under salt stress, found out that many proteins related to antioxidant defense were overexpressed in response to salinity. Damaris et al. (2016), also assessing proteomic alterations induced by salt stress in two rice cultivar, reported that salinity leads to an upregulation of several redox and stress-responsive enzymes, such as Cu/Zn Sod, Mn-SOD, V-ATPase, and pyruvate-decarboxylase.

Sorghum (*Sorghum bicolor* L. Moench) is a versatile annual grass species with C4 metabolism that possesses moderate tolerance to abiotic stresses such as drought and salinity (Miranda *et al.*, 2016). It is widely used as animal and human food (Yan *et al.*, 2012). This investigative study aimed to assess the modulation of proteome profile in leaves of sorghum plants affected by salinity as subjected to two inorganic nitrogen sources, namely nitrate and ammonium.

MATERIALS AND METHODS

Seeds of *Sorghum bicolor* L. Moench genotype CSF 20 were surface sanitized, washed, and sown in plastic cups containing moisturized vermiculite. After germination, ten uniform seedlings were transferred to hydroponic systems of 10 L plastic trays containing one-third Hoagland nutrient solution modified to include inorganic nitrogen at 5.0 mM, provided as either nitrate or ammonium. It was constantly aerated, renewed every three days, and pH values were daily monitored and adjusted to 6.0 with 1.0 M HCl or 1.0 M NaOH whenever necessary. After 7 days, sorghum seedlings were transferred to hydroponic systems of 3 L plastic containers and submitted to saline treatment with NaCl at 0 mM (control treatment) or 75 mM, applied in daily doses of 37.5 mM to avoid osmotic stress. Five plants from control and salt treatments of each nitrogen source were harvested 10 days after salinity exposure (10 DAS), and had their leaves stored at -20 ºC for proteomic analyses.

Total soluble protein was extracted from fresh frozen leaves of each treatment according to Mesquita *et al.* (2012), and 2-DE gels were scanned using a gel densitometer

DS-6000 (Loccus, Cotia, SP, Brazil). Image analysis was conducted by ImageMaster 2D Platinum software (version 7.05, GE Life Sciences) through LabScan v. 5.0 program (GE-Healthcare), according to manufacturer's instructions. At least three gels from each treatment (nitrate control; nitrate salt; ammonium control; and ammonium salt) were used for the analysis, and the spots were considered to be "differential" according to the following criteria: reproducible differences in relative abundance intensity % (1.5-fold) and p-value < 0.05. Selected protein spots with reproducible differences in relative abundance intensity % (1.5-fold and p-value < 0.05) were manually excised from the gel and digested with sequencing-grade trypsin as described by Shevchenko *et al*. (2006).

Extracted tryptic fragments were analyzed by capillary liquid chromatography/nanoelectrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) using a Q-ToF mass spectrometer (Waters Corporation, Milford, MA, USA) coupled with a Water Nano high-performance liquid chromatography (UPLC) unit. Peptides identification was performed with peptide mass fingerprinting (PMF) and MS/MS data obtained from ESI-Q-TOF via MASCOT Daemon (Matrix Science; www. matrixscience.com/search_form_select.html). The National Center for Biotechnology Information (NCBI) non-redundant and the SwissProt (www.uniprot.org) databases.

The experimental design was completely randomized design in a 2 × 2 factorial, composed of two nitrogen sources (NO_3^- and NH_4^+) and two salt treatments (0 and 75 mM NaCl). Thus, 4 distinct plant groups were formed: a) NH_4^+-fed plants without NaCl (AC); b) NH_4^+-fed plants in presence of 75 mM NaCl (AS); c) NO_3^--treated plants without NaCl (NC); and d) NO_3^--treated plants in presence of 75 mM NaCl (NS) with five repetitions each. For gel analyses, the significance of abundance protein spot differences was assessed by Student's t-test using the ImageMasterTM 2-D Platinum software. Protein spots with a significant difference ($p < 0.05$) of abundance change (up or down) and percentual volume ratio (% vol) ≥ 1.5 were considered as 'differentially regulated proteins'.

