UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

FRANCISCO DALTON BARRETO DE OLIVEIRA

ANÁLISE FISIOLÓGICA E PROTEÔMICA DE PLANTAS DE SORGO SOB ESTRESSE SALINO E NUTRIDAS COM DIFERENTES FONTES DE NITROGÊNIO

FORTALEZA
2017

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica do Departamento de Bioquímica e Biologia Molecular, da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Mestre em Bioquímica. Área de concentração: Bioquímica Vegetal

Orientador: Enéas Gomes Filho

Coorientador: Rafael de Souza Miranda

FORTALEZA

2017

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

O47a Oliveira, Francisco Dalton Barreto de.
Análise fisiológica e proteômica de plantas de sorgo sob estresse salino e nutridas com diferentes fontes de nitrogênio. / Francisco Dalton Barreto de Oliveira. – 2017.
104 f. : il. color.

Dissertação (mestrado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica, Fortaleza, 2017.
Orientação: Prof. Dr. Enéas Gomes Filho.
Coorientação: Prof. Dr. Rafael de Souza Miranda.

1. Crescimento. 2. Estresse Salino. 3. Nutrição Nitrogenada. 4. Perfil Proteico. 5. Sorghum bicolor. I. Título.

CDD 572

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Aprovada em:____/____/_____.

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A minhas queridas irmãs Maitê (*in memorian*) e Dayse; a meu pai Maurílio, a minha mãe Terezinha e a meu primo Eliezer Bruno (*in memorian*).

...com amor.

AGRADECIMENTOS

A Deus, por me fornecer força e perseverança para continuar nestes dois anos e meio de caminhada;

Ao Prof. Dr. Enéas Gomes Filho, do Laboratório de Fisiologia Vegetal, do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, por ter me acolhido, me orientado e me apoiado durante este período e na realização deste trabalho;

Ao Dr. Rafael de Souza Miranda (coorientador), elemento fundamental na realização deste trabalho, por sua solicitude, conhecimentos transmitidos, auxílio e coorientação na realização deste trabalho;

À Profa. Dra. Dirce Fernandes de Melo, do Laboratório de Bioenergética, do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará (UFC), por ter aceitado constituir a banca examinadora, além de toda a tutoria, conselhos e ensinamentos durante a graduação;

À Profa. Dra. Ana Cristina de Oliveira Monteiro Moreira e à Dra. Marina Duarte Pinto Lobo, da Universidade de Fortaleza (UNIFOR), pela colaboração e solicitude prestadas na etapa final deste trabalho, com as análises de espectrometria de massas dos *spots* protéicos;

Ao Prof. Dr. Humberto Henrique de Carvalho do Laboratório de Fisiologia Vegetal, do Departamento de Bioquímica e Biologia Molecular (UFC), por ter aceito compor a banca examinadora, pelo convívio e pelos conselhos prestados;

À Profa. Dra. Maria Raquel Alcântara de Miranda, do Laboratório de Bioquímica e Fisiologia de Frutos, do Departamento de Bioquímica e Biologia Molecular da UFC, e seus orientandos, por ter nos auxiliado em parte dos experimentos;

À Profa. Dra. Ilka Maria Vasconcelos, do Laboratório de Toxinas Vegetais (DBBM – UFC), por todo o conhecimento, carinho e apoio adquiridos na disciplina de Bioquímica e durante todo o mestrado;

Ao Prof. Dr. Bruno Anderson Matias da Rocha, coordenador do programa de pós-graduação em Bioquímica da UFC, assim como os secretários Pedro Alencar e Vanda Livino pela compreensão e colaboração, que permitiram a elaboração deste trabalho;

Aos amigos Gyedre dos Santos Araújo e Daniel Gomes Coelho, outros componentes cruciais na elaboração desse trabalho, pelo companheirismo, pelo auxílio imensurável na execução desse trabalho, pelo agradável convívio, pelas sugestões, ensinamentos e pelos bons momentos vividos no Laboratório de Fisiologia Vegetal;

Ao Dr. Carlos Eduardo Braga de Abreu, do Departamento de Bioquímica e Biologia Molecular (UFC), pelo companheirismo, convívio, risadas e principalmente pelos auxílios prestados no início deste trabalho;

A meus pais, José Maurílio de Oliveira e Terezinha Araújo Barreto, pelo carinho, por sempre acreditarem na minha capacidade, pelo direcionamento e pelo apoio em cada decisão tomada por mim durante toda a minha vida;

À minha companheira e amiga Raissa Barros Rodrigues Neves, pelo amor, carinho, compreensão, conselhos, apoio, bons momentos e auxílios prestados nesses quase dois anos de convívio;

À minha irmã de criação Tereza Garcia de Morais e meu sobrinho, João Henrique Sávio Deodoro Filho, pelo carinho e convívio diário;

Aos demais membros e agregados do Laboratório de Fisiologia Vegetal (LABFIVE - UFC): Igor Moura, Luckas Huriel Duarte, Daniel de Oliveira, Paulo André Freitas, Stelamaris Paiva, Joanna Rocha, Cibelle Gadelha, Isa Mary Pereira, Camila Domingues, Lineker Lopes, Karollyny Roger, Cinthia Queiroz, Valeria Chaves, Viviane Ruppenthal e Valdineia Soares de Freitas, pelo convívio, risadas, aprendizado e experiências compartilhadas nestes dois anos;

Aos membros e agregados do Laboratório de Genômica Funcional (UFC): Prof. Dr. José Hélio Costa, Thais Andrade, Clesivan Pereira, André Luiz Maia, Sarah Gomes, Lyndefania Melo, Thays Aquino, Beatriz Lima, Katia Saraiva, Camila Freitas e à querida mascote Severina, pelas conversas, momentos e apoio prestados na elaboração deste trabalho;

Aos grandes amigos que este Mestrado me proporcionou, Ms. Felipe Barros e Ms. Roberto Nascimento (UFC), pelas experiências, conhecimentos trocados, convívio, consideração e apoio nos momentos mais delicados deste período;

Aos amigos de longa data Marcos Paulo Oliveira (primo), Tony Jeimison Matos, Diego Pereira, Caio Araújo, José Araujo, Bruno Damasceno, Rafael Vieira, Anderson Cavalcanti, Fellipe Cavalcante, Rainer Leal e Marcelo Farias, por estes quase 13 anos de risadas, saídas e companheirismo;

A meu terapeuta, Mateus Costa, pela disponibilidade, compreensão, diálogos e reflexões que temos tido e que me ajudaram a concluir este trabalho;

Aos demais amigos e familiares que de alguma forma me ampararam nas horas difíceis ou propiciaram bons momentos vividos: Carlos Eduardo Barreto, Jessika Barreto, Eduardo Barreto Neto, Diana Barreto, Eduardo Vasconcelos e Lucas Sampaio (primos e

agregados); a meus tios; a Raissa Bret e Handerson Ribeiro (DBBM – UFC); Samara e Rebeca Brito; Luiz Gonzaga Figueiredo (amigo do peito de longa data); Fernando Serafim Sales, Kamile Girão e Amanda Sombra; Deborah Doublas (Biotecnologia – UFC); aos membros do curso BRASAS Fernando Henrique Sales (*teacher Henry*), Karine Moura (*teacher Bunny*) e Germana Frota (*teacher Ge*) e a meus alunos; aos membros do time Instinct Pici e a todos que passaram pela minha vida e contribuíram de alguma forma para meu crescimento pessoal;

À Secretária do Departamento de Bioquímica e Biologia Molecular, Sra. Maria Gildemar de Oliveira e ao funcionário Daniel Farias, pela consideração e auxílios prestados;

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela bolsa concedida, e ao Instituto Nacional de Ciência e Tecnológia em Salinidade (INCTSal), pelo indispensável apoio financeiro para execução desse trabalho

*“*É o que as pessoas dizem [que o tempo muda tudo]. Não é verdade. Fazer coisas é o que muda algo. Não fazer nada deixa as coisas do jeito que eram”.

Dr. Gregory House

RESUMO

A salinidade é um fator abiótico que acarreta danos significativos à produtividade de inúmeras culturas em todo o mundo, principalmente nas regiões áridas e semiáridas, como é o caso do Nordeste brasileiro. Nesse quadro, a busca por estratégias de cultivo e a elucidação de mecanismos de tolerância a esse estresse têm se tornado cada vez mais importantes para a produção agrícola em solos salinos. Nos últimos anos, tem sido demonstrado que o fornecimento de amônio (NH_4^+), como fonte de nitrogênio, ativa mecanismos cruciais para a aclimatação de plantas de sorgo ao estresse salino. No presente estudo, foi conduzida uma investigação detalhada do proteoma de plantas de sorgo forrageiro (*Sorghum bicolor* L. Moench), genótipo CSF20, submetidas ao estresse salino e nutridas com nitrato (NO_3^-) ou NH_4^+, buscando identificar proteínas responsivas à salinidade e correlacioná-las com os mecanismos de tolerância ao estresse. A apresentação desse trabalho foi dividida em dois capítulos: no primeiro, Capítulo I, é apresentada uma revisão de literatura com o estado da arte dos principais temas abordados ao longo do estudo; e no segundo, Capítulo II, o qual se encontra na forma de um artigo completo para submissão, são apresentados os dados de crescimento, as respostas fisiológicas e o estudo proteômico das folhas das plantas de sorgo. Os resultados demonstraram claramente que as plantas de sorgo nutridas com NH_4^+ apresentaram melhor desempenho sob estresse salino que as nutridas com NO_3^-, dados os maiores valores de biomassa, tais como área foliar, massa seca da parte aérea e das raízes. A maior sensibilidade das plantas tratadas com NO_3^- foi associada a decréscimos nas massas secas de raízes e partes aéreas, acúmulo excessivo de Na^+ e baixa relação K^+/Na^+ nos tecidos. O estudo proteômico revelou que um grande de número de proteínas foi modulado diferencialmente pelo estresse salino e que essa regulação foi bastante influenciada pela fonte de nitrogênio. De maneira geral, 115 proteínas apresentaram algum tipo de alteração pelo estresse, tanto pelo aumento ou redução na abundância, como pela repressão ou síntese *de novo*. Sob estresse salino, plantas nutridas com NO_3^- apresentaram regulação em 67 proteínas, sendo 28 reguladas positivamente, 23 moduladas negativamente, 6 reprimidas e 10 sintetizadas *de novo*. A identificação, por LC-ESI-MS-MS, das proteínas alteradas pela salinidade revelou que essas moléculas estão envolvidas principalmente com o processo fotossintético/metabolismo do carbono (52%), o metabolismo energético (21%), a resposta ao estresse (9%), ao sistema de defesa antioxidativo (5%) e a outros processos celulares (13%). Em contraste, plantas nutridas com NH_4^+ mostraram alteração na expressão de 53 proteínas,

sendo 25 reguladas positivamente pela salinidade, 10 moduladas negativamente, 4 reprimidas e 14 sintetizadas *de novo*. Desse total de proteínas, 42% estão relacionadas com o processo fotossintético/metabolismo do carbono, 28% com o metabolismo energético, 13% com a resposta ao estresse, 2% com o sistema antioxidativo e 15% a outros processos celulares. A comparação do perfil proteico das plantas sob estresse salino (NO_3^- sal × NH_4^+ sal) mostrou que 35 proteínas foram expressas diferencialmente nas folhas; sendo 13 com maior abundância nos tecidos das plantas nutridas com NH_4^+, 11 mais abundantes naquelas tratadas com NO_3^-, 8 exclusivas do tratamento com NH_4^+ e 3 específicas do tratamento com NO_3^-. Os dados sugerem que, sob salinidade, o melhor desempenho das plantas tratadas com NH_4^+ está associado não somente à expressão de proteínas de resposta ao estresse, mas também ao acionamento de um metabolismo energético mais eficiente, o qual fornece energia para os mecanismos de defesa das plantas. Tais respostas possibilitam a manutenção da eficiência da maquinaria fotossintética e são fundamentais para a maior tolerância ao estresse salino. Os achados do presente estudo fornecem indícios de como a modulação do proteoma pode resultar em uma melhor aclimatação ao estresse salino. Esses resultados proporcionam novas perspectivas para o desenvolvimento de estratégias biotecnológicas a fim de se adquirir genótipos mais tolerantes à salinidade.

Palavras-chave: Crescimento. Estresse salino. Fotossíntese. Íons inorgânicos. Nutrição nitrogenada. Perfil proteico. *Sorghum bicolor*. Tolerância.

ABSTRACT

Salinity is an abiotic stress that causes significant damages to several crops yield around the world, especially in arid and semiarid regions, like the Brazilian Northeast. In this context, a search for cultivation strategies and the elucidation of the mechanisms of tolerance to that stress have become more and more important for agricultural production in saline soils. Lately, it has been shown that ammonium (NH_4^+) provided as nitrogen source activates pivotal mechanisms to the acclimation of sorghum plants to salt stress. In this work a detailed investigation in the proteome of forage sorghum (*Sorghum bicolor* L. Moench) genotype CSF20 under salt stress and fed with either nitrate (NO_3^-) or NH_4^+ was performed, aiming to identify salt-responsive proteins and correlate them with tolerance mechanisms to the stress. The presentation of this work was divided in two chapters. The first one, chapter I, exhibits a review with the state-of-the-art of the main subjects addressed throughout the study. In chapter II, in the form of article, growth data, physiological responses and the proteomic study of leaves of sorghum plants are presented. The results clearly demonstrated that NH_4^+-fed sorghum plants displayed better performance under salt stress than those ones supplied with NO_3^-, given higher biomass values, such as leaf area and root and shoot dry mass. The greater sensitivity of nitrate-grown plants was associated with massive decreases in roots and shoots dry masses, excessive Na^+ accumulation and low K^+/Na^+ ratio in tissues. The proteomic study revealed that a large number of proteins are differentially modulated by salt stress, and this regulation is highly influenced by the nitrogen source. In general, 115 proteins underwent some sort of alteration in reponse to the stress, like increase or decrease in the abundance, repression or *de novo* synthesis. Under salt stress, NO_3^--fed plants showed regulation in 67 proteins being 28 positively regulated, 23 negatively regulated, 6 repressed and 10 *de novo* synthesized. The identification of these proteins by LC-ESI-MS-MS revealed that they are involved mainly in carbon/photosynthetic metabolism (52%), energetic metabolism (21%), response to stress (9%) antioxidant defense system (5%) and other cellular processes (13%). In contrast, NH_4^+-grown plants displayed alteration in the expression of 53 proteins, being 25 positively salt-regulated, 10 negatively regulated, 4 suppressed and 14 *de novo* synthesized. Out of this total of proteins, 42% are associated with carbon/photosynthetic metabolism, 28% with energetic metabolism, 13% with response to stress, 2% with the antioxidant defense system and 15% other cellular processes. The comparison of the protein profile of plants under salt stress (NO_3^- salt × NH_4^+ salt) showed that 35 proteins are differentially expressed in

leaves, being 13 with greater abundance in tissues of NH_4^+-fed plants, 11 in those grown with NO_3^-, 8 NH_4^+-growth exclusive and 3 NO_3^- -treated specific. Data suggest that, under salinity, the better performance of NH_4^+ is related not only to the expression of stress-responsive proteins, but also to the activation of a more efficient energetic metabolism, which provides energy to defense mechanisms of those plants. Such results allow the maintenance of the photosynthetic apparatus efficiency and are crucial for the greater tolerance to salt stress. These findings give signs of how proteome modulation can result in a better acclimation to salt stress. The results offer new perspectives to the development of biotechnological strategies in order to acquire more salt-tolerant genotypes.

Keywords: Growth. Inorganic ions. Nitrogen nutrition. Protein profile. Salt stress. *Sorghum bicolor*. Tolerance.

LISTA DE ABREVIATURAS

APX	peroxidase do ascorbato
CAT	catalase
DHAR	redutase do desidroascorbato
GOGAT	glutamato-sintase (glutamina-oxoglutarato-aminotransferase)
GPX	peroxidase da glutationa
GR	redutase da glutationa
GS	glutamina-sintetase
HKT	proteína transportadora K^+de alta afinidade
H^+-PPase	pirofosfatase inorgânica
MDHAR	redutase do monodesidroascorbato
NHX	proteína antiporte vacuolar trocadora de Na^+/H^+
NiR	redutase do nitrito
NR	redutase do nitrato
NRT	transportadores simporte de nitrato (*Nitrate Transporters*)
P-ATPase	ATPase de membrana plasmática
POD	peroxidase
ROS	espécies reativas de oxigênio (*Reactive Oxygen Species*)
SOD	dismutase do superóxido
SOS1	proteína antiporte de sódio de membrana plasmática (*Salt Overly Sensitive 1*)
V-ATPase	ATPase de tonoplastos (membrana vacuolar)
2D	eletroforese bidimensional

SUMÁRIO

1 INTRODUÇÃO

A salinização dos solos é um importante fator que causa a perda de produtividade de solos cultivados. Sabe-se que, no mundo, as áreas com solos afetados pela salinidade têm aumentado, e este fenômeno é particularmente intenso em solos irrigados. Estima-se que 20% das áreas irrigadas (45 milhões de hectares), as quais produzem um terço dos alimentos mundiais, sejam afetadas pelo estresse salino (SHRIVASTAVA; KUMAR, 2015; MACHADO; SERRALHEIRO, 2017). No Brasil, estima-se que cerca de 20 a 25% das áreas irrigadas exibam algum grau de salinidade, sendo que a maior parte dessas terras se localiza na região do semiárido brasileiro (CASTELLANOS *et al.*, 2016).

O sorgo (*Sorghum bicolor* L. Moench) é uma cultura conhecida por possuir grande tolerância a altas temperaturas e ao déficit hídrico (BERENGUER; FACI, 2001; TERRA *et al.*, 2010). Essas características, associadas com o eficiente processo fotossintético, torna seu plantio possível nas regiões semiáridas e subtropicais, nas quais, além das elevadas temperaturas, são baixas as precipitações pluviométricas anuais (SMITH, FREDERIKSEN, 2000; BARCELOS *et al.*, 2011). O sorgo também é caracterizado por sua moderada tolerância ao estresse salino (LACERDA *et al*., 2005), sendo esta característica fundamental para utilização dessa espécie com rendimentos economicamente aceitáveis, principalmente quando o cultivo em condições salinas é inevitável (AQUINO; LACERDA; GOMES-FILHO, 2007).