RESULTS AND DISCUSSION

In general, 256 proteins were detected in leaves of nitrate-grown plants, of which 67 were characterized as differentially modulated in response to salinity. A total of 28 proteins was upregulated and 23 downregulated by salt stress, whereas 6 proteins were repressed under NaCl stress and 10 were found to be de novo synthesis (Fig. 1A). 2-DE gels from leaves of

ammonium–grown plants revealed a total of 318 protein spots, of which 53 showed differential expressions in response to salt stress. Under salinity, 25 proteins/peptides were upregulated, 10 showed downregulation; 4 were repressed and 14 were de novo synthesized (Fig. 1B). Comparing 2-DE gels from salt treatments of both nitrogen sources, a total of 334 protein spots were registered, being 33 proteins found as differentially regulated as affected by nitrate or ammonium treatments. Considering nitrate salt gel as a reference, ammonium salt gel showed 14 proteins upregulated, 11 downregulated, 2 proteins were exclusive to nitrate salt treatment, while 6 were found to be exclusively expressed in ammonium-grown plants (Fig. 1C). The differentially expressed protein spots were divided into five categories: photosynthesis/carbon metabolism, energetic metabolism, antioxidant enzymes, response to stress and other categories (Figs. 2A, B, C).

In nitrate-grown sorghum plants, after identification, the proteins differentially regulated were related to photosynthesis/carbon metabolism (52%), energetic metabolism (21%), response to stresses (9%), antioxidative system (5%), and other cellular processes, 13% (Fig. 2A). In ammonium-fed plants, 42% of all differentially expressed proteins were related to photosynthesis/carbon metabolism; 28% were related to energetic metabolism; 13% to respond to stresses; 2% to antioxidative action; and 15% of them to other cellular processes (Fig. 2B). Comparing nitrate and ammonium treatments under salt stress, it was observed that the proteins differentially modulated, and affected by nitrogen source were mainly involved in energetic metabolism (40%), photosynthesis/carbon metabolism (30%), response to stress (21%) and other cellular processes (9%) (Fig. 2C).

In an attempt to overcome the salt harmful effects, nitrate-fed plants upregulated numerous proteins related to energetic metabolism and a group of proteins of response to stress and other cellular processes; but failed to activate at least part of the antioxidant system. Our results are in accordance with Zhang *et al.* (2013), who assessed the effects of salinity on antioxidant enzymes in roots and leaves of *Broussonetia papyrifera* and showed plant growth inhibition. On the other hand, ammonium-fed plants displayed a specific modulation of the proteome that resulted in a more efficient response to salt stress. Firstly, salt-stressed plants synthesized de novo some thylakoid structural proteins as well as upregulated diverse enzymes of photosynthetic/carbon metabolism. In addition, ammonium-fed plants activated (exclusively or by upregulation) several proteins involved in energetic metabolism. Miranda et al. (2016) also verified that NH_4^+-fed plants displayed higher biomass values (root and shoot dry mass), higher root and shoot K^+/Na^+ ratios, higher free amino acids content and greater CO_2 assimilation under salt stress in comparison to nitrate nutrition.

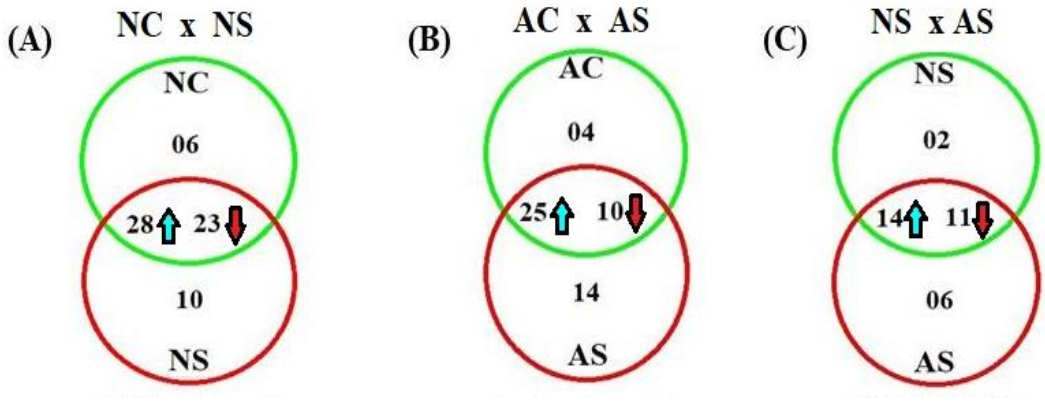

Figure 1. Venn diagrams represent different subsets of proteins of sorghum cv. CSF20 leaves. Subsets to (A) NH_4^+-fed plants without NaCl (NC) and NH_4^+-fed plants in presence of 75 mM NaCl (NS) treatments, (B) NO_3^--treated plants without NaCl (AC) and NO_3^--treated plants in presence of 75 mM NaCl (AS) treatments, and (C) NS and AS) treatments, where NS was taken as reference.

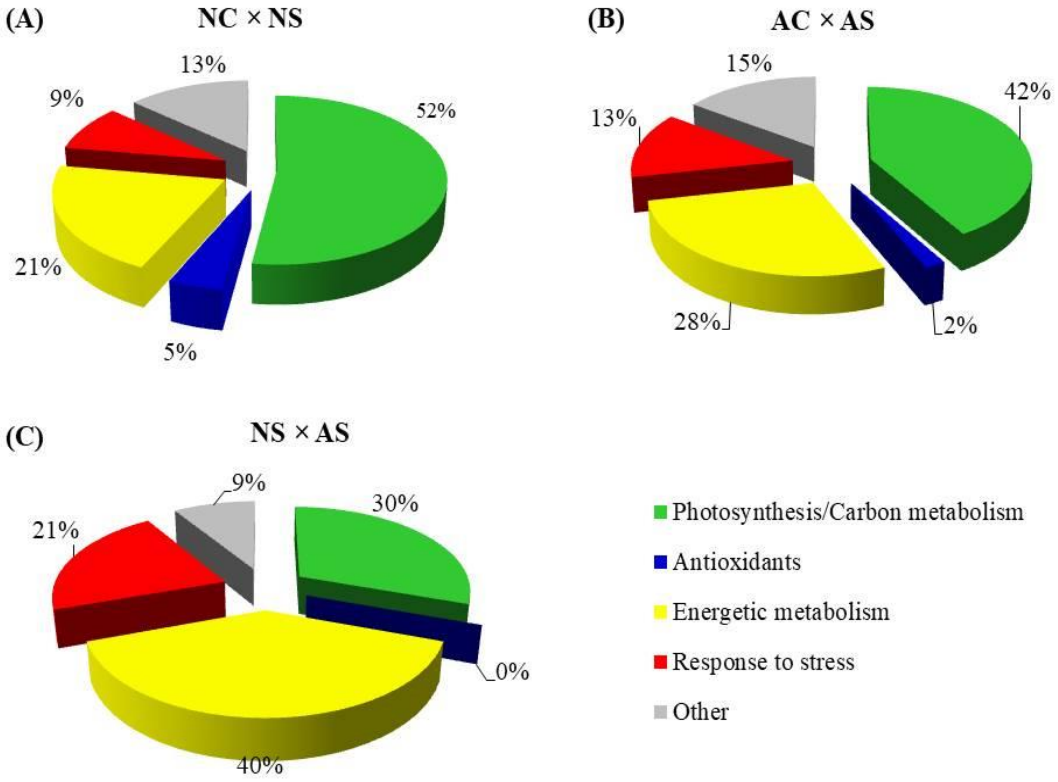

Figure 2. Functional characterization of all differential spots identified in leaves of *Sorghum bicolor* L. Moench cv. CSF 20. (A) nitrate-grown sorghum plants grown after 10 days in control conditions (NC) and salt stress with 75 mM-NaCl (NS). (B) ammonium-fed sorghum plants grown after 10 days in control conditions (AC) and salt stress with 75 mM-NaCl (AS). (C) comparison between differentially expressed proteins in leaves of sorghum plants 10 days after salinity as affected by nitrogen source, nitrate (NS) and ammonium (AS).