De modo geral, a aclimatação de plantas ao estresse salino depende dos ajustamentos osmótico, iônico e bioquímico, que incluem: o acúmulo seletivo ou a exclusão de íons; o controle da absorção de íons pelas raízes e seu transporte para as folhas; a compartimentalização de íons ao nível celular e de planta inteira; a síntese de solutos compatíveis, mudanças nas vias fotossintéticas, indução de enzimas e compostos antioxidativos e de hormônios vegetais, dentre outros (MUCHATE *et al*., 2016). No nível molecular, essas regulações podem envolver vários genes associados a múltiplas vias metabólicas de percepção, sinalização e resposta ao estresse (KURUSU e*t al.*, 2015).

A proteômica tornou-se um dos enfoques mais promissores para compreender os mecanismos de tolerância das plantas aos estresses ambientais, tais como a salinidade (AHMAD *et al*., 2016). Por meio dessa abordagem, o padrão de proteínas responsivas aos estresses ambientais tem sido investigado, utilizando-se de técnicas de eletroforese e espectrometria de massas (CARUSO *et al*., 2008; AGRAWAL *et al*., 2013). Além disso, os estudos envolvendo aplicações biotecnológicas têm contribuído para melhorar a compreensão

das proteínas e processos metabólicos envolvidos nas respostas ao estresse em plantas (SILVEIRA; CARVALHO, 2016). Recentemente, vários trabalhos abordaram o estudo proteômico em plantas sob condições de salinidade, sendo verificado que diversas proteínas diferencialmente expressas pelo estresse pertenciam a importantes processos fisiológicos, tais como o metabolismo do carbono ou às vias de defesa antioxidantes, bem como a outras importantes vias metabólicas (SOBHANIAN; AGHAEK; KOMATSU, 2011; ABREU *et al*., 2014; MOSTEK *et al*., 2015).

Miranda *et al.* (2013), estudando a tolerância de plantas de sorgo à salinidade, observaram que ela dependia da fonte de nitrogênio presente no meio de crescimento. Segundo esses autores, as plantas nutridas com amônio (NH_4^+) exibiram melhores características fisiológicas que as nutridas com nitrato (NO_3^-). Recentemente, Miranda *et al*. (2017) comprovaram que o NH_4^+ como única fonte de nitrogênio ativa mecanismos, que envolvem a expressão gênica de bombas de H^+ e transportadores antiporte Na^+/H^+ de membrana plasmática e de tonoplasto, que controlam o acúmulo de Na^+ na planta inteira e conferem maior tolerância das plantas de sorgo à salinidade. No entanto, apesar desses avanços não se conhece o conjunto das proteínas que são diferencialmente alteradas sob condições de estresse salino e na presença de diferentes fontes de nitrogênio (nitrato e amônio), cujo conhecimento pode contribuir para o esclarecimento das vias metabólicas envolvidas com a tolerância aos sais.

Diante do exposto, o objetivo do presente trabalho foi estudar as alterações fisiológicas, especialmente aquelas no proteoma, de folhas de plantas de sorgo (espécie com genoma completo conhecido e disponível) sob estresse salino e expostas a diferentes fontes de nitrogênio. Espera-se com isso, contribuir para uma melhor compreensão da aclimatação de plantas ao estresse salino, bem como entender melhor o papel da fonte de nitrogênio na tolerância ao estresse salino.

2 OBJETIVOS

2.1 Objetivo geral

Investigar as alterações fisiológicas, com ênfase no perfil proteico de folhas, de plantas *Sorghum bicolor* sob estresse salino em função da fonte de nitrogênio, bem como relacioná-las aos mecanismos de tolerância ao estresse salino

2.2 Objetivos específicos

- ✓ Determinar os parâmetros de crescimento (área foliar, massa seca da parte aérea e das raízes) de plantas de sorgo nutridas com nitrato ou amônio e submetidas ao estresse salino;
- ✓ Mensurar o acúmulo dos íons K^+ e Na^+ nos tecidos da parte aérea e das raízes de plantas de sorgo estressadas com NaCl;
- ✓ Investigar as alterações ocorrentes nas taxas de assimilação de CO_2 e na eficiência quântica máxima e efetiva do fotossistema II de plantas de sorgo estressadas com NaCl e tratadas com diferentes regimes de nitrogênio inorgânico;
- ✓ Elaborar mapas proteômicos reprodutíveis e de qualidade das proteínas de folhas, avaliando a influência dos tratamentos sobre os padrões eletroforéticos bidimensionais;
- ✓ Analisar os mapas proteômicos e identificar as proteínas diferencialmente expressas nas folhas das plantas de sorgo dos diferentes tratamentos;
- ✓ Realizar um estudo da ontologia das proteínas diferencialmente expressas e identificar possíveis vias metabólicas de aclimatação ao estresse salino.

3 FUNDAMENTAÇÃO TEÓRICA

3.1 Estresse salino e seus efeitos sobre as plantas

A salinidade em terras cultiváveis é um problema crescente e limita o desenvolvimento das plantas, culminando em perda de produtividade. De acordo com Pedrotti *et al*. (2015), a área total de solos salinos corresponde a 7% de toda a superfície terrestre, equivalente a aproximadamente 10.000.000 km^2. No Brasil, o problema da salinização é verificado em todo país, principalmente na região Nordeste, onde aproximadamente 25% das áreas irrigadas estão salinizadas (PEDROTTI *et al*., 2015).

A salinidade induz uma série de respostas morfológicas, fisiológicas e bioquímicas nas plantas decorrentes de mudanças nos processos moleculares (GUPTA; HUANG, 2014). Essas respostas variam amplamente entre as espécies e dependem do tipo e concentração do sal, do tempo e número de exposições ao estresse, do estado fenológico da planta, bem como de outros fatores (SHINOZAKY *et al.*, 2015).

Os efeitos adversos da salinidade sobre as plantas podem ser ocasionados pelo estresse osmótico, toxicidade iônica e/ou deficiência nutricional (ZHU, 2002). Durante a exposição ao estresse, em um curto intervalo de tempo, apenas o efeito osmótico atua sobre a planta, induzindo variações instantâneas (MUNNS, 2002). Nessa condição, os sais na solução do solo baixam seu potencial hídrico em decorrência da diminuição do potencial osmótico do solo, o que acarreta na dificuldade da absorção de água pelas plantas. Já os problemas de toxicidade normalmente surgem quando os íons do solo são acumulados nas plantas em demasia, ficando dessa maneira dissolvidos no citosol e em outras organelas onde acabam interferindo em inúmeras reações enzimáticas e provocando danos ao seu crescimento (MUNNS, 2002; MUNNS; TESTER, 2008; PRISCO; GOMES-FILHO; MIRANDA, 2016). Em adição a esses efeitos diretos da salinidade sobre as plantas, inúmeros efeitos secundários também podem surgir. Por exemplo, o estresse salino também provoca um desbalanço no estado redox das células, gerando um estresse oxidativo através da produção excessiva de espécies reativas de oxigênio (ROS), tais como o peróxido de hidrogênio oxigênio (H_2O_2), o oxigênio singleto ($^1O_2$) e os radicais livres superóxido ($\bullet O_2^-$) e hidroxil (HO•). Essas ROS são altamente reativas e podem alterar o metabolismo celular normal através da oxidação de biomoléculas (DEMIDCHIK, 2015).

A inibição do crescimento em decorrência desses efeitos ocorre na maioria das

espécies cultivadas, mesmo em baixas concentrações de sais (CHINNUSAMY; JAGENDORF; ZHU, 2005). Contudo, o grau no qual o crescimento das plantas é reduzido dependerá das diferenças nos níveis de tolerância entre as espécies e entre cultivares de uma mesma espécie (PARIDA; DAS, 2005). Nesse contexto, as plantas têm sido classificadas em halófitas e glicófitas, de acordo com a tolerância ao estresse salino. As halófitas são plantas que crescem em solos salinos e toleram altas concentrações de sais, enquanto que as glicófitas, que compreendem a maioria das plantas cultivadas, são sensíveis aos sais e não toleram longa ou breve exposição a ambientes salinos (CHINNUSAMY; JAGENDORF; ZHU, 2005). Durante o estresse salino, os principais processos metabólicos da planta, como a fotossíntese, a respiração, a síntese de proteínas, as relações hídricas e as reações enzimáticas, são afetados e a primeira resposta é a redução da taxa de crescimento foliar, seguida de supressão do crescimento quando o estresse se acentua (MUNNS, 2002; PARIDA; DAS, 2005).

O acúmulo de Na^+ nos tecidos tem sido considerado determinante para a maior sensibilidade ao estresse salino em diversas espécies de plantas (HAMAMOTO *et al.*, 2015). Entretanto, as plantas desenvolveram mecanismos para prevenir e aliviar os danos provocados pelo excesso de sais, a fim de restabelecer as condições homeostáticas, especialmente a homeostase da relação K^+/Na^+, retomando seu crescimento, ainda que com taxas reduzidas (ZHU, 2001; ZHANG; SHI, 2013). De modo geral, nas glicófitas, a tolerância das plantas à salinidade depende da habilidade em controlar o acúmulo e o transporte de Na^+, envolvendo: i) seletividade na absorção pelas células das raízes; ii) exclusão ou compartimentalização de Na^+ nas células das raízes; iii) carregamento do K^+ no xilema em detrimento do Na^+; iv) descarregamento do Na^+ da seiva do xilema; v) minimização da translocação dos íons Na^+ e Cl^- para a parte aérea em crescimento (MUNNS; TESTER, 2008; MAATHUIS; AHMAD; PATISHTAN, 2014). Além disso, as plantas devem ser capazes de alterar a estrutura da membrana plasmática; induzir a expressão e a atividade de enzimas antioxidativas; e alterar seu balanço hormonal, a fim de restabelecer sua homeostase celular e, finalmente, retomar seu crescimento e desenvolvimento (ZHU, 2001; PARIDA; DAS, 2005; MUNNS; TESTER, 2008).

Assim, nas situações em que a salinidade do solo não pode ser mantida em um nível aceitável para as culturas exploradas economicamente, por meio de um manejo adequado do solo e da água de irrigação, uma alternativa viável é a seleção criteriosa de espécies ou cultivares que, ao mesmo tempo, sejam mais tolerantes aos danos causados pelos

sais e possam ter produções economicamente satisfatórias.

3.2 Mecanismos de tolerância ao estresse salino

Muitos fatores determinam como as plantas respondem ao estresse salino, e como suas interações culminam em tolerância ou susceptibilidade à salinidade (SHINOZAKI *et al*., 2015). A tolerância ao estresse salino pode ser definida como a capacidade das plantas de crescerem e completarem seu ciclo de vida, mesmo sob altos níveis de sais no meio (YADAV *et al*., 2012). Como referido anteriormente, as glicófitas para sobreviverem ao estresse salino necessitam ativar complexas redes regulatórias, as quais envolvem mudanças fisiológicas, bioquímicas, morfológicas e moleculares integradas que não são transmitidas para as gerações futuras. Esse processo é conhecido como aclimatação e envolve o restabelecimento da homeostase celular em organismos individuais (CABELLO; LODEYRO; ZURBRIGGEN, 2014).

3.2.1 Ajustamento osmótico e iônico

Para restabelecer o gradiente de potencial hídrico e a consequente absorção de água, as plantas precisam tornar o potencial hídrico das raízes menor que o do solo. Tal processo é denominado ajustamento osmótico e é considerado um mecanismo importante para a aclimatação de plantas à seca e à salinidade, pois possibilita as plantas manter o turgor e processos relacionados, tais como: a abertura estomática, o crescimento e o alongamento celular (SINGH *et al*., 2015).

O ajustamento osmótico ocorre através da absorção de íons e da síntese de solutos orgânicos como: açúcares, polióis, aminoácidos, amidas e compostos de amônio quaternário (FLOWERS; MUNNS; COLMER, 2015). Esses solutos compatíveis não são tóxicos às células, mesmo sob altas concentrações e não possuem carga líquida em pH fisiológico (FAROOQ *et al*., 2017). Embora nem todas as culturas sejam capazes de sintetizar essas moléculas, a maioria das plantas tolerantes ao estresse salino tendem a acumulá-las sob essas condições, mantendo o teor relativo de água nos tecidos e o consequente crescimento da planta (SAXENA *et al*., 2013; NASCIMENTO *et al*., 2015; FAN *et al*., 2016).

Em adição ao efeito dos solutos orgânicos, muitos estudos têm evidenciado que plantas tolerantes à salinidade também podem alcançar o ajustamento osmótico através da

distribuição de íons entre o vacúolo e o citosol (HASEGAWA, 2013; NASCIMENTO *et al*., 2015; FAN *et al*., 2016). Fan *et al*. (2016), ao determinarem o transcriptoma de plantas de *Paulownia tomentosa* submetidas ao estresse salino, destacou a expressão aumentada dos transcritos associados a uma complexa rede regulatória para manter a homeostase intracelular, tanto osmótica quanto iônica, o que culminou no aumento da tolerância dessa espécie à salinidade.

Além do seu papel no ajustamento osmótico, é essencial que, sob condições de estresse salino, as células regulem o fluxo iônico, para manter baixa a concentração de íons tóxicos, como o Na^+ e alta a concentração de íons essenciais, como o K^+. Esse processo é conhecido por ajustamento iônico, ou seja, plantas tolerantes ao estresse salino são aquelas capazes de manter alta razão K^+/Na^+ citosólica, especialmente nos tecidos fotossintetizantes (FAROOQ *et al*., 2017).

Como citado anteriormente, os principais mecanismos relacionados ao restabelecimento da homeostase iônica envolvem: o efluxo de Na^+ de volta para o meio de crescimento ou para o apoplasto; a compartimentação de Na^+ no vacúolo; o controle do carregamento do xilema; a retenção de Na^+ nas células do caule; a recirculação de Na^+ pelo floema; e a alocação de sais para as folhas mais velhas (YAMAGUCHI; HAMAMOTO; UOZUMI, 2013). Esses processos são mediados por canais e transportadores associados à membrana plasmática, ou ao tonoplasto, e são fortemente regulados (SHINOZAKY *et al*., 2015).

Os principais transportadores são o SOS1 (*Salt Overly Sensitive 1*), o NHX (*Na^+/H^+ exchanger*) e, em menor escala, o HKT (*High-affinity K^+ Transporter*). Tais proteínas catalisam o transporte de Na^+ à custa da formação de um gradiente de potencial eletroquímico, gerado por bombas de prótons na membrana plasmática (P-ATPases) e no vacúolo (V-ATPases e H^+-PPases), caracterizando, assim, um transporte ativo secundário. O SOS1 é localizado na membrana plasmática e é responsável pelo efluxo de Na^+ a partir do citosol, enquanto que NHX é encontrado no tonoplasto e é responsável pela compartimentação desse íon no vacúolo. Já aos transportadores HKT tem sido atribuído a recirculação de Na^+ entre os órgãos da planta, contribuindo para a menor exposição dos tecidos fotossintetizantes à toxicidade desse íon (KHAN; AHMAD; KHAN, 2015). Assim, diversas pesquisas têm evidenciado que o aumento da expressão de genes ou da atividade fisiológica dos transportadores e bombas contribuem para a regulação da homeostase K^+/Na^+

e, consequentemente, para o aumento da tolerância das plantas à salinidade (LU *et al*., 2014; MANCARELLA *et al*., 2016; MIRANDA *et al*., 2017).

Em adição ao seu papel no restabelecimento do gradiente de potencial hídrico planta-solo, o acúmulo de osmólitos compatíveis pode desempenhar funções protetivas, uma vez que atuam também como antioxidantes, e com isso, contribuem para a manutenção da integridade das macromoléculas e para a eliminação de várias ROS. Além disso, esses osmoprotetores estabilizam enzimas e proteínas (FAROOQ *et al*., 2017).

3.2.2 O estresse oxidativo e o ajustamento redox

As plantas, sob condições ótimas de crescimento, mantêm baixo nível de ROS, porém em condições de estresse, como o salino, por exemplo, essas moléculas têm suas concentrações elevadas, gerando o chamado estresse oxidativo (VAIDYANATHAN *et al*., 2003; KAUR; KAUR; ARORA 2016). Nesse caso, as plantas se ajustam com relação a seu estado de óxido/redução de modo a manter o equilíbrio entre a produção e a eliminação das ROS, sendo esse processo essencial para a proteção oxidativa das células vegetais (NOCTOR; FOYER, 2016). De acordo com esses autores, as plantas possuem mecanismos de defesa oxidativa, os quais podem ser enzimáticos e não enzimáticos. O primeiro é constituído principalmente pelas enzimas catalase (CAT), peroxidase do ascorbato (APX), peroxidase da glutationa (GPX), dismutase do superóxido (SOD), redutase da glutationa (GR), redutase do monodesidroascorbato (MDHAR), redutase do desidroascorbato (DHAR), peroxidases (que utilizam fenóis para eliminar o H_2O_2) (POD) dentre outras (NOCTOR; FOYER, 2016); enquanto o segundo é formado por compostos de baixo peso molecular, que podem ser de natureza lipofílica, como os tocoferóis e carotenoides, ou hidrofílica, como o ascorbato e a glutationa. A coordenação entre esses sistemas está intimamente relacionada com a tolerância das plantas à salinidade (MUCHATE *et al*., 2016; NOCTOR; FOYER, 2016; SHAFI *et al*., 2017).

Sob condições de estresse salino, a regulação positiva de enzimas antioxidantes, bem como dos níveis de compostos antioxidantes, pode ser considerada como uma resposta proativa de aclimatação, que resulta em níveis mais baixos de ROS e maior tolerância ao estresse salino (HOSSAIN *et al*., 2016). Nesse contexto, Hossain *et al*. (2017) demonstraram que a tolerância de plantas de mangue ao estresse salino está diretamente relacionada com o aumento de atividade das enzimas CAT, APX, SOD, GPX e MDHAR, principalmente nas

raízes. Nessa mesma linha de raciocínio, Chakraborty *et al*. (2016), estudando seis genótipos de amendoim com tolerância diferencial ao estresse salino, observaram que a atividade da POD foi significativamente aumentada nos genótipos tolerantes, o que contribuiu para a redução no conteúdo de H_2O_2 nesses genótipos. Segundo esses autores, os resultados encontrados sustentam a hipótese de que a tolerância do amendoim ao estresse salino pode ser atribuída a uma melhor capacidade de defesa antioxidante. Adicionalmente, a superexpressão dos genes *CuZnSOD* e *APX* em plantas de batata doce sensíveis à salinidade promoveu uma eficiente limpeza de ROS e o crescimento das raízes nas plantas transgênicas em comparação às selvagens (YAN *et al*., 2016).

3.3 Proteômica como ferramenta para elucidação dos mecanismos de tolerância à salinidade

O estudo sistemático global das proteínas, conhecido como proteômica, aliado a ensaios fisiológicos e moleculares, tornou-se uma poderosa ferramenta para a identificação de proteínas e de mecanismos envolvidos nas respostas da tolerância aos estresses ambientais, pois permite avaliar simultaneamente o perfil proteico e, em paralelo, a identificação de alterações pós-traducionais induzida por estresses, inclusive o salino (WILKINS *et al*., 1996; SOBHANIAN *et al*., 2010; ABREU *et al*., 2014).

Pesquisas destinadas à comparação de diversos proteomas são, na maior parte, dominadas pela separação das proteínas por eletroforese bidimensional em gel de poliacrilamida e, em seguida, por identificação dessas proteínas por espectrometria de massas (CARUSO *et al*., 2008; AGRAWAL *et al*., 2013). A eletroforese bidimensional (2D) permite a separação das proteínas pelo ponto isoelétrico e pela massa molecular e, após a confecção de mapas proteicos bidimensionais, as proteínas podem ser analisadas através de softwares específicos para a análise da imagem dos géis 2D. Logo após, apenas as proteínas que apresentarem alterações significativas reprodutíveis são selecionadas para a identificação por espectrometria de massas (ROGOWSKA-WRZESINSKA *et al*., 2013).

A espectrometria de massas é uma técnica analítica extremamente valiosa, pois apresenta acurado grau de sensibilidade. Nela, as moléculas presentes na amostra são convertidas em íons em fase gasosa, que são subsequentemente separados no espectrômetro de massas de acordo com a relação massa/carga (m/z) (DASS, 2001; CUNHA; CASTRO; FONTES, 2006). Essa técnica é crucial nos estudos proteômicos, principalmente nos que

empregam uma abordagem comparativa ocasionada por certos estímulos, tais como mudanças no estádio de desenvolvimento e estresses ambientais. Assim, os passos centrais no emprego da proteômica em plantas são: (a) extração e solubilização das proteínas; (b) separação das proteínas por focalização isoelétrica e massa molecular (eletroforese 2D); (c) digestão das proteínas com uma enzima proteolítica, tal como a tripsina; (d) determinação das massas dos fragmentos peptídicos por espectrometria de massas; e (e) análise das proteínas de interesse em bancos de dados (RAMPITSCH; SRINIVASAN, 2006).

Recentemente, vários estudos empregando abordagens proteômicas foram realizados em diferentes cultivares e espécies de plantas sob condições de salinidade. Em plântulas de sorgo (*Sorghum bicolor* L.), Swami *et al*. (2011) avaliaram as mudanças no proteoma em resposta ao tratamento com NaCl e identificaram um total de 21 proteínas diferencialmente sintetizadas pelo sal. A maioria das proteínas identificadas pertencia a vias de transdução de sinal, transporte de íons inorgânicos e metabolismo.

Ghaffari *et al*. (2014), por sua vez, em estudos com folhas de arroz (*Oryza sativa* L.), demostraram que dos 864 *spots* detectados de forma reprodutível, 67 *spots* mostraram regulação diferencial sob estresse salino. Os autores observaram que as proteínas que apresentaram alterações estavam envolvidas em importantes processos fisiológicos, tais como fotossíntese, síntese e processamento de proteínas e transdução de sinal. Além disso, a síntese de proteínas relacionadas com o mecanismo de defesa antioxidante, conhecidas como eliminadoras de ROS, também sofreram alterações com a salinidade. De modo similar, Huerta-Ocampo *et al*. (2014) investigaram as mudanças no perfil de expressão de proteínas em raízes de *Amaranthus cruentus* L. submetidas à salinidade. Na ocasião, os autores identificaram 77 proteínas diferencias em resposta ao estresse salino, que foram agrupadas em diferentes categorias de processos biológicos, tais como aquelas envolvidas com o metabolismo dos carboidratos, a biossíntese e metabolismo dos aminoácidos e ácidos graxos e as relacionadas com as respostas de defesa. Já a comparação do proteoma de dois genótipos contrastantes à salinidade de *Brassica juncea* L. mostrou que 42 proteínas eram responsivas à salinidade, sendo relacionadas a inúmeros processos celulares, incluindo: fotossíntese, homeostase redox, metabolismo do nitrogênio, síntese de ATP e síntese e degradação de proteínas (YOUSUF *et al*., 2017).

No geral, existe um grande número de informações que os estudos proteômicos podem oferecer capazes de esclarecer os efeitos da salinidade na gama de proteínas relacionadas a processos fisiológicos, bioquímicos e moleculares essenciais. Adicionalmente,

com o auxílio de ferramentas da bioinformática e de técnicas particulares de proteômicas, tais como a proteômica redox e a fosfoproteômica, estudos voltados para a integração do proteoma, transcriptoma, fisiologia e bioquímica poderão constituir uma importante estratégia para o melhor entendimento dos mecanismos que governam as respostas à salinidade. (STRÖHER *et al*., 2006; LV *et al*., 2014; SILVEIRA; CARVALHO, 2016).

3.4 Fonte de nitrogênio e tolerância ao estresse salino

O nitrogênio (N) é um dos elementos mais importante para o crescimento e produtividade das plantas, sendo constituinte de biomoléculas essenciais, como ATP, NADH, NADPH, clorofila, proteínas, enzimas, nucleotídeos (ácidos nucléicos) e muitos outros metabólitos (HARPER, 1994; MASCLAUX-DAUBRESSE *et al*., 2010). Este elemento apresenta-se disponível na solução do solo principalmente nas formas dos íons NO_3^- e NH_4^+, que podem ser absorvidos pela planta através da membrana plasmática das células da epiderme e do córtex da raiz, principalmente nas regiões mais jovens (região apical) (ZHONGHUA *et al*., 2011).

Após ser absorvido pelas raízes, o NO_3^- pode ser reduzido a NH_4^+ através das enzimas do metabolismo do nitrogênio ou ser transportado para a parte aérea, via xilema, para ser assimilado nas folhas, uma vez que somente parte do NO_3^- absorvido é assimilado nas raízes (CRAWFORD; GLASS, 1998; TAIZ; ZEIGER, 2013). No interior da célula, o nitrato é reduzido a nitrito pela ação da redutase do nitrato (NR), o qual, por ser tóxico, é rapidamente convertido em amônio pela atividade da redutase do nitrito (NiR), seguido pela assimilação do NH_4^+ em aminoácidos, os quais podem ser translocados para as outras partes da planta (WANG *et al.*, 2012; KRAPP, 2015). Em contrapartida, a absorção direta de NH_4^+ pode ocorrer nas raízes, com rápida assimilação pela atividade das enzimas sintetase da glutamina (GS) e sintase do glutamato (GOGAT), no chamado ciclo GS/GOGAT, resultando num processo com menor custo energético de absorção e assimilação, quando comparado à absorção do NO_3^- (XU; FAN; MILLER, 2012). A absorção de nitrato ocorre majoritariamente por meio de transporte ativo secundário mediado pelo simporte 2 H^+/NO_3^- NRT (do inglês *Nitrate Transporter*). Nesse processo, NO_3^- é transportado passivamente, mas depende de um gradiente de prótons no apoplasto gerado pela P-ATPase, o qual envolve a hidrólise de ATP. Contudo, a absorção de amônio pode se dar tanto por transporte ativo secundário, por

mecanismo similar àquele observado para o nitrato, ou por transporte passivo, quando a amônia (NH_3) é a forma predominante (XU; FAN; MILLER, 2012).

Em condições de salinidade, as plantas estão sujeitas a impactos que afetam a aquisição de nutrientes do solo, devido a competição dos íons Na^+ e Cl^-, que inibem a absorção de importantes íons como o K^+ e o NO_3^- (MUNNS; TESTER, 2008). Estudo com plântulas de *Arabdopsis* destaca os efeitos negativos da salinidade na absorção de nitrato e assimilação do amônio (DEBOUBA *et al.*, 2013), respostas comumente induzidas pelo estresse salino na maioria das glicófitas, como a inibição da atividade de transportadores e enzimas relacionados ao metabolismo do nitrogênio nas plantas (DEBOUBA *et al.*, 2007; SHAO *et al*, 2015). Assim sendo, recentes estudos apontam que a modificação da fonte de nitrogênio, com substituição parcial ou total de NO_3^- por NH_4^+, pode favorecer o desenvolvimento de algumas espécies, principalmente quando estas estão sujeitas a condições de estresse (KANT *et al.*, 2007; BYBORDI, 2012; HU *et al.*, 2015). Segundo Zhonghua *et al.* (2011), plantas de *Catharanthus roseus* mostraram-se mais resistentes ao estresse salino quando crescidas em meio nutritivo com quantidades equimolares das formas de NO_3^- e NH_4^+. Para os autores, a nutrição com as formas combinadas de nitrogênio induziram menor acúmulo de Na^+ nos tecidos, maior acúmulo de açúcares solúveis e de aminoácidos livres e manutenção da fotossíntese líquida em reposta ao estresse salino, resultando na mitigação dos efeitos deletérios da salinidade no crescimento das plantas.

Apesar da vantagem energética, um dos fatores que limitam o uso do íon NH_4^+ é sua toxicidade à célula. Alguns dos sintomas de toxidez comum entre as espécies envolvem inibição da absorção de cátions, como K^+, Mg^{2+} ou Ca^{2+}; acidificação do meio de crescimento; desbalanço hormonal e aumento do estresse oxidativo (BRITTO; KRONZUCKER, 2002; BITTSÁNSZKY *et al.*, 2015; ESTEBAN *et al.*, 2016), embora o mesmo não se aplique para algumas espécies, como o tomate (NIEVES-CORDONES *et al.*, 2007), o sorgo (MIRANDA *et al.*, 2013) e citrus (FERNÁNDEZ-CRESPO *et al.*, 2014). Para as espécies *Carrizo citrange*, *Spartina alterniflora* e *Sorghum bicolor*, a nutrição com NH_4^+, como fonte exclusiva de nitrogênio, foi determinante para mitigar os danos resultantes do estresse salino.

Segundo Fernández-Crespo, Camañes e García-Agustín (2012), a nutrição com NH_4^+ atuou acionando os sistemas de defesa antioxidante, bem como promoveu a redução do acúmulo dos íons Cl^- nos tecidos fotossintéticos, contribuindo para a aclimatação das plantas de citrus em condições de salinidade. De forma semelhante, o feito *priming* foi observado em

halófita *S. alterniflora*, com intensificação das respostas antioxidantes que reduziram os danos de membrana mesmo em altas concentrações de NaCl a 500 mM (HESSINI *et al.*, 2013). Em sorgo, a nutrição com NH_4^+ promoveu o menor acúmulo de Na^+ na planta, através da indução da atividade e da expressão gênica das bombas P-ATPase e V-ATPase e transportadores SOS1 e NHX de membrana plasmática e de tonoplasto (ALVAREZ-PIZARRO *et al.*, 2011; MIRANDA *et al.*, 2013; 2017).

Também podem ser atribuídas à nutrição com amônio as reduções nos danos na fotossíntese causados pela salinidade (MIRANDA *et al.*, 2014). Embora nos tecidos fotossintetizantes o NH_4^+ possa interferir negativamente no transporte de elétrons da fotofosforilação (SCHORTEMEYER; STAMP; FEIL, 1997), Miranda *et al.* (2014) forneceram evidências que as reduções na fotossíntese induzidas pela salinidade foram mitigadas em plantas de sorgo nutridas com NH_4^+, resultando na alta taxa fotossintética em comparação com as plantas nutridas com NO_3^- sob estresse salino.

O grupo de pesquisa de Fisiologia Vegetal da Universidade Federal do Ceará tem demonstrado que a nutrição com NH_4^+ ativa diversas rotas metabólicas, que constituem mecanismos determinantes para a aclimatação de plantas de sorgo ao estresse salino (ALVAREZ-PIZARRO *et al*., 2011; MIRANDA *et al.*, 2013; 2014; 2016; 2017). No entanto, ainda não se sabe quais proteínas são moduladas pela fonte de nitrogênio prevalecente no meio de crescimento de plantas de sorgo, bem como não existem relatos de como essas respostas ocorrem quando as plantas estão expostas ao estresse salino.

3.5 O Sorgo

O sorgo (*Sorghum bicolor* L. Moench) é uma gramínea anual, pertencente à família *Poaceae* (gramínea) e com metabolismo fotossintético do tipo C4. É uma cultura bastante versátil e eficiente no aspecto fotossintético, sendo tolerante à seca e a temperaturas elevadas (BERENGUER; FACI, 2001; BARCELOS *et al.*, 2011; TERRA *et al.*, 2010). Sua significância no mundo deve-se à sua grande aplicabilidade (MEKI *et al.* 2013), sendo originário da África, onde é empregado na alimentação básica humana, assim como na China e Índia. Já nos Estados Unidos, Austrália e América do Sul ele é utilizado principalmente na dieta animal (BELTON; TAYLOR, 2004; BARCELOS *et al.*, 2011). É uma cultura moderadamente tolerante à salinidade, sendo o quinto cereal mais cultivado no mundo, estando atrás apenas do arroz, trigo, milho e cevada, respectivamente (FAO, 2012).

Tem sido relatado, na literatura, vários genótipos de sorgo em estudos envolvendo o estresse salino, como por exemplo, o CSF 20, o qual é moderadamente tolerante (LACERDA *et al.*, 2003; OLIVEIRA; GOMES-FILHO, 2009) e, portanto, de grande importância para estudos de fisiologia, visando o esclarecimento dos mecanismos de tolerância à salinidade em plantas exposta a diferentes fontes de nitrogênio.

4 A DETAILED INVESTIGATION OF NITROGEN SOURCE-REGULATED SALINITY RESPONSIVE PROTEINS IN SORGHUM LEAVES

(Artigo a ser submetido)

4.1 Abstract

This work aimed to evaluate the modulation of proteome and physiological responses of sorghum plants [*Sorghum bicolor* (L.) Moench] under salinity as affected by inorganic nitrogen (N) sources. Five days after sowing, plants were transferred to complete nutrient solutions modified to contain 5 mM N, supplied as either NO_3^- or NH_4^+. Seven days after acclimation in the nutrient solutions, plants were subjected to salt stress with 75 mM NaCl, which was applied in two doses of 37.5 mM. Plants were harvested ten days after salinity (10 DAS). Salinity promoted significant reductions in leaf area, root and shoot dry mass of sorghum plants, regardless of the nitrogen source; however, a higher growth was observed in ammonium-grown plants. The better performance of ammonium-fed stressed plants was associated with increased CO_2 assimilation and higher K^+/Na^+ homeostasis under salinity. Proteomic study revealed that, under salt stress, nitrogen source modulates proteins mainly related to photosynthesis/carbon metabolism, energetic metabolism, response to stress and other cellular processes. Nitrate-fed plants induced a series of proteins of thylakoidal electron transport chain, structural and carbon assimilation enzymes, but these mechanisms seemed to be not enough to mitigate salt damage in photosynthetic performance. Additionally, plants from nitrate treatments possibly failed in activating the antioxidant system, once some enzymes were downregulated under stress, resulting in severe complications in plant metabolism such as excessive reactive oxygen species generation. In contrast, the greater tolerance to salinity of ammonium-grown plants may arised from different mechanisms: i.) *de novo* synthetis of tilacoidal electron transport chain, structural proteins and upregulation of enzymes from photosynthetic/carbono metabolism, which resulted in better CO_2 assimilation rates under NaCl-stress; ii.) activation (exclusively or by upregulation) of proteins involved in

energetic metabolism which make available energy salt responses, most likely by H^+-ATPases proton pumps and Na^+/H^+ antiporters; and iii.) reprogramating of proteins involved in response to stress and other metabolic processes, constituting intricate pathways of salt responses. Consequently, the ponctual modulation of proteome in ammonium-fed plants seem to be more effective to lead a better acclimation to salinity. Overall, our findings not only provide new insights of molecular basis of salt tolerance in sorghum plants induced by ammonium nutrition, but also give new perspectives to develop biotechnological strategies to generate more salt-tolerant crops.

Keywords: Ammonium. Nitrate. Proteomics. Salt stress. Salt tolerance. *Sorghum bicolor*.

4.2 Introduction

Soil salinization is a significant environmental factor that restricts plant growth, thus causing serious loss of productivity of cultures around the world, especially in arid and semiarid regions where land irrigation is widely used. This phenomenon can be caused by many factors like poor water management, inappropriate drainage systems, weather conditions, soil features and others. Countries like Australia, India, Pakistan, and the United States, which face significant salinity and drainage problems that affect between 15 and 36% of their irrigated lands, are devoting substantial resources toward this problem. Worldwide, approximately one-third of the irrigated areas that provides 40% of the global food production is affected by salinization (El-Mageed et al., 2018). This is a worrisome issue, for around 0.3 to 1.5 million of hectares of total arable lands are getting unusable every year (FAO, 2015). In Northeastern region of Brazil, around 25% of irrigated lands are affected by salinity, which is equivalent to 9 million hectares of arable soils (Freire *et al.*, 2014). Salt harmful effects on plant growth and metabolism may arise from two components, the osmotic that causes water deficit; and the ionic, which is characterized by a severe accumulation of Na^+ and Cl^- toxic ions. Also, salinity may disrupt numerous cellular events, such as enzyme activities, generation of reactive oxygen species and others. Both aspects can cause growth inhibition as well as impairment of nutrient uptake, membrane degradation, and cell and tissue death (Munns and Tester, 2008).

Nitrogen is a crucial macronutrient for plant growth, and its main sources typically occurring in the soil are nitrate (NO_3^-) and ammonium (NH_4^+). However, when there

is low NO_3^- availability in the soil, ammonium is taken up by plant roots (Zhong et al., 2018). It has been reported that nitrogen availability can be an efficient approach to generate acclimation to salinity in plants (Fernández-Crespo *et al.*, 2012; Miranda *et al.*, 2014). Although NH_4^+ at high levels becomes toxic to most plants, some species display some tolerance to this ion, such as rice (Wu et al., 2018), citrus (Fernandez-Crespo *et al.*, 2012) and sorghum (Miranda *et al*, 2013; Miranda *et al.*, 2016). Ammonium absorption by plant cells is more energetically cost-effective than nitrate (Xu *et al*., 2012), and it has been reported that salinity can inhibit NO_3^- uptake through competition with Cl^- ions (Zhonghua *et al.*, 2011). In addition, NH_4^+-treated citrus plants had reduced salt-induced oxidative stress by raising polyamine levels and activating hormone pathways, such as abscisic acid and salicylic acid (Fernandez-Crespo *et al*., 2012). In sorghum, NH_4^+ supply was able to partially 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). Mrid *et al.* (2016), studying the effects of nitrogen source and concentration in sorghum roots, found that at high nitrogen levels, especially ammonium, sorghum roots showed an increase in biomass accompanied by an increase in protein content, including some nitrogen metabolism enzymes such as glutamine synthetase and aspartate aminotransferase, which had their activities raised as well.

Ammonium nutrition has proved to be efficient in alleviating the toxic effects of salinity in others species. Rajaie and Motieallah (2018), studying the effect of ammonium ion in lemon seedlings under salt stress, observed that ammonium nutrition not only increased shoot dry weight even under saline conditions, but also increased shoot contents of macronutrients (N, P and K) and some micronutrients as well (such as Cu, Fe, Mn, and Zn), which contributed to alleviate the harmful effects of salinity in this plant. Moreover, ammonium nutrition has also been stated to atenuate not only salt stress, but also other abiotic factors like drought and heavy metals. Pereira et al. (2018), analyzing the effects of ammonium growth in *Guzmania monostachia* plants under drought stress, reported significant increases in malic acid and citric acid contents, as well as increases in phosphoenolpyruvate-carboxylase and malate dehydrogenase enzyme activities, as well as increases in sugar contents (sucrose, fructose and glucose) and antioxidant enzyme activities (such as superoxide dismutase, catalase, ascorbate peroxidase) in plants treated with ammonium as a N source. Cheng et al (2016) reported that ammonium nutrition enhanced root length and surface and biomass of both species in comparison to the nitrate one under cadmium stress and increased

cadmium accumulation in *Carpobrotus rossii* and *Solanum nigrum*, two hyperaccumulators. This proves that ammonium nutrition can also be used to improve strategies like phytoremediation.

Proteomics approaches have also been used to further understand how plants adjust to different N sources. For example, the response of whole plant proteome to different nitrate levels has been studied in maize, *Hordeum vulgare*, and wheat (Wang et al., 2012). However, due to the small number of proteins identified from these studies, regulatory mechanisms of plant nitrogen responses at the protein level remain poorly understood (Wang *et al.*, 2012). In addition, in sorghum, the alteration of proteomic profile in response to stress modulated by nitrogen nutrition remains to be elucidated.

Sorghum (*Sorghum bicolor* L. Moench) is an annual grass species with C4 photosynthetic metabolism. It is a versatile crop with a great adaptability to growth in stressful environments, being moderately tolerant to abiotic stresses like high temperatures, salinity, and drought (Venuto and Kindiger, 2008; Meki *et al.*, 2013; Miranda *et al.*, 2016). Sorghum plants and seeds are widely employed for animal and human food (Yan *et al.*, 2012) and sugar and ethanol production (Whitfield *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.

4.3 Materials and Methods

4.3.1 Plant material and growth conditions

Seeds of *Sorghum bicolor* L. Moench, CSF 20 genotype supplied from Instituto Agronômico de Pernambuco (IPA), Recife, PE, Brazil, were surface sterilized in sodium hypochlorite at 3% (v/v) for 5 min, and then exaustively rinsed with distilled water and subsequently sown in plastic cups contained vermiculite umedeced with distilled water. After germination, ten uniform seedlings were transferred to 10 L plastic trays containing one-third Hoagland's nutrient solutions modified to contain inorganic nitrogen at 5.0 mM, provided as either nitrate or ammonium (Miranda *et al.*, 2012). The nutrient solutions were kept under constant aeration, being renewed every three days. The 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 3 L plastic containers and submitted to saline treatment with

NaCl at 0 mM (control treatment) or 75 mM. This concentration was chosen due to the fact that it can inhibit growth higher than 50% in sorghum plants (Silva et al., 2003). The final NaCl concentration was achieved in a daily dose of 37.5 mM NaCl, in order to avoid osmotic stress. Five plants from control and salt treatments of each nitrogen source were harvested 10 days after salinity (10 DAS). Five plants of each treatment had their leaves stored at -20 ºC for proteomic analyses and the rest was used for growth parameters, CO_2 assimilation and ions determination.

The experiment was conducted in a greenhouse, and the environmental conditions were the following: mean air temperature of 32.0 ± 2.0 °C during the day and 27.5 ± 2.0 °C at night; mean air relative humidity of 65.6 ± 9.2 % and an average midday photosynthetic photon flux density (PPFD) of approximately 1,300 μmol m^{-2} s^{-1}.

4.3.2 CO_2 assimilation rate and photosystem II efficiency

Before harvesting, CO_2 assimilation and photosystem II efficiency of sorghum plants was measured in fully expanded leaves using a leaf chamber fluorometer (6400-40, LI-COR, Lincoln, NE, USA) coupled to a portable photosynthesis system (LI-6400-XT, LI-COR, Lincoln, NE, USA). Analyses were done under constant CO_2 concentration of 380 μmol CO_2 mol^{-1} air and photosynthetic photon flux density of approximatelly 1,200 μmol photons m^{-2} s^{-1} on days with full sun. The parameters measured were CO_2 assimilation rate (*A*), photosystem II maximum efficiency (Fv/Fm) and effective quantum yield of PSII (Φ PSII).

4.3.3 Growth parameters

During harvesting, plants were initially separated in roots and shoots and the leaf area was determined employing an area meter (LI – 3100, Li-Cor, Lincoln, NE, USA). Then, plant material was divided in two groups: the first one was stored at -20 for protein extraction and 2-DE analysis; and the latter was dried by lyophilization and the dry mass of shoot and roots was measured. The material was finely powdered and stored for further analysis.

4.3.4 Determination of inorganic ions

Extracts for ion inorganic determination were prepared after incubation of 18 mg of powdered root and shoot samples in 1.8 mL of deionized water in water bath at 40 ºC for 1.0 h. The homogenate was centrifuged at 3.000 × g for 10 min and the supernatant was colleted, filtered in filter papers, and then used for determination of K^+ and Na^+ using a flame photometer, according to Malavolta *et al.* (1989).

4.3.5 Protein extraction

Total soluble protein was extracted from fresh frozen leaves of each treatment according to Mesquita *et al.* (2012). Approximately 200 mg of leaf samples were homogeneized in 10 mL of extraction buffer solution composed by 40 mM Tris-HCL (pH 7.5), 250 mM sucrose, 10 mM EDTA, 1.0 mM PMSF, 1.0 mM DTT, 1% Triton-X 100 (v/v), 2% 2-mercaptoethanol (v/v) and 200 mg of PVPP. The homogenate was kept under constant agitation for 1.0 h at 4 ºC, being vortexed every 15 min. Thereafter, the material was centrifuged at 6.000 × g for 15 min, at 4 ºC. The supernatant was collected and submitted to precipitation with 10 mL of 10% (w/v) TCA in cold acetone for 12 h at -20 ºC. Then, the homogenate was centrifuged at 6.000 × g for 10 min at 4 ºC, the supernatants were discarded, and the pellets were submitted to several washes with cold acetone and finally with 80% ethanol (v/v). The resulting pellet was vacuum dried and dissolved in 400 uL lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS(w/v), 2% IPG buffer 4-7 (v/v) and 1% DTT (w/v)) and kept in constant agitation for 1.0 h. Finally, the suspension was centrifuged at 12.000 × g for 10 min, at 25 ºC, and the supernatant was collected and used as proteic extract for bidimensional electroforesis (2-DE) assays. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (Sigma-Aldrich, USA) as standard.

4.3.6 SDS-PAGE

Protein samples were submitted to electrophoresis under non-reducing conditions on 12.5% polyacrylamide gels (SDS-PAGE) according to the method described by Laemmli (1970), in order to verify protein integrity. A 0.1% coomasie brilliant blue R-250 (w/v) was

used to stain protein bands for at least 2 h (Neuhoff et al., 1988). After dying, a solution of 7% acetic acid (v/v) and 40% methanol (v/v) was used for gel discoloration.

4.3.7 Bidimensional electrophoresis (2-DE) and image analysis

Proteic extracts were diluted with rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 2% IPG buffer (pH 4–7) (v/v), 0.3% DTT (w/v) and 0.002% bromophenol blue (w/v) (GE Healthcare: Life sciences, USA). Approximately 500 μg of protein was loaded onto a 13-cm, pH 4–7 linear gradient IPG strip (GE Healthcare, USA). Isoelectric focusing (IEF) was conducted in an Ettan™ IPGPhor™ (GE Healthcare) according to the manufacturer's instructions. Briefly, focusing was performed at 20 ºC with the following steps: 200 V during 1 h; 500 V until 500 Vhr; gradient at 1,000 V until 800 Vhr, gradient at 8,000 V until 11,300 V; 8,000 V during 0.5 h; and finally, 500 V during 2 h.

After IEF, the strips with proteins were submitted to denaturation with an initial equilibration buffer [75 mM Tris–HCl (pH 8.8), 6 M urea, 29.3% glycerol (v/v), 2% SDS (w/v), 0.002% bromophenol blue (w/v) and 1% DTT (w/v)] for 30 min; and then alkylated by a second equilibration buffer with 2.5 % iodoacetamide (w/v) instead of DTT for 30 min in the dark, at room temperature. The second dimension of electrophoresis was performed on 12.5% polyacrylamide gels (w/v) using a SE 600 Ruby electrophoresis unit (GE-Healthcare). The system was powered by an EPS 3501 XL power supply (GE-Healthcare). Alkylated strips were placed on the top of SDS-PAGE gels and then sealed with 0.5% molten agarose solution (v/v) (containing 0.002% v/v bromophenol blue). Electrophoresis was run at 10 mA per gel for one hour and followed by 40 mA per gel until the bromophenol tracking dye ran off the gel. After SDS-PAGE, gels were fixed overnight with a solution of 10% acetic acid (v/v) and 40% ethanol (v/v). Then, gels were stained for at least 48 h with 0.1% colloidal coomasie blue G-250. Finally, gels were kept in conservation solutions of 5% (v/v) acetic acid until further analyses.

2-DE gels were scanned at 300 dpi 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. Background subtraction, landmark identification, matching of spots within gels, quantification of matched

spots and spot analysis were performed according to the software guide. Before spots matching, one of the gel images was chosen as reference gel. Quantitative image analysis was performed to display protein spots with reproductible differences in relative abundance intensity % (1.5-fold and p value < 0.05). Experimental molecular weight (MW) of each protein was deducted using 2-DE markers (LMW Calibration Kit for SDS Electrophoresis, GE-Healthcare, UK), and the experimental isoelectrical point was determined by the migration of the markers on the IPG strip.

5.3.8 In-gel digestion

Selected protein spots with reproductible 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). Briefly, gel pieces (around 1 mm^2) were destained by washing three times with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN) (v/v), pH 8.8. The microtubes with the spots were vortexed every wash, but after the second one, gel pieces were kept in contact with the destaining buffer overnight. After the third wash, gel pieces were dehydrated twice with 100% ACN for five min. At the end of this process, ACN was removed and the gel pieces were dried out in a vacuum concentrator machine (SpeedVac Concentrator, Eppendorf). Gel pieces were rehydrated again, for 45 min at 4 ºC with a 40 mM ammonium bicarbonate and 10% ACN containing 20 ng μL^{-1} trypsin (Sequencing Grade Modified Trypsin, Promega). Protein digestion was conducted at 37ºC for 16 h, with a dry bath Peltier system (Thermomixer, Bioer). Digested peptides were extracted from the gel pieces using 5% fomic acid (v/v) in 50 % (v/v) ACN in three repetitive steps with an ultrasonicator (UltraCleaner 1400A, Unique, SP, Brazil). Samples were transferred to sterilized microtubes, vacuum-concentrated (SpeedVac Concentrator, Eppendorf) and used for protein identification by mass spectrometry analysis.

4.3.9 Mass spectrometry analysis

Extracted tryptic fragments were analyzed by capillary liquid chromatografy/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. Product ion spectra collected were processed by Protein Lynx Global Server 2.0 software (Waters Corporation, MA, USA), being converted to peak-list text files for database searching.

4.3.10 Protein identification

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 SwisProt (www.uniprot.org) databases were utilized under the taxon Viridiplantae (Green Plants). All peptides were hypothesized to be monoisotopic and $[M+H]^+$ (protonated molecular ions). Other searched parameters were: a mass accuracy of ± 1.2 Da, complete carbamidomethylation of cysteine residues and partial oxidation of methionine residues.

To identify a protein by LC–ESI–MS/MS, these criteria were adopted: a minimum of three independent matching peptides, significant protein scores given by MASCOT ($p < 0.05$), and a minimum of 2% coverage of protein by matching peptides. The peptides were also researched for identification by allignment against UniProt/Monocots database (http://www.uniprot.org/?tab=blast) and also the NCBI/Reference protein/ Monocots database, (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi), with $p < 0.05$ and max identity = 100%.

4.3.11 Experimental design and statystical analysis

Experimental design was completely randomized design in a 2 × 2 factorial, composed by 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 (NH_4^+ control treatment - AC); b) NH_4^+-fed plants in presence of 75 mM NaCl (NH_4^+ salt treatment - AS); c) NO_3^--treated plants without NaCl (NO_3^- control treatment - NC); and d) NO_3^--treated plants in presence of 75 mM NaCl (NO_3^- salt treatment - NS). For growth, CO_2 assimilation, photosystem II effciency and ion accumulation, the experiments were performed using five repetitions, being each repetition composed by one plant. The data were submitted to a two-way analysis of variance (two-way ANOVA) and, when significative at $p < 0.05$ level, the

main values were analyzed by Tukey test. For gel analyses, spots intensities of differential proteins in a 2D-gel were calculated from three spots in three replicated gels. 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'.

4.4 Results

4.4.1 Salt tolerance indicators

In nitrate-grown plants, salinity caused significant decreases in leaf area (LA) (↓65%), root dry mass (RDM) (↓56%) and shoot dry mass (SDM) (↓47%) as compared to control (Fig. 1A; Table 1). The reduced growth was closely related to reductions in K^+/Na^+ ratio in roots (↓95%) and shoots (↓86%). Similarly, ammonium-fed plants showed significant reductions by salinity in LA (↓40%), RDM (↓29%), SDM (↓17%), K^+/Na^+ ratio in root (↓77%) and shoots (↓75%) (Fig. 1B; Table 1). However, no significant alteration was reported in CO_2 assimilation, photosystem II maximum efficiency (Fv/Fm) and effective quantum yield of PSII (Φ PSII) in both nitrate and ammonium-grown plants (Fig. 1A, B; Table 1).

By comparing both salt-stressed groups, the data clearly evidence that ammonium-grown plants displayed a better performance under salt stress (Fig. 1C; Table 1). Under salinity, the values of LA, RDM and SDM of ammonium-grown stressed plants were 50, 42 and 46% higher than those of nitrate-grown stressed plants, respectively. In a similar way, the K^+/Na^+ ratio of ammonium-grown plants was found to be 141 and 89% greater than the ones of nitrate treatments, in roots and shoots, respectively (Fig. 1C; Table 1). However, no significant alteration was reported in CO_2 assimilation, photosystem II maximum efficiency (Fv/Fm) and effective quantum yield of PSII (Φ PSII) in both nitrate and ammonium-salt plants.

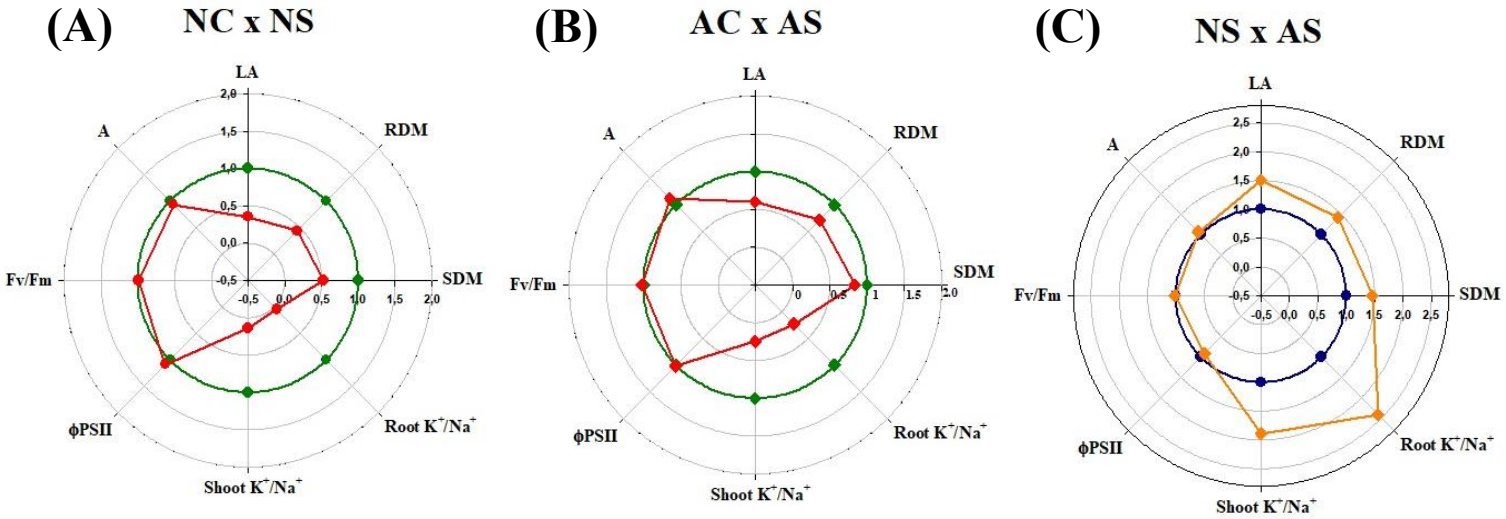

Figure 1. Overall characterization of sorghum physiological responses to NaCl stress (red line). Comparison between nitrate-grown sorghum plants without (NC) and under (NS) 75 mM-NaCl stress (A); ammonium-grown plants without (AC) and under (AS) 75 mM-NaCl stress (B), and nitrate (NS) and ammonium-grown plants (AS) under 75 mM-NaCl stress (C). In (C), NS is the blue line and was taken as reference, while the orange line represents AS plants. Leaf area (LA), root dry mass (RDM), shoot dry mass (SDM), root K^+/Na^+ ratio, shoot K^+/Na^+ ratio, effective quantum yield of photosystem II (Φ PSII), photosystem II maximum efficiency (Fv/Fm) and CO_2 assimilation (A) of ammonium-grown plants. Values of control plants were normalized and taken as reference (control treatment, green line).

Table 1. Comparison between nitrate-grown sorghum plants without (NC) and under (NS) 75 mM-NaCl stress; ammonium-grown plants without (AC) and under (AS) 75 mM-NaCl stress, and nitrate (NS) and ammonium-grown plants (AS) under 75 mM-NaCl stress (NS x AS). Shoot dry mass (SDM; g $plant^{-1}$), root dry mass (RDM; g $plant^{-1}$), leaf area (LA; cm^2), net photosynthesis (*A*; μmol CO_2 m^{-2} s^{-1}), maximum quantum efficiency of PSII (Fv/Fm), effective quantum yield of PSII (ΦPSII), shoot K^+/Na^+ ratio and root K^+/Na^+ ratio in roots and shoots.

Variable	NC x NS		AC x AS		NS x AS	
	NC	NS	AC	AS	NS	AS
SDM	1.353 a	0.713 b	1.250 a	1.039 b	0.713 b	1.039 a
RDM	0.344 a	0.152 b	0.302 a	0.216 b	0.152 b	0.216 a
LA	227.676 a	80.180 b	199.890 a	120.548 b	80.180 b	120.548 a
A	39.279 a	36.693 a	35.109 a	39.431 b	36.693 a	39.431 b
Fv/Fm	0.762 a	0.756 a	0.755 a	0.772 a	0.756 a	0.772 a
ΦPSII	0.352 a	0.375 a	0.344 a	0.350 a	0.375 a	0.350 a
Shoot K^+/Na^+	12.350 a	1.693 b	12.897 a	3.197 b	1.693 b	3.197 a
Root K^+/Na^+	7.890 a	0.382 b	4.023 a	0.921 b	0.382 b	0.921 a

Note: Data are means of five repetitions. Different lowercase letters indicate significant differences due to salt stress to each comparison (NC x NS, AC x AS or NS x AS) according to Tukey's test ($p \leq 0.05$).

4.4.2 2D electrophoresis profile, protein identification and level of regulation of proteome

To detect the modulation of proteome of sorghum leaves, 2-DE patterns from control and salt treatments from both nitrogen sources were analyzed. Regulation of proteomic profile by salt stress in nitrate-grown plants is shown in Fig. 2. In general, 256 proteins were detected in leaves of nitrate-grown plants, of which 67 were characterized as differentially modulated in response to salinity (Fig. 3; Table 2). A total of 28 proteins was upregulated and 23 downregulated by salt stress, whereas six proteins were repressed under NaCl stress and 10 were found to be *de novo* synthesis (Fig. 3; Table 3). In this treatment, after identification (Table 2), 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. 4).

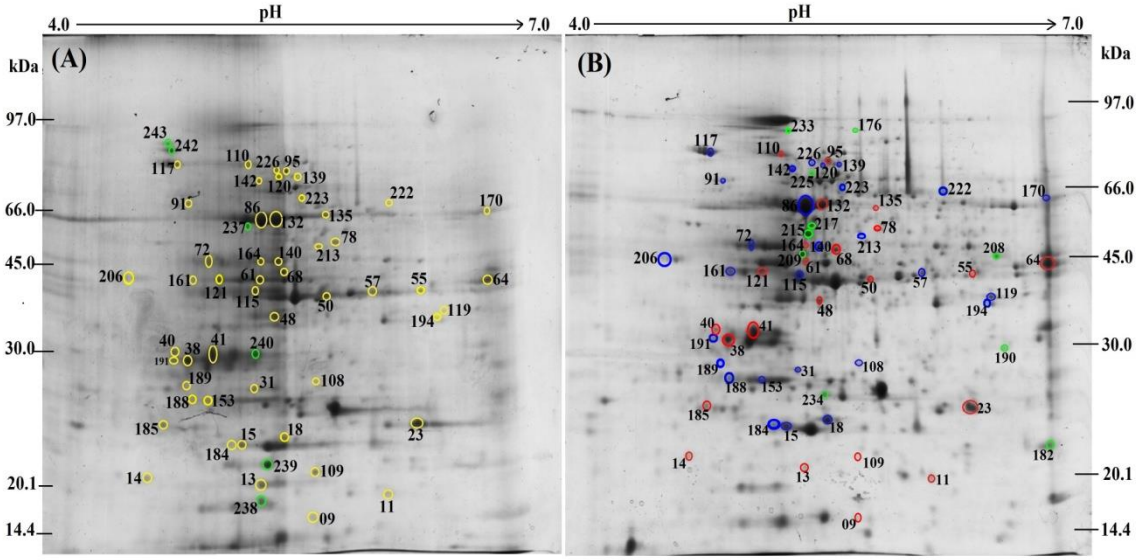

Figure 2. Coomasie brilliant blue G 250-stained two-dimensional gels from leaves of nitrate-fed sorghum plants in control conditions (A) and in salt stress with 75 mM NaCl (B). Proteins (500 μg) were separated in 13 cm IPG strips (linear 4-7 pH) in isoelectrical focusing and in SDS-PAGE on 12.5% polyacrylamide gels. Differentially modulated protein spots (intensity % ≥ 1.5-fold) are numbered. Spots marked with yellow circles indicate those ones present in the control treatment that underwent differential protein expression in relation to the salt treatment. Spots marked with blue and red circles represent proteins that were significantly upregulated (blue) or downregulated (red); spots with green circles in control plants indicates proteins whose expression was suppressed by salinity, whereas green circles in the salt gel point out that those spots suffered *de novo* synthesis under stress. Spots identified are statistically significant to a level of 95% per group (Student's t-test) using biological and analytical replicates (n = 3).

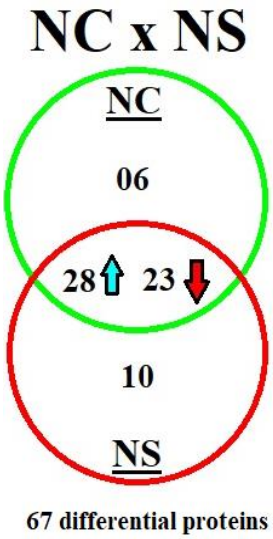

Figure 3. Venn diagram representation of different subsets of nitrate-fed *Sorghum bicolor* L. Moench cv. CSF 20 leaves, displaying their changes in control conditions (NC) and under salt-stress with 75 mM NaCl (NS).

Table 2 – List of differentially regulated proteins in leaves of nitrate-grown sorghum plants under salt stress (75 mM NaCl). Differential spots were excised from 2DE gels, comparison NC × NS (see Fig. 3), and the proteins were identified by LC–ESI–MS/MS.

Spot	Protein (Species)	Access number (NCBI/SwissProt)	Peptide sequences (MS/MS)	Sequence coverage (%)	Score	Biological process (localization)	pI/MM (kDa) Theoretical	pI/MM (kDa) Experimental
9	Cytochrome b6-f complex iron-sulfur subunit [*Zea mays*]	ACG28186.1	GDPTYLVVEQDK GPAPLSLALVHADVDDGK VLFVPWVETDFR	18	275	Photosynthesis; electron transporter, transferring electrons within cytochrome b6/f complex of photosystem II activity (Chloroplast)	8.52 24	5.65 13
11	Superoxide dismutase [Cu-Zn]	C0HK70.1	AVAVLGSSEGVK	7	71	Cellular response to oxidative stress (Cytosol)	5.48 15	6.05 19
13	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial [*Zea mays*]	ANP22077.1	VTPKLGVPPEEAGAAVAAESSTGTWTTVWT-DGLTSLDR	24	87	Carbon fixation (Chloroplast)	5.17 17	5.20 20
14	FHA domain containing protein [*Sorghum bicolor*]	XP_002442729.1	WILDPAGDGDWR VARPGAIEIISDDAVTVGR VADKADIVLPIATVSGTHAR KGGSLLVTDLDSTNGTYINER	31	449	Regulation of developmental growth; stamen filament development mRNA binding (Nucleus)	8.31 25	4.46 20
15	PEPcase - C4 phosphoenolpyruvate carboxylase [*Saccharum officinarum*]	CAC08829.1	VSEELIQYDALLVDR FLDILQDLHGPSLR HGDFSDEGSATTESDIEETLKR	5	400	Carbon Fixation (Cytosol)	5.94 109	5.09 23

18	Light-harvesting complex I chlorophyll a-b binding protein 1B-21 [*Triticum urartu*]	EMS45291.1	DFGFDPLGLATVPENFER KYPGGAFDPLGFSK	15	103	Photosynthesis, light harvesting (Chloroplast)	4.93 22	5.37 23
23	Oxygen-evolving enhancer protein 2 [*Sorghum bicolor*]	XP_002461438.1	TNTEYIAYNGDGFK KTITEYGSPEEFLSQVDFLLGK QAFGGSTDSEGGFETGAVATANVLESSTPVV-DGK QYYSVSVLTR TADGDEGGKHQLITATVSDGK HQLITATVSDGK	38	466	Photosynthesis; light reaction; calcium ion binding (Chloroplast)	8.63 28	6.24 24
31	L-ascorbate peroxidase 2 [*Sorghum bicolor*]	XP_002463451.1	LAWHSAGTFDVATK NPAEQAHGANAGLEIAVR LPDATQGSDHLR QVFSTQMGLSDQDIVALSGGHTLGR SGFEGAWTSNPLIFDNSYFTELLSGEK EGLLQLPSDK ALLSDPSFRPLVDK	48	600	Cellular response to oxidative stress (Cytosol)	5.18 27	5.17 26
38	Soluble inorganic pyrophosphatase 6 [*Sorghum bicolor*]	XP_002452886.1	AQGQPETLDYR KVSPWHDVPLR AGDGVFHFVVEIPK MEVATDEAFTPIK VKPLAALAMIDEGELDWK ASLVNDVDDVEKHFPGTLTAIR VIEETNESWEK	38	696	Phosphate-containing compound metabolic process; response to salt stress (Chloroplast)	5.78 32	4.73 29
40	Plastid-lipid-associated protein 6 [*Sorghum bicolor*]	XP_002449783.1	LLSAVSGLNR GLAASQEDLDRADAAAR LLPVTLGQVFQR GSLAQLPPLEVPR VPDNLRPPSSSSNAGSGEGEFEVTYLDDDTR	32	525	Chloroplast organization. Resistance to multiple stresses (Chloroplast)	8.84 31	4.64 29
41	Oxygen-evolving enhancer protein 1 [*Sorghum bicolor*]	XP_021305066.1	RLTYDQIQSLTYMEVK GTGTANQCPTIDGGVEAFPFK KLCLEPTSFTVK LTYTLDEIEGPLEVGSDGTLKFEEK FEEKDGIDYAAVTVQLPGGER DGIDYAAVTVQLPGGER QLVATGKPESFGGPFLVPSYR GGSTGYDNAVALPAGGR GDEEELLKENIK NAAASTGNITLSVTK SNPETGEVIGVFESVQPSDTDLGAK	54	1071	Photosystem II assembly and stabilization (Chloroplast)	5.91 35	4.89 29

48	Glyoxylase 1 [*Saccharum hybrid cultivar*]	AHF18211.1	AETPEPLCQVMLR YTIAMLGYADEDK GNAYAQVAIGTNDVYK IASFVDPDGWK VVLVDNTDFLR	21	380	Detoxification of methylglyoxal; Response to abiotic stresses (Cytosol)	5.44 33	5.31 35
50	Fructose-bisphosphate aldolase [*Sorghum bicolor*]	XP_002442893.1	LASIGLENTEANR IVDILVEQGIVPGIK YAAISQDNGLVPIVEPEILLDGEHGIER VTAAQDALLLR	17	319	Glycolytic process; Reduction (Calvin cycle) (Chloroplast)	6.39 42	5.65 38
55	Ribulose bisphosphate carboxylase/oxygenase activase [*Sorghum bicolor*]	XP_002451328.1	VPLILGIWGGK MSCLFINDLDAGAGR VPIIVTGNDFSTLYAPLIR TDGVDEEHVVQLVDTFPGQSIDFFGALR YLNEAALGAANEDAMK	20	382	Rubisco activation (Chloroplast)	6.97 49	6.26 40
57	Ribulose 1,5-bisphosphate carboxylase/oxygenase activase precursor [*Zea mays*]	AAG22094.3	VPLILGIWGGK MSCLFINDLDAGAGR VPIIVTGNDFSTLYAPLIR TDGVDEEHVVQLVDTFPGQSIDFFGALR	23	378	Rubisco activation (Chloroplast)	6.43 34	5.96 39
61	ATP synthase beta subunit [*Radula tasmanica*]	AHZ11983.1	TVLIMELINNIAK VALVYGQMNEPPGAR MVQAGSEVSALLGR	21	223	ATP biosynthesis (Chloroplast)	5.77 21	5.22 42
64	Glyceraldehyde-3-phosphate dehydrogenase 1 [*Sorghum bicolor*]	XP_021305022.1	VALQSEDVELVAVNDPFITTDYMTYMFK TLLFGQKPVTVFGIR NPEEIPWGEAGADYVVESTGVFTDK KVVISAPSK DAPMFVVGVNEDKYTSDINIVSNASCTTNCL-APLAK VIHDNFGIIEGLMTTVHAITATQK VPTVDVSVVDLTVR GIMGYVEEDLVSTDFVGDSR LVSWYDNEWGYSNR	55	906	Glycolytic process (Cytosol)	7.05 36	6.70 42
68	Ribulose 1,5-bisphosphate carboxylase/oxygenase activase precursor [*Zea mays*]	AAG22094.3	VPLILGIWGGK VPIIVTGNDFSTLYAPLIR TDGVDEEHVVQLVDTFPGQSIDFFGALR	19	225	Rubisco activation (Chloroplast)	6.43 34	5.36 44

72	Phosphoglycerate kinase [*Sorghum bicolor*]	XP_002441313.1	SVGDLTEADLEGK ADLNVPLDENQNITDDTR LSELLGIQVQK LVDALPIGGVLLLENVR LASLADLYVNDAFGTAHR ELDYLVGAVSSPK RPFAAIVGGSK IGVIESLLEK GVSLLLPTDVVIADK VGVADVMSHISTGGGASLELLEGK	34	1037	Reductive pentose-phosphate cycle (Chloroplast)	5.89 50	4.87 46
78	S-adenosylmethionine synthase OS [*Dendrobium crumenatum*]	METK_DENCR	TQVTVEYR FVIGGPHGDAGLTGR	5	90	S-adenosylmethionine biosynthetic process (Cytosol)	5.42 44	5.70 53
86	ATP synthase CF1 beta subunit [*Sorghum bicolor*]	YP_899414.1	TNPTTSRPGVSTIEEK IDQIIGPVLDITFPPGK LPYIYNALIVK DTADKQINVTCEVQQLLGNNR QINVTCEVQQLLGNNR AVAMSATDGLMR GMEVIDTGTPLSVPVGGATLGR IFNVLGEPIDNLGPVDTSATFPIHR SAPAFIELDTK TVLIMELINNIAK AHGGVSVFGGVGER TREGNDLYMEMK VALVYGQMNEPPGAR VGLTALTMAEYFR DVNKQDVLLFIDNIFR FVQAGSEVSALLGR MPSAVGYQPTLSTEMGSLQER KGSITSIQAVYVPADDLTDPAPATTFAHLDA-TTVLSR GIYPAVDPLDSTSTMLQPR IVGNEHYETAQR YKELQDIIAILGLDELSEEDR	68	2288	ATP synthesis coupled proton transport (Chloroplast)	5.31 54	5.22 61
91	Rubisco large subunit-binding protein subunit alpha [*Sorghum bicolor*]	XP_002440887.1	LANAVGVTLGPR NVVLDEYGSPK AIELYDPMENAGAALIR TNDSAGDGTTTASVLAR LGLLSVTSGANPVSLK TVQGLIQELENK AVLQDIAIVTGAEFLAK DLGLLVENATEAQLGTAR VTIHQTTTTLIADAASKDEIQAR ELAETDSVYDTEK VGAATETELEDR	34	953	Protein refolding (Chloroplast)	5.07 61	4.74 67

			IKDSVWEVGYNAMTDTYENLIEAGVIDPAK					
95	Phosphoglucomutase 1 [*Zea mays*]	PGMC1_MAIZE	YNMGNGGPAPESVTDK SMPTSAALDVVAK FFEVPTGWK YDYENVDAGAAK DSQDALAPLVDVALK	11	177	Glucose metabolic process (Cytosol)	5.46 63	5.38 77
108	Photosystem II stability/assembly factor HCF136 [*Sorghum bicolor*]	XP_021318830.1	LETSRPENSGLSAALDLLTEAK GGPISFADLIQFAAQSALK TDYEVDLITTLTK	15	214	Cellular response to oxidative stress (Chloroplast)	8.43 38	5.73 27
109	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Nasturtium officinale*]	ALF41107.1	VTPQPGDPPEEAGAAVAAESSTGTWTTVWT-DGLTSLDR TFQGPPHGIQVER	30	167	Reductive pentose-phosphate cycle; photorespiration (Chloroplast)	5.75 19	5.64 21
110	Luminal-binding protein [*Saccharum hybrid cultivar* R570]	AGT15890.1	ITPSWVAFTDSER VFSPEEISAMILGK DAGVIAGLNVAR IINEPTAAAIAYGLDK VEIESLFDGTDFSEPLTR FEELNNDLFR SQIHEIVLVGGSTR	14	518	Endoplasmic reticulum protein complex assembly (Endoplasmic reticulum)	5.14 73	5.24 74
115	Fructose-bisphosphate aldolase [*Sorghum bicolor*]	XP_002450383.1	GILAMDESNATCGK RLDSIGLENTEANR IVDVLNEQGIVPGIK GLVPLAGSNNESWCQGLDGLASR EAAYYQAGAR YAAISQENGLVPIVEPEILLDGEHGIER ATPEQVAEYTLK AAQDALLLR	32	730	Glycolytic process; Reduction (Calvin cycle) (Chloroplast)	5.85 42	5.34 69
117	Heat shock 70 kDa protein 6 [*Zea mays*]	ONM07984.1	VVGIDLGTTNSAVAAMEGGKPTIVTNAEGAR QFAAEEISAQVLR AVITVPAYFNDSQR IINEPTAASLAYGFEK NDEGIDLLKDK AKFEELCSDLLDR DIDEVILVGGSTR	16	611	Protein folding (Chloroplast)	5.14 73	4.78 75

119	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Zea mays*]	RBL_MAIZE	LTYYTPEYETK DTDILAAFR VTPQLGVPPEEAGAAVAAESSTGTWTTVWT-DGLTSLDR TFQGPPHGIQVER GGLDFTKDDENVNSQPFMR DDENVNSQPFMR FVFCAEAIYK GHYLNATAGTCEEMIKR	24	394	Reductive pentose-phosphate cycle; photorespiration (Chloroplast)	6.33 53	6.50 37
120	V-type proton ATPase catalytic subunit A [*Sorghum bicolor*]	XP_002451639.1	LAADTPLLTGQR TTLVANTSNMPVAAR DMGYNVSMMADSTSR EDDLNEIVQLVGK NIIHFNTLANQAVER FEDPAEGEEALVAK	13	457	ATP hydrolysis coupled proton transport (Vacuole)	5.37 69	5.44 73
121	Sedoheptulose-1,7-bisphosphatase [*Triticum aestivum*]	S17P_WHEAT	LLICMGEAMR LLFEALEYSHVCK LTGVTGGDQVAAAMGIYGPR DCPGTHEFLLLDEGK MFSPGNLR YTGGMVPDVNQIIVK GIFTNVTSPTAK FEETLYGSSR	26	355	Reductive pentose-phosphate cycle (Chloroplast)	6.04 42	5.15 39
132	ATP synthase CF1 beta subunit [*Saccharum hybrid cultivar*]	YP_024386.1	RTNPTTSRPGVSTIEEK IDQIIGPVLDITFPPGK QINVTCEVQQLLGNNR AVAMSATDGLMR GMEVIDTGTPLSVPVGGATLGR IFNVLGEPIDNLGPVDTSATFPIHR TVLIMELINNIAK AHGGVSVFGGVGER VALVYGQMNEPPGAR VGLTALTMAEYFR DVNKQDVLLFIDNIFR FVQAGSEVSALLGR MPSAVGYQPTLSTEMGSLQER KGSITSIQAVYVPADDLTDPAPATTFAHLDA-TTVLSR GIYPAVDPLDSTSTMLQPR IVGNEHYETAQR YKELQDIIAILGLDELSEEDR	61	1704	ATP synthesis coupled proton transport (Chloroplast)	5.31 54	5.53 56
135	Probable NADP-dependent malic enzyme [*Gossypium arboreum*]	KHG05054.1	LLNDEFYIGLR	2	70	Pyruvate and malate metabolic process (Cytosol)	5.92 59	5.64 63

139	Glutamine synthetase [*Zea mays*]	GLNAC_MAIZE	LEQLLNMDTTPYTDK TISKPVEDPSELPK AILNLSLR HDLHISAYGEGNER	12	162	Glutamine biosynthetic process (Chloroplast)	6.42 46	5.45 76
140	Phosphoenolpyruvate carboxylase 3 [*Sorghum bicolor*]	XP_002438521.2	HLLATGFSEISEDAVFTK IEEFLEPLELCYK AIADGSLLDLLR HTDVIDAITTHLGIGSYR RPLLPPDLPMTEEIADVIGAMR	8	351	Photosynthesis; carbon fixation (Cytosol)	6.12 116	5.40 47
142	ATP-dependent zinc metalloprotease FTSH 2 [*Sorghum bicolor*]	XP_021304746.1	KVDLFENGTIAIVEAISPELGNR AQGGLGGPNGPGFPLGFGQSR FQMEPNTGVTFDDVAGVDEAK AKENAPCIVFVDEIDAVGR QVSVDVPDVR TPGFSGADLANLLNEAAILAGR IVAGMEGTVMTDGK AAEEVIFGEPEVTTGAAGDLQQITGLAK QLSDEAYEIALR ETLTGDEFR AILSEFVEIPVENR	28	895	Protein catabolic process (Chloroplast)	5.68 72	5.27 72
153	Triosephosphate isomerase [*Sorghum bicolor*]	XP_002462733.1	IEVSAQNVWIGK LIACIGELLEER VATPEQAQEVHAAVR TNVSPEVASSTR IIYGGSVNAANCAELAK KEDIDGFLVGGASLK APDFATIVNSVTAK	31	663	Reductive pentose-phosphate cycle (Chloroplast)	6.45 33	4.95 26
161	Phosphoglycerate kinase [*Sorghum bicolor*]	XP_002441313.1	LVDALPIGGVLLLENVR LASLADLYVNDAFGTAHR ELDYLVGAVSSPK IGVIESLLEK GVTTIIGGGDSVAAVEK	15	330	Reductive pentose-phosphate cycle (Chloroplast)	5.89 50	4.90 40
164	Probable Glutamine synthetase [*Lactuca sativa*]	GLNA_LACSA	DTNWPLGWPLGGFPGPQGPYYCGIGADK	7	40	Glutamine biosynthetic process (Chloroplast)	5.24 40	5.33 46
170	ATP synthase CF1 beta subunit [*Triticum turgidum*]	ASI07736.1	SAPAFIELDTK VGLTALTMAEYFR DVNKQDVLLFIDNIFR	8	197	ATP synthesis coupled proton transport (Chloroplast)	4.99 54	6.94 62

176	Chaperone protein ClpC1, OS=*Oryza sativa subsp. japonica*	CLPC1_ORYSJ	VLESLGADPNNIR VPEPTVDETIQILR AIDLIDEAGSR	4	153	Protein metabolic process (Chloroplast)	6.14 101	5.73 83
182	Photosynthetic NDH subunit of lumenal location 5, chloroplastic [*Sorghum bicolor*]	XP_002439092.1	VYFDISIGNPVGK IVIGLYGDDVPQTAENFR VISDFMIQGGDFDK HVVFGQVLEGMDIVK	35	427	NAD(P)H dehydrogenase complex assembly; protein folding (Chloroplast)	9.26 26	6.94 21
184	Putative C4 phosphoenolpyruvate carboxylase [*Saccharum officinarum*]	CAC08829.1	VSEELIQYDALLVDR HGDFSDEGSATTESDIEETLKR	3	166	Carbon fixation (Chloroplast)	5.94 109	5.18 23
185	28 kDa ribonucleoprotein [*Sorghum bicolor*]	XP_002461966.1	LAQLFEQAGVVEVAEVIYNR GFGFVTMSTVEEADKAVEMLHR GFGFVTMATQDELDDAIAALDGQSLDGR	23	221	mRNA processing (Chloroplast)	4.60 32	4.94 23
188	Triosephosphate isomerase [*Sorghum bicolor*]	XP_002462733.1	IEVSAQNVWIGK LIACIGELLEER APDFATIVNSVTAK	12	220	Reductive pentose-phosphate cycle (Chloroplast)	6.45 33	4.90 27
189	ATP synthase CF1 beta subunit [*Licania membranacea*]	YP_009265111.1	FVQAGSEVSALLGR YKELQDIIAILGLDELSEEDR FLSQPFFVAEVFTGSPGRYVGLAETIR	12	297	ATP synthesis coupled proton transport (Chloroplast)	5.16 54	4.84 28
190	Glyceraldehyde 3-phosphate dehydrogenase, partial [*Salvia officinalis*]	AIZ00507.1	VPTVDVSVVDLTVR	6	99	Reductive pentose-phosphate cycle (Chloroplast)	6.67 21	6.65 30
191	Soluble inorganic pyrophosphatase 6 [*Sorghum bicolor*]	XP_002452886.1	AQGQPETLDYR VIEETNESWEK	7	137	Phosphate-containing compound metabolic process; response to salt stress (Chloroplast)	5.78 32	4.79 31

194	Ferredoxin--NADP reductase, leaf isozyme [*Glycine soja*]	KHN01599.1	LYSIASSAIGDFGDSK	4	92	Photosynthetic electron transport chain (Chloroplast)	8.04 41	6.54 35
206	Peptidyl-prolyl cis-trans isomerase [*Sorghum bicolor*]	XP_002444271.1	LAVGLEELQR IVLDGYNAPVTAGNFIDLVER FYDGMEIQR TVPLEIMVDGDKAPVYGETLEELGR APVYGETLEELGR ESELTPSNANILDGR	19	441	Cell differentiation (Chloroplast)	4.74 46	4.50 42
208	Glycerate dehydrogenase [*Sorghum bicolor*]	KXG29243.1	TILSVDDILALIGDR NGIAVGNTPGVLTETTAELAASLTLSAAR GQTVGVIGAGR MNLIYYDLYQATR EADVISLHPVLDK TTYHLINPER EGMATLTALNVLGK	27	485	Oxidative photosynthetic carbon pathway (Peroxisome)	6.04 42	6.59 42
209	Ribulose bisphosphate carboxylase/oxygenase activase [*Sorghum bicolor*]	XP_002451328.1	GLAYDVSDDQQDITR VPLILGIWGGK MGIIPIMMSAGELESGNAGEPAK MSCLFINDLDAGAGR VPIIVTGNDFSTLYAPLIR TDGVDEEHVVQLVDTFPGQSIDFFGALR YLNEAALGAANEDAMK	28	550	Rubisco activation (Chloroplast)	6.97 49	5.37 43
213	NADP-dependent malic enzyme [*Sorghum bicolor*]	XP_002455030.1	NVQVICVTDGER LLNDEFYIGLR AVFASGSPFAPVEYDGK VHEDMLLAASAALADQATEENFVTGSIFPPF-TNIR	11	285	Pyruvate and malate metabolic process (Chloroplast)	6.23 70	5.74 46
215	Elongation factor Tu [*Sorghum bicolor*]	XP_002452390.1	TKPHVNIGTIGHVDHGK TTLTAALTMVLASVGGSAPK KYDEIDAAPEER GITINTATVEYETETR HYAHVDCPGHADYVK NMITGAAQMDGAILVVSGADGPMPQTK KDMVDDEELLELVELEVR ALEALMANPALK QTDLPFLLAVEDVFSITGR VGDTVDIVGIR TMDDAMAGDNVGLLLR	39	1208	Protein synthesis (Chloroplast)	6.07 51	5.40 47
217	Putative ADP-glucose pyrophosphorylase small subunit [*Sorghum bicolor*]	ABK97538.1	SSQTCLDPDASTSVLGIILGGGAGTR IYVLTQFNSASLNR AMMVDTTILGLDDVR KPVPDFSFYDR SCISEGAIIEDTLLMGADYYETEADKK ILNADNVQEAAR	20	491	Starch biosynthetic process (Chloroplast)	6.65 57	5.42 49

222	ATP synthase subunit alpha [*Sacharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR IAQIPVSEAYLGR LIESPAPGIISR SVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK GYLDSLEIEQVKK EAIQEQLER	21	581	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	6.26 61
223	ATP synthase subunit alpha [*Sacharum* sp]	Q6L3A1.2	VVQVGDGIAR IIGLGEIMSGELVEFAEGTR SVYEPLQTGLIAIDSMIPIGR HTLIIYDDLSK GYLDSLEIEQVKK	14	357	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.62 62
225	2,3-bisphosphoglycerate-independent phosphoglycerate mutase [*Sorghum bicolor*]	XP_021311469.1	ESFESGTLHLIGLLSDGGVHSR GIDAQIASGGGR	6	123	Carbon metabolism (glycolysis and carbon fixation) (Cytosol)	5.41 60	5.42 68
226	V-type proton ATPase catalytic subunit A [*Sorghum bicolor*]	XP_002438797.1	LAADTPLLTGQR TTLVANTSNMPVAAR DMGYNVSMMADSTSR LAEMPADSGYPAYLAAREDDLNEIVQLVGK DALAESDKITLETAK FEDPAEGEEALVGK	16	526	ATP hydrolysis coupled proton transport (Vacuole)	5.31 69	5.41 71
233	Pyruvate phosphate dikinase [*Sorghum bicolor*]	KXG21960.1	FLDMFGNVVMDIPR AVFNSWESPR IAVDMVSEGLVER AETSPEDVGGMHAAAGILTER QPLSPPALSGDLGTFMSWVDDVRK VLANADTPEDALAAR TEHMFFASDER LAEVNPMLGFR	14	648	Photosynthesis; (Chloroplast)	5.09 96	5.27 83

			IGTMIEIPR					
234	Oxygen-evolving enhancer protein 2 [*Dichanthelium oligosanthes*]	OEL30643.1	TADGDEGGKHQLITATVSDGK HQLITATVSDGK	8	136	Photosynthesis; light reaction. (Chloroplast)	8.61 27	5.44 25
237	Chaperone protein ClpC1 [*Zea mays*]	AQK40544.1	VIGQDEAVVAISR NTLLIMTSNVGSSVIEK IGFDLDSDEKDSSYSR AKDINLQVTEK EGDSAIVDVDSEGK	18	364	Chloroplast organization; regulation of chlorophyll biosynthetic process (Chloroplast)	5.15 43	5.13 59
238	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial [*Zea mays*]	ANP22077.1	VTPKLGVPPEEAGAAVAAESSTGTWTTVW-TDGLTSLDR TFQGPPHGIQVER	33	172	Carbon fixation (Chloroplast)	5.17 17	5.21 18
239	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial [*Zea mays*]	ANP22077.1	VTPKLGVPPEEAGAAVAAESSTGTWTTVW-TDGLTSLDR TFQGPPHGIQVER	33	163	Carbon fixation (Chloroplast)	5.17 17	5.26 21
240	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial [*Zea mays*]	ANP22077.1	VTPKLGVPPEEAGAAVAAESSTGTWTTVWT-DGLTSLDR TFQGPPHGIQVER	24	59	Carbon fixation (Chloroplast)	5.17 17	5.19 29

242	Heat shock 70 kDa protein 6 [*Zea mays*]	ONM07984.1	QFAAEEISAQVLR IINEPTAASLAYGFEK AKFEELCSDLLDR DIDEVILVGGSTR NQAESVIYQTEK	9	383	Protein folding (Chloroplast)	5.14 73	4.63 84
243	Heat shock 70 kDa protein 6 [*Zea mays*]	ONM32478.1	QFAAEEISAQVLR IAGLEVLR IINEPTAASLAYGFEK FEELCSDLIDR LSVSDLDEVILVGGSTR IPAVQELVR NQADSVVYQTEK	13	526	Protein folding (Chloroplast)	4.81 66	4.60 86

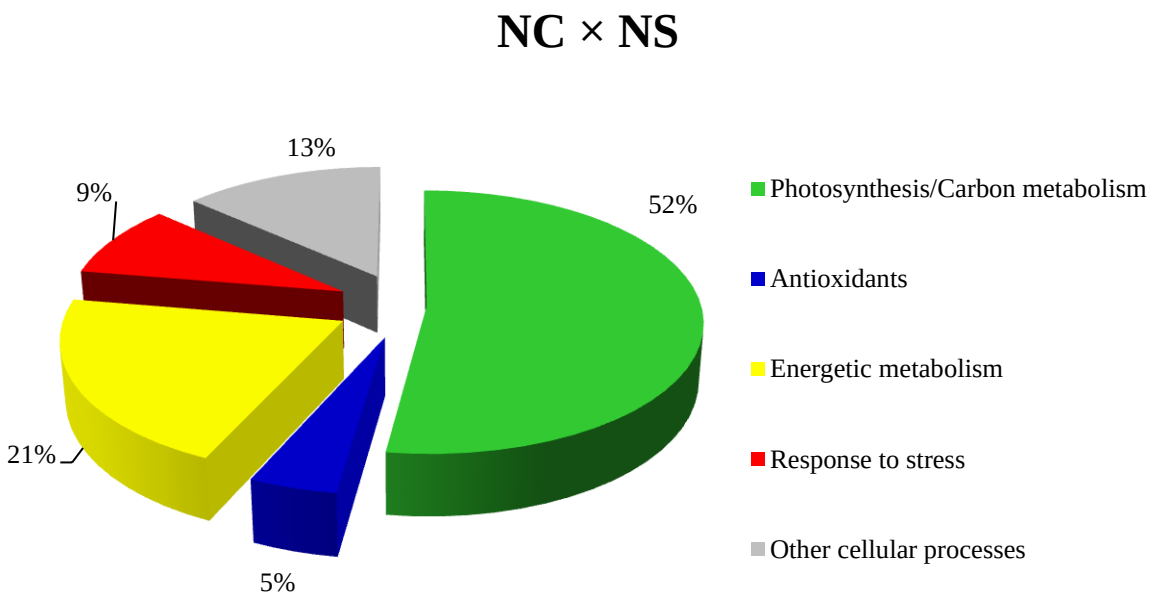

Figure 4. Functional characterization of all differential spots identified in leaves of nitrate-grown sorghum plants, after 10 days in control conditions (NC) and salt stress with 75 mM-NaCl (NS). Five categories were created according to the results.

Herein, table 2 lists all differentially regulated proteins identified in leaves of nitrate-grown plants and table 3 shows the level of variation as affected by salt treatment. Interestingly, in photosynthetic/carbon metabolism of nitrate-grown plants, two rubisco large subunits were upregulated by salinity, two showed downregulation and three of them were suppressed by stress. Also, three proteins of phosphoenolpyruvate carboxylase (PEPCase) were *de novo* synthesized in response to salt stress. In energetic metabolism, whereas the ATP synthase CF1 beta subunit was reduced by salinity, the alpha subunit displayed an increase; and V-type proton ATPase catalytic subunits were upregulated (Table 3).

In response to stress, glycerate dehydrogenase and chloroplastic chaperone protein ClpC1 had *de novo* synthesis, while plastid-lipid-associated protein 6 and S- adenosylmethionine synthase were inhibited under salinity (Table 3). Additionally, the antioxidant enzymes cytosolic superoxide dismutase (cSOD) and glyoxylase 1 were downregulated by salt stress, while cytosolic ascorbate peroxidase 2 (cAPX2) was upregulated. In terms of other processes, two chloroplastic heat shock 70 kDa protein 6 were suppressed under salinity, whereas chloroplastic elongation factor Tu was *de novo* synthesized in response to the stress.

Table 3. Level of regulation of proteomic profile from leaves of sorghum plants subjected to salt stress. Comparisons were done as following: nitrate control × nitrate salt (NC × NS), ammonium control × ammonium salt (AC × AS) and nitrate salt × ammonium salt (NS × AS). Numbers highlighted in blue bars indicate upregulation in response to salinity (NC × NS or AC × AS) or higher protein abundance in ammonium treatments (NS × AS); whereas numbers highlighted in red bars denote downregulation by salt stress (NC × NS or AC × AS) or bigger expression in nitrate treatments (NS × AS). Green cells indicate proteins exclusive of a specific treatment in that comparison, it being repressed by salt stress (AC or NC) or *de novo* synthesized (NS or AS) in comparisons NC × NS or AC × AS; as well as a specific protein from nitrogen treatments (NS or AS) under salt stress, in the comparison NS × AS. The level of modulation was obtained in ImageMaster 2D Platinum software, considering significant differences in relative abundance intensity ≥ 1.5-fold and p value < 0.05.

ID	Protein	NC x **NS**	AC x **AS**	NS x **AS**
PHOTOSYNTHESIS/CARBON METABOLISM				
ACG28186.1	Cytochrome b6-f complex iron-sulfur subunit	1,7		1,56
EMS45291.1	Light-harvesting complex I chlorophyll a-b binding protein 1B-21	1,75		
AND01108.1	Light-harvesting complex I chlorophyll a/b binding protein 3		AS	
CB22_MAIZE	Light-harvesting complex I chlorophyll a/b binding protein		AS	2,82
CB3_ARATH	Light-harvesting complex I chlorophyll a/b binding protein 3		1,97	
XP_021304159.1	Light-harvesting complex I chlorophyll a/b binding protein 8		AS	
XP_021318830.1	Photosystem II stability/assembly factor	1,74		
AGT15890.1	Luminal-binding protein	1,55		
KHN01599.1	Ferredoxin-NADP reductase, leaf isozyme, chloroplastic	2,89		
AFO59573.1	Ferredoxin-NADP reductase			1,5
XP_002461438.1	Oxygen-evolving enhancer protein 2	1,56		
XP_021305066.1	Oxygen-evolving enhancer protein 1	1,61		
XP_021305066.1	Oxygen-evolving enhancer protein 1, chloroplastic		3,43	
XP_021305066.1	Oxygen-evolving enhancer protein 1, chloroplastic		1,87	
OEL30643.1	Oxygen-evolving enhancer protein 2, chloroplastic	NS		
XP_002439092.1	Photosynthetic NDH subunit of lumenal location 5	NS		
ANP22077.1	Rubisco (large subunit)	3,49		
XP_002440887.1	Rubisco (alpha subunit)	1,7	1,68	
ALF41107.1	Rubisco (large subunit)	2,9		
RBL_MAIZE	Rubisco (large subunit)	1,56		
ANP22077.1	Rubisco (large subunit)	NC		
ANP22077.1	Rubisco (large subunit)	NC		
ANP22077.1	Rubisco (large subunit)	NC		
CAC08829.1	Phosphoenolpyruvate carboxylase (PEPCase)	1,59	1,56	
XP_002438521.2	Phosphoenolpyruvate carboxylase 3 (PEPCase 3)	1,61		1,66
CAC08829.1	Phosphoenolpyruvate carboxylase (PEPCase)	2,43	1,62	
CAC08829.1	Phosphoenolpyruvate carboxylase (PEPCase)		AS	
XP_002451328.1	Rubisco activase	1,65		
AAG22094.3	Rubisco activase	2,56		
AAG22094.3	Rubisco activase	1,53		
XP_002451328.1	Rubisco activase	NS		
AAG22094.3	Rubisco activase		1,7	
KHG05054.1	NADP-dependent malic enzyme	2,56	1,76	
XP_002455030.1	NADP-dependent malic enzyme, chloroplastic	1,78		

XP_021312698.1	Carbonic anhydrase, chloroplastic isoform X1			1,76
XP_002442893.1	Fructose-bisphosphate aldolase	2,03		
XP_002450383.1	Fructose-bisphosphate aldolase	1,59		
XP_002441313.1	Phosphoglycerate kinase, chloroplastic	1,91	1,5	
XP_002441313.1	Phosphoglycerate kinase, chloroplastic	1,72	1,51	
XP_002441313.1	Phosphoglycerate kinase, chloroplastic		1,81	
S17P_WHEAT	Sedoheptulose-1,7-bisphosphatase	1,66	3,46	
XP_002462733.1	Triosephosphate isomerase, chloroplastic	1,84	2,04	
XP_002462733.1	Triosephosphate isomerase, chloroplastic	2,52	5,58	
ABK97538.1	ADP-glucose pyrophosphorylase small subunit	NS		
XP_021311469.1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	NS		
XP_021304041.1	Pyruvate phosphate dikinase 1		1,5	
KXG21960.1	Pyruvate phosphate dikinase	NS	2,06	
XP_021313843.1	Phosphoribulokinase, chloroplastic		1,78	
XP_002463921.1	Glucose-1-phosphate adenylyltransferase large subunit		AS	
ANTIOXIDANTS				
C0HK70.1	Superoxide dismutase cytosolic	1,52		
XP_002463451.1	APX2 - Ascorbate peroxidase 2	2,25		
AHF18211.1	Glyoxylase 1	1,65		
XP_002445671.1	Superoxide dismutase [Cu-Zn], chloroplastic		AC	
ENERGETIC METABOLISM				
AHZ11983.1	ATP synthase beta subunit	3,16		
YP_899414.1	ATP synthase CF1 beta subunit, chloroplast	1,5		
YP_024386.1	ATP synthase CF1 beta subunit, chloroplast	2,09		
ASI07736.1	ATP synthase CF1 beta subunit, chloroplast	1,63		
YP_009265111.1	ATP synthase CF1 beta subunit, chloroplast	2,08		
YP_899414.1	ATP synthase CF1 beta subunit, chloroplast			3,68
Q6L3A1.2	ATP synthase subunit alpha, chloroplastic	2,16	4,22	
Q6L3A1.2	ATP synthase subunit alpha, chloroplastic	2,19		
ATPA_MAIZE	ATP synthase subunit alpha, chloroplastic		2,16	3,38
Q6L3A1.2	ATP synthase subunit alpha, chloroplastic		2,25	1,98
ATPA_MAIZE	ATP synthase subunit alpha, chloroplastic		1,54	
NP_043022.1	ATP synthase CF1 alpha subunit chloroplast		AS	3,52
YP_054628.1	ATP synthase CF1 alpha subunit chloroplast			1,92
Q6L3A1.2	F-ATP synthase subunit alpha, chloroplastic		AS	
Q6L3A1.2	F-ATP synthase subunit alpha, chloroplastic		5,77	AS
Q6L3A1.2	F-ATP synthase subunit alpha, chloroplastic		2,75	AS
Q6L3A1.2	F-ATP synthase subunit alpha, chloroplastic		AS	1,61
Q6L3A1.2	F-ATP synthase subunit alpha, chloroplastic		AS	1,6
Q6L3A1.2	F-ATP synthase subunit alpha, chloroplastic		AS	
CAB99314.1	F0-F1 ATPase alpha subunit, mitochondrial		1,86	1,51
ATPM_MAIZE	ATP synthase subunit alpha, mitochondrial		2,46	
XP_021308049.1	ATP synthase subunit gamma, chloroplastic		AS	
XP_002451639.1	V-type proton ATPase catalytic subunit A	1,5		
XP_002438797.1	V-type proton ATPase catalytic subunit A	1,55		
XP_002452886.1	Soluble inorganic pyrophosphatase 6	1,84		
XP_002452886.1	Soluble inorganic pyrophosphatase 6, chloroplastic	1,65		1,52
XP_021305022.1	Glyceraldehyde-3-phosphate dehydrogenase 1	1,52		
AIZ00507.1	Glyceraldehyde 3-phosphate dehydrogenase	NS		
XP_021318665.1	Glyceraldehyde-3-phosphate dehydrogenase A			1,64
PGMC1_MAIZE	Phosphoglucomutase 1	1,69		
XP_002437265.1	Fructose-bisphosphate aldolase 5, cytosolic		4,27	

RESPONSE TO STRESS				
XP_002449783.1	Plastid-lipid-associated protein 6	1,97	1,59	
METK_DENCR	S-adenosylmethionine synthase	2,1	1,79	
XP_021304746.1	ATP-dependent zinc metalloprotease FTSH 2	1,66		
CLPC1_ORYSJ	Chaperone protein ClpC1, chloroplastic	NS	AS	
AQK40544.1	Chaperone protein ClpC1 chloroplastic	NC	AC	
KXG29243.1	Glycerate dehydrogenase	NS	2,43	
XP_021301730.1	Cysteine synthase		AS	
XP_021302102.1	Polyamine oxidase-like			2,65
XP_021304577.1	Aldehyde dehydrogenase mitochondrial			1,54
XP_002443073.2	beta-D-glucosidase 2, chloroplastic			2,09
XP_002465329.1	Ricin B-like lectin R40C1			1,62
XP_021312414.1	Cytosolic isocitrate dehydrogenase [NADP]-like		AS	AS
OTHER				
XP_002442729.1	FHA domain containing protein	1,62		
ONM07984.1	Heat shock 70 kDa protein 6	1,56		
ONM07984.1	Heat shock 70 kDa protein 6 chloroplastic	NC	AC	
ONM32478.1	Heat shock 70 kDa protein 6 chloroplastic	NC	AC	
GLNAC_MAIZE	Glutamine synthetase	2,6		
GLNA_LACSA	Glutamine synthetase	1,59		
XP_002461966.1	28 kDa ribonucleoprotein,	1,53		
XP_002444271.1	Peptidyl-prolyl cis-trans isomerase, chloroplastic	1,5	1,62	
XP_002452390.1	Elongation factor Tu, chloroplastic	NS		1,85
XP_002448238.1	Elongation factor G-2, chloroplastic		2,82	1,5
XP_021301657.1	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase 1		1,62	
XP_021301657.1	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase 1		1,52	
XP_002467242.1	GDP-mannose 3,5-epimerase 1		1,82	
XP_002459396.1	Uncharacterized protein		1,62	

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 (Fig. 5). Under salinity, 25 proteins/peptides were upregulated, 10 showed downregulation; four were repressed and 14 were *de novo* synthesized (Fig. 6). In this treatment, after identification (Table 4), the proteins differentially regulated were related to photosynthesis/carbon metabolism representing 42% of all spots; energetic metabolism, 28%; response to stresses, 13%; antioxidative action 2%; and other cellular processes 15% (Fig. 7).

In general, several important proteins of photosynthetic/carbon metabolism were differentially regulated by salt stress, including structural proteins, enzymes of CO_2 assimilation and other metabolic pathways (Tables 3 and 4). Also, a large number of proteins involved in energetic metabolism and response to stress (for instance, chloroplastic ATP synthase alpha subunits, glycerate dehydrogenase, cysteine synthase, cytosolic $NADP^+$-dependent isocitrate dehydrogenase, among others) were upregulated or *de novo* synthesized in ammonium-grown plants exposed to NaCl stress.

Surprisingly, in antioxidant activity, the chloroplastic superoxide dismutase (chlSOD) was repressed by salinity in ammonium-supplied plants (Fig. 5 and Table 3).

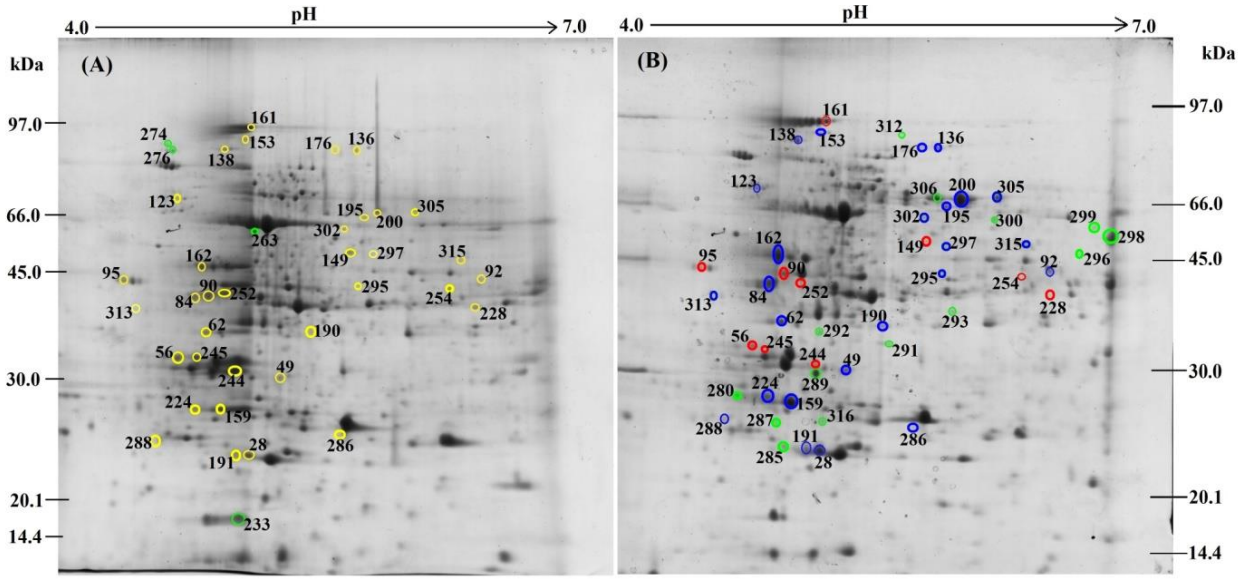

Figure 5. Coomasie Brilliant Blue G 250-stained two-dimensional gels of leaves of ammonium-fed sorghum plants in control conditions (A) and in salt stress with 75 mM NaCl (B). Spots marked with blue and red circles represent proteins that were significantly upregulated (blue) or downregulated (red); Spots with green circles in control plants indicates proteins whose expression was suppressed by salinity, whereas green circles in the salt gel point out that those spots suffered *de novo* synthesis under stress. Spots identified are statistically significant to a level of 95% per group (Student's t-test) using biological and analytical replicates (n = 3).

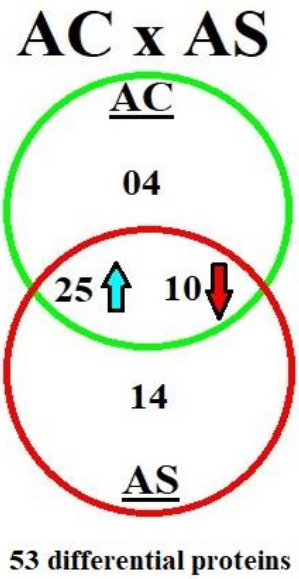

Figure 6. Venn diagram representation of different subsets of ammonium-fed *Sorghum bicolor* L. Moench cv. CSF 20 leaves, displaying their changes in control conditions (AC) and under salt-stress with 75 mM NaCl (AS).

On the other hand, in other processes, numerous proteins presented differential regulation under salt stress, including transcription factors, heat shock proteins and others (Fig. 5 and Table 3). For all cases, table 4 lists all proteins identified in leaves of ammonium-fed plants and table 3 shows the level of modulation

as affected by salt treatment.

Table 4 – List of differentially regulated proteins in leaves of ammonium-grown sorghum plants under salt stress (75 mM NaCl). Differential spots were excised from 2DE gels, comparison AC × AS (see Fig. 6), and the proteins were identified by LC–ESI–MS/MS.

Spot	Protein (Species)	Access number (NCBI/SwissProt)	Peptide sequences (MS/MS)	Sequence coverage (%)	Score	Biological process (localization)	pI/MM (kDa) Theoretical	pI/MM (kDa) Experimental
28	PEPcase - C4 phosphoenolpyruvate carboxylase [*Saccharum officinarum*]	CAC08829.1	VSEELIQYDALLVDR FLDILQDLHGPSLR HGDFSDEGSATTESDIEETLKR	5	400	Carbon fixation (Cytosol)	5.94 109	5.20 23
56	Plastid-lipid-associated protein 6 [*Sorghum bicolor*]	XP_002449783.1	LLSAVSGLNR GLAASQEDLDRADAAAR LLPVTLGQVFQR GSLAQLPPLEVPR VPDNLRPPSSSSNAGSGEGEFEVTYLDDDTR	32	525	Chloroplast organization (Chloroplast)	8.84 31	4.78 32
162	Phosphoglycerate kinase [*Sorghum bicolor*]	XP_002441313.1	SVGDLTEADLEGK ADLNVPLDENQNITDDTR LSELLGIQVQK LVDALPIGGVLLLENVR LASLADLYVNDAFGTAHR ELDYLVGAVSSPK RPFAAIVGGSK IGVIESLLEK GVSLLLPTDVVIADK VGVADVMSHISTGGGASLELLEGK	34	1037	Reductive pentose phosphate pathway (Chloroplast)	5.89 50	4.93 47
149	S-adenosylmethionine synthase [*Dendrobium crumenatum*]	METK_DENCR	TQVTVEYR FVIGGPHGDAGLTGR	5	90	Carbon fixation (Cytosol)	5.42 44	5.80 51
123	Rubisco large subunit-binding protein subunit alpha [*Sorghum bicolor*]	XP_002440887.1	LANAVGVTLGPR NVVLDEYGSPK AIELYDPMENAGAALIR TNDSAGDGTTTASVLAR LGLLSVTSGANPVSLK TVQGLIQELENK AVLQDIAIVTGAEFLAK DLGLLVENATEAQLGTAR VTIHQTTTTLIADAASKDEIQAR ELAETDSVYDTEK VGAATETELEDR	34	953	Chloroplast organization (Chloroplast)	5.07 61	4.77 69

			IKDSVWEVGYNAMTDTYENLIEAGVIDPAK					
302	Probable NADP-dependent malic enzyme [*Gossypium arboreum*]	KHG05054.1	LLNDEFYIGLR	2	70	Reductive pentose phosphate pathway (Chloroplast)	5.92 59.	5.71 57
159	Triosephosphate isomerase [*Sorghum bicolor*]	XP_002462733.1	IEVSAQNVWIGK LIACIGELLEER VATPEQAQEVHAAVR TNVSPEVASSTR IIYGGSVNAANCAELAK KEDIDGFLVGGASLK APDFATIVNSVTAK	31	663	Reductive pentose-phosphate cycle (Chloroplast)	6.45 33	5.05 26
84	Phosphoglycerate kinase [*Sorghum bicolor*]	XP_002441313.1	LVDALPIGGVLLLENVR LASLADLYVNDAFGTAHR ELDYLVGAVSSPK IGVIESLLEK GVTTIIGGGDSVAAVEK	15	330	Reductive pentose phosphate pathway (Chloroplast)	5.89 50	4.88 41
312	Chaperone protein ClpC1 [*Oryza sativa subsp. Japonica*]	CLPC1_ORYSJ	VLESLGADPNNIR VPEPTVDETIQILR AIDLIDEAGSR	4	153	Protein metabolic process (Chloroplast)	6.14 101	5.60 85
191	Putative C4 phosphoenolpyruvate carboxylase [*Saccharum officinarum*]	CAC08829.1	VSEELIQYDALLVDR HGDFSDEGSATTESDIEETLKR	3	166	Carbon fixation (Cytosol)	5.94 109	5.12 23
224	Triosephosphate isomerase [*Sorghum bicolor*]	XP_002462733.1	IEVSAQNVWIGK LIACIGELLEER APDFATIVNSVTAK	12	220	Reductive pentose-phosphate cycle (Chloroplast)	6.45 33	4.89 26

95	Peptidyl-prolyl cis-trans isomerase [*Sorghum bicolor*]	XP_002444271.1	LAVGLEELQR IVLDGYNAPVTAGNFIDLVER FYDGMEIQR TVPLEIMVDGDKAPVYGETLEELGR APVYGETLEELGR ESELTPSNANILDGR	19	441	Cell differentiation; Chaperone-mediated protein folding (Chloroplast)	4.74 46	4.46 44
92	Glycerate dehydrogenase [*Sorghum bicolor*]	KXG29243.1	TILSVDDILALIGDR NGIAVGNTPGVLTETTAELAASLTLSAAR GQTVGVIGAGR MNLIYYDLYQATR EADVISLHPVLDK TTYHLINPER EGMATLTALNVLGK	27	485	Oxidative photosynthetic carbon pathway (Peroxisome)	6.04 42	6.61 42
305	ATP synthase subunit alpha [*Sacharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR IAQIPVSEAYLGR LIESPAPGIISR SVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK GYLDSLEIEQVKK EAIQEQLER	21	581	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	6.10 65
153	Pyruvate phosphate dikinase [*Sorghum bicolor*]	KXG21960.1	FLDMFGNVVMDIPR AVFNSWESPR IAVDMVSEGLVER AETSPEDVGGMHAAAGILTER QPLSPPALSGDLGTFMSWVDDVRK VLANADTPEDALAAR TEHMFFASDER LAEVNPMLGFR IGTMIEIPR	14	648	Photosynthesis (Chloroplast)	5.09 96	5.19 89
49	ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR IAQIPVSEAYLGR VINALAKPIDGR LIESPAPGIISR SVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK	19	531	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.42 28
62	Uncharacterized protein [*Sorghum bicolor*]	XP_002459396.1	VLATSAVFGEEIWPAK VDGDGDLTEKEIIR IEAGLSALEEALK KSEEFEPLLK NYDESFINENAINAIK	19	406		6.01 38	4.94 34

90	Phosphoribulokinase [*Sorghum bicolor*]	XP_021313843.1	GGNPDSNTLISDTTTVICLDDYHSLDR DGQAVEKPIYNHVTGLLDPPELITPPK KPDFDAYIDPQK FFNPVYLFDEGSSISWVPCGR FAYGPDTYFGK HADFPGSNNGTGLFQTIIGLK	28	423	Reductive pentose-phosphate cycle (Chloroplast)	6.09 46	4.96 41
136	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1 [*Sorghum bicolor*]	XP_021301657.1	FALESFWDGK YIPSNTFSYYDQVLDTTAMLGAVPER ALGVDTVPVLIGPVSYLLLSKPAK TLTSLSGVTAYGFDLVR GTQTLGLVTSAGFPAGK YLFAGVVDGR LVVSTSCSLMHTAVDLVNETK GMLTGPVTILNWSFVR VIQIDEAALR IPSTEEIADR	21	770	Homocysteine metabolic process; Response to salt stress (Cytosol)	5.61 84	5.84 83
138	Elongation factor G-2 [*Sorghum bicolor*]	XP_002448238.1	EDIPVAVTGDIVALAGLK VEVITPEEHLGDVIGDLNSR	4	141	Protein synthesis (Chloroplast)	5.42 85	5.05 86
161	Pyruvate phosphate dikinase 1 [*Sorghum bicolor*]	XP_021304041.1	FLDMFGNVVMDIPR AVFNSWESPR SINQITGLVGTAVNVQSMVFGNMGNTSGT-GVLFTR IAVDMVSEGLVER VIATGLPASPGAAVGQIVFTAEDAEAWHA-QGK AETSPEDVGGMHAAAGILTER TVAIGDHVLSEGEWLSLNGSTGEVILGK VLANADTPEDALAAR ELCAETGANEEEALER LAEVNPMLGFR LGISYPELTEMQAR IGTMIEIPR	24	1116	Photosynthesis (Chloroplast)	5.54 103	5.21 94

			VGLDYVSCSPFR					
176	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase 1 [*Sorghum bicolor*]	XP_021301657.1	YIPSNTFSYYDQVLDTTAMLGAVPER GTQTLGLVTSAGFPAGK YLFAGVVDGR LVVSTSCSLMHTAVDLVNETK ELNLPVLPTTTIGSFPQTVELR GMLTGPVTILNWSFVR VIQIDEAALR YGAGIGPGVYDIHSPR	18	635	Homocysteine metabolic process; Response to salt stress (Cytosol)	5.61 84	5.76 83
190	ATP synthase subunit alpha [*Zea mays*]	ATPA_MAIZE	VGIENIGR VVQVGDGIAR LIESPAPGIISR	5	125	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.61 34
195	ATP synthase subunit alpha [*Zea mays*]	ATPAM_MAIZE	VVSVGDGIAR VVDALGVPIDGK TAIAIDTILNQK NILSTINPELLK	9	160	ATP synthesis coupled proton transport (Mitochondrion)	5.85 55	5.92 62
200	F0-F1 ATPase alpha subunit, partial [*Sorghum bicolor*]	CAB99314.1	VVSVGDGIAR VVDALGVPIDGK TAIAIDTILNQK	7	190	ATP synthesis coupled proton transport (Mitochondrion)	5.96 48	5.96 66
228	Ribulose 1,5-bisphosphate carboxylase/oxygenase activase precursor, partial [*Zea mays*]	AAG22094.3	TDGVDEEHVVQLVDTFPGQSIDFFGALR	9	76	Rubisco activation (Chloroplast)	6.43 34	6.59 38
280	Chlorophyll a-b binding protein [*Zea mays*]	CB22_MAIZE	AKPAAASGSPWYGPDR VLYLGPLSGEPPSYLTGEFPGDYGWDTAG- LSADPETFAK ELEVIHCR GPLENLADHIADPVNNNAWAYATNFVPGK	34	138	Photosynthesis; light harvesting (Chloroplast)	5.29 28	4.71 26

285	Putative C4 phosphoenolpyruvate carboxylase [*Saccharum officinarum*]	CAC08829.1	VSEELIQYDALLVDR HGDFSDEGSATTESDIEETLKR	3	122	Carbon fixation (Cytosol)	5.94 109	4.94 22
286	ATP synthase subunit alpha [*Zea mays*]	ATPA_MAIZE	QSQSNPLPVEEQVATIYTGTR EAIQEQLER	5	79	ATP synthesis coupled proton transport (Chloroplast)	5.87 55	5.73 24
287	Light-harvesting complex I chlorophyll a/b binding protein 3, partial [*Saccharum officinarum*]	AND01108.1	FAMLGAAGAIAPEVFGK LGIIPPETALPWFK	11	115	Photosynthesis; light harvesting (Chloroplast)	8.96 29	4.94 24
288	Chlorophyll a-b binding protein 3 [*Arabidopsis thaliana*]	CB3_ARATH	INGLDGVGEGNDLYPGGQYFDPLGLADD-PVTFAELK	13	82	Photosynthesis; light harvesting (Chloroplast)	4.96 29	4.64 24
289	ATP synthase CF1 alpha subunit [*Zea mays*]	NP_043022.1	VVQVGDGIAR IIGLGEIMSGELVEFAEGTR NVGIVLMGDGLMIQEGSFVK IAQIPVSEAYLGR VINALAKPIDGR LIESPAPGIISR RSVYEPLQTGLIAIDSMIPIGR SVYEPLQTGLIAIDSMIPIGR HTLIIYDDLSK	23	737	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.15 28
291	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	VVQVGDGIAR IIGLGEIMSGELVEFAEGTR LIESPAPGIISR SVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK	16	459	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.55 32

292	ATP synthase subunit gamma [*Sorghum bicolor*]	XP_021308049.1	VALVVLTGER QLGLQYSVISVGK SDPIIQTLLPMSPK ALQESLASELAAR	13	343	ATP biosynthetic process (Chloroplast)	7.52 40	5.17 33
293	Cysteine synthase [*Sorghum bicolor*]	XP_021301730.1	IGYSMITDAEEK IDALVSGIGTGGTITGTGR EGLLVGISSGAAAAAAVR LFVVVFPSFGER YLSSVLFQSIK	22	361	Cysteine biosynthetic process; Response to cadmium (Cytosol)	5.67 34	5.88 36
295	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	VVQVGDGIAR IIGLGEIMSGELVEFAEGTR TAVATDTILNQK EAYPGDVFYLHSR	10	296	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.81 42
296	Cytosolic isocitrate dehydrogenase [NADP]-like [*Sorghum bicolor*]	XP_021312414.1	AFAEASMTTAYEK SHYLNTEEFIDAVATELR	6	172	Response to salt stress (Cytosol)	8.26 52	6.59 46
297	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR LIESPAPGIISR TAVATDTILNQK	8	220	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.83 48
298	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR NVGIVLMGDGLMIQEGSFVK VINALAKPIDGRGEIVASESR LIESPAPGIISR RSVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK EAYPGDVFYLHSR	28	996	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	6.78 51

299	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR IAQIPVSEAYLGR LIESPAPGIISR	8	267	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	6.65 55
300	Glucose-1-phosphate adenylyltransferase large subunit [*Sorghum bicolor*]	XP_002463921.1	TVVAVILGGGAGTR VYILTQFNSQSLNR WFQGTADAVR AYLFNDYWEDIGTIK NVIISNSEGVVEADR	13	383	Starch biosynthetic pathway (Chloroplast)	8.33 56	6.08 56
306	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	VVQVGDGIAR LIESPAPGIISR TAVATDTILNQK HTLIIYDDLSK GYLDSLEIEQVKK IIGLGEIMSGELVEFAEGTR SVYEPLQTGLIAIDSMIPIGR	19	597	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.78 65
313	Phosphoglycerate kinase [*Sorghum bicolor*]	XP_002441313.1	LVDALPIGGVLLLENVR LASLADLYVNDAFGTAHR ELDYLVGAVSSPK	9	227	Reductive pentose phosphate pathway (Chloroplast)	5.89 50	4.51 38
315	GDP-mannose 3,5-epimerase 1 [*Sorghum bicolor*]	XP_002467242.1	ISITGAGGFIGSHIAR FEMWGDGLQTR SFTFIDECVEGVLR TQGVDIAAYGSSK VVSTQAPVQLGSLR	17	369	L-ascorbic acid biosynthetic process; response to oxidative stress (cytosol)	5.94 43	6.40 46
316	Chlorophyll a-b binding protein 8 [*Sorghum bicolor*]	XP_021304159.1	FAMLGAAGAIAPEVFGK LGIIPPETALPWFK	9	196	Photosynthesis, light harvesting (Chloroplast)	9.34 36	5.17 24

233	Superoxide dismutase [Cu-Zn] [*Sorghum bicolor*]	XP_002445671.1	GTSEVEGVVTLTQDDDGPTTVNVR ITGLTPGLHGFHLHEFGDTTNGCISTGPHF-NPNNLTHGAPEDEVR HAGDLGNIVANAEGVAEATIVDTQIPLSGP-NSVVGR AFVVHELEDDLGKGGHELSLSTGNAGGR GGHELSLSTGNAGGR	64	537	Cellular response to oxidative stress; cellular response to salt stress (Chloroplast)	5.30 21	4.78 88
244	Oxygen-evolving enhancer protein 1 [*Sorghum bicolor*]	XP_021305066.1	GTGTANQCPTIDGGVEAFPFK KLCLEPTSFTVK LCLEPTSFTVK LTYTLDEIEGPLEVGSDGTLK LTYTLDEIEGPLEVGSDGTLKFEEK FEEKDGIDYAAVTVQLPGGER DGIDYAAVTVQLPGGER QLVATGKPESFGGPFLVPSYR GGSTGYDNAVALPAGGR NAAASTGNITLSVTK SNPETGEVIGVFESVQPSDTDLGAK	46	893	Photosynthesis; light reaction (Chloroplast)	5.91 35	5.20 28
245	Oxygen-evolving enhancer protein 1 [*Sorghum bicolor*]	XP_021305066.1	LCLEPTSFTVK DGIDYAAVTVQLPGGER QLVATGKPESFGGPFLVPSYR GGSTGYDNAVALPAGGR NAAASTGNITLSVTK	24	450	Photosynthesis; light reaction (Chloroplast)	5.91 35	4.99 31
252	V-type proton ATPase catalytic subunit A [*Sorghum bicolor*]	XP_002451639.1	LAADTPLLTGQR TTLVANTSNMPVAAR DMGYNVSMMADSTSR EDDLNEIVQLVGK NIIHFNTLANQAVER FEDPAEGEEALVAK	13	457	ATP hydrolysis coupled proton transport (Vacuole)	5.37 68	5.05 41
254	Fructose-bisphosphate aldolase 5 [*Sorghum bicolor*]	XP_002437265.1	LSSINLENVESNR YALICQEYGLVPIVEPEILTDGGHDIK	11	153	Glycolytic process; Gluconeogenesis (Cytosol)	7.56 38	6.45 42
263	Chaperone protein ClpC1 [*Zea mays*]	AQK40544.1	VIGQDEAVVAISR NTLLIMTSNVGSSVIEK IGFDLDSDEKDSSYSR AKDINLQVTEK EGDSAIVDVDSEGK	18	364	Protein metabolic process (Chloroplast)	5.15 43	5.26 56

274	Heat shock 70 kDa protein 6 [*Zea mays*]	ONM07984.1	QFAAEEISAQVLR IINEPTAASLAYGFEK AKFEELCSDLLDR DIDEVILVGGSTR NQAESVIYQTEK	9	383	Protein folding; response to abiotic stresses (Chloroplast)	5.14 73	4.75 85
276	Heat shock 70 kDa protein 6 [*Zea mays*]	ONM32478.1	QFAAEEISAQVLR IAGLEVLR IINEPTAASLAYGFEK FEELCSDLIDR LSVSDLDEVILVGGSTR IPAVQELVR NQADSVVYQTEK	13	526	Protein folding; response to abiotic stresses (Chloroplast)	4.81 66	4.72 86

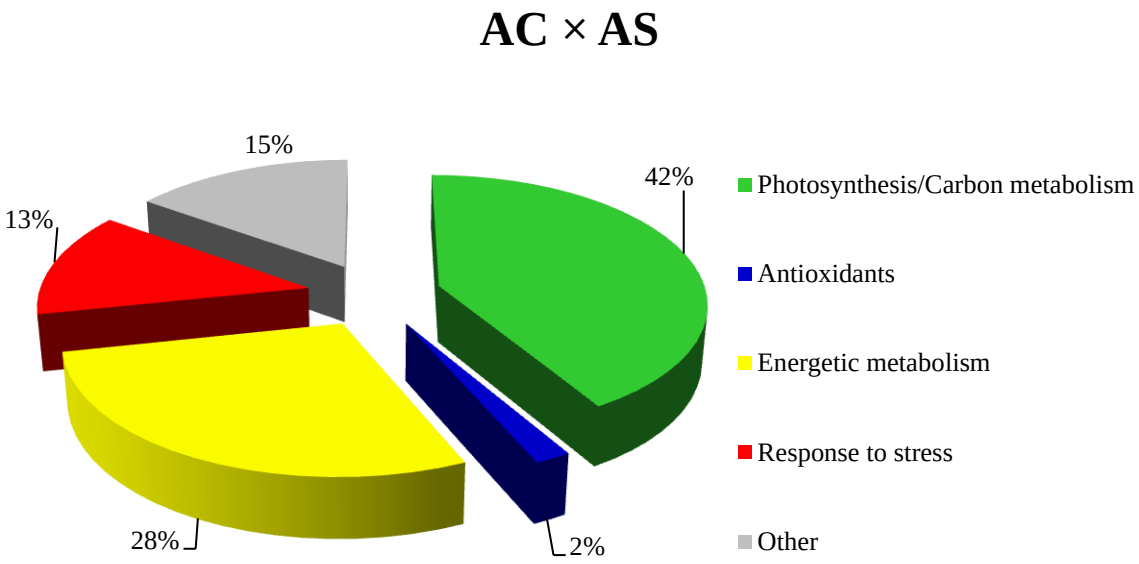

Figure 7. Functional characterization of all differential spots identified in leaves of ammonium-grown plants, after 10 days in control conditions (AC) and salt stress with 75 mM NaCl (AS). Five categories were created according to the results.

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 (Fig. 8). Considering nitrate salt gel as reference, ammonium salt gel showed 14 proteins upregulated, 11 downregulated, two proteins were exclusive to nitrate salt treatment, while six were found to be exclusively expressed in ammonium-grown plants (Fig. 9).

After identification (Table 5), it was observed that the proteins differentially expressed as 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. 10). The level of nitrogen-mediated regulation of proteins, including the exclusive proteins, is showed in table 3.

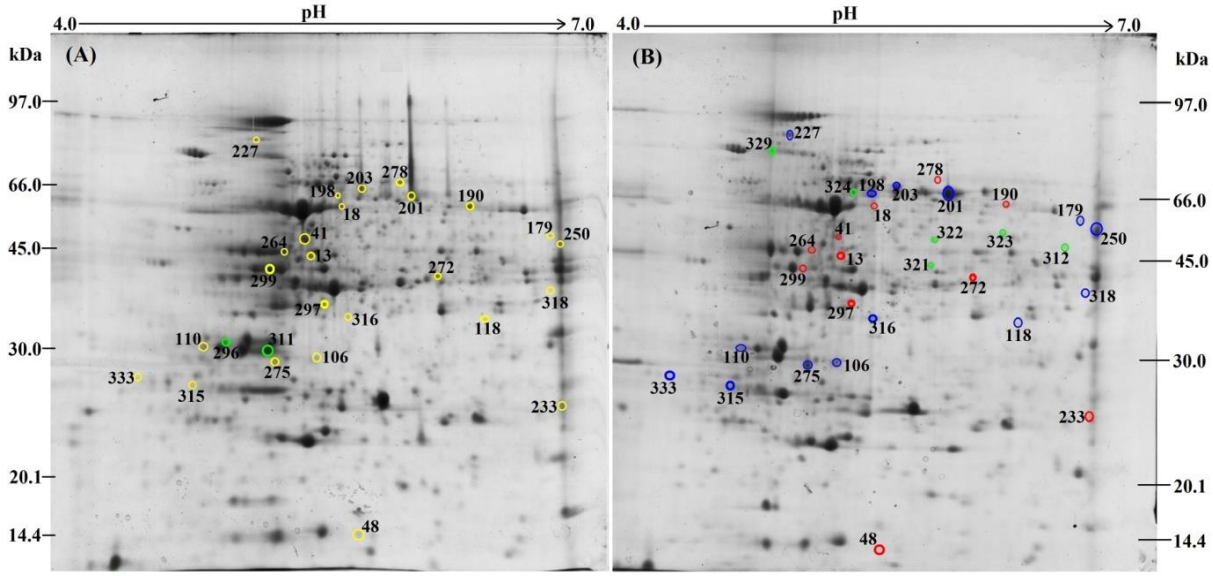

Figure 8. Coomasie Brilliant Blue G 250-stained two-dimensional gels of leaves from sorghum plants grown in nutrient solutions with nitrate (A) or ammonium (B), after 10 days of 75 mM-NaCl stress. Spots marked with yellow circles indicate those ones present in the control treatment that underwent differential protein expression in relation to the salt treatment. Spots marked with blue and red circles represent proteins that were significantly upregulated (blue) or downregulated (red); Spots with green circles in indicates proteins whose expression was found only in the respective treatment. Spots identified are statistically significant to a level of 95% per group (Student's t-test) using biological and analytical