CONCLUSION

This study showed that the external nitrogen source can modify responses to salinity in the sorghum plant by reprogramming especially several proteins involved in energetic metabolism and in response to stresses. In all cases, ammonium nutrition seems to be the most efficient in leading to a better acclimation to salinity. These data display important information for a better understanding of molecular mechanisms of response to salinity in sorghum plants mediated by the nitrogen source.

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REFERENCES

DAMARIS, R. N.; LI, M.; LIU, Y.; CHEN, X.; MURAGE, H.; YANG, P. A proteomic analysis of salt stress response in seedlings of two African rice cultivars. **Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics,** v. 1864, p. 1570-1578, 2016.

HELALI, S. M.; NEBLI, H.; KADDOUR, R..; MAHMOUDI, H.; LACHAÂL, M.; OUERGHI, Z. Influence of nitrate—ammonium ratio on growth and nutrition of Arabidopsis thaliana. **Plant Soil**, v. 336, p. 65-74, 2010.

MESQUITA, R. O.; SOARES, E. A.; BARROS, E. G.; LOUREIRO, M. E. Method optimization for proteomic analysis of soybean leaf: improvements in identification of new and low-abundance proteins. **Genetics and Molecular Biology**, North Carolina, v. 35, p. 353-361, 2012.

MIRANDA, R. S.; ALVAREZ-PIZARRO, J. C.; ARAÚJO, C. M. S.; PRISCO, J. T.; GOMES-FILHO, E. Influence of inorganic nitrogen sources on K+/Na+ homeostasis and salt tolerance in sorghum plants. **Acta Physiologiae Plantarum**, v. 35, p.841-852, 2012.

MIRANDA, R. S.; GOMES-FILHO, E.; PRISCO, J. T.; ALVAREZ-PIZARRO, J. C. Ammonium improves tolerance to salinity stress in Sorghum bicolor plants. **Plant Growth Regulation**, Berlin, v. 78. p. 121-131, 2016.

PEDROTTI, A.; CHAGAS, R. M.; RAMOS, V. C.; PRATA, A. P. N.; LUCAS, A. A. T.; SANTOS, P. B. Causas e consequências do processo de salinização dos solos. **Revista Eletrônica em Gestão, Educação e Tecnologia Ambiental**, Santa Maria, v. 19, p. 1308-1324, 2015.

SHEVCHENKO, A.; TOMAS, H.; HAVLIS, J.; OLSEN, J. V.; MANN, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. **Nature Protocols**, London, v. 1, p. 2856-2880, 2006.

SHINOZAKY, K.; UEMARA, M.; BAILEY-SERRES, J.; BRAY, E. A.; WERETILNYK, E. Responses to abiotic stresses. In: BUCHANAN, B.; GRUISSEM, W.; JONES, R. L. **Biochemistry and Molecular Biology of Plants**, Hoboken, 2 ed. Rockville: American Society of Plant Physiologists, p. 1051-1100, 2015.

SWAMI, A. K.; ALAMB, S. I.; SENGUPTAB, N.; SARINA, R. Differential proteomic analysis of salt stress response in *Sorghum bicolor* leaves. **Environmental and Experimental Botany**, Amsterdam, v. 71, p. 321-328, 2011.

XU, G.; FAN, X.; MILLER, A. J. Plant nitrogen assimilation and use efficiency. **Annual Review of Plant Biology**, v. 63, p.153–182, 2012.

YAN, K.; CHEN, P.; SHAO, H.; ZHAO, S.; ZHANG, L.; ZHANG, L.; XU, G.; SUN, J. Responses of photosynthesis and photosystem II to higher temperature and salt stress in sorghum. **Journal of Agronomy and Crop Science**, Hoboken, v.198, p. 218-225, 2012.

ZHANG, M.; FANG, Y.; JI, Y.; JIANG, Z.; WANG, L. Effects of salt stress on ion content, antioxidant enzymes and protein profile in different tissues of *Broussonetia papyrifera*. **South African Journal of Botany,** Amsterdam, v. 85, p. 1-9, 2013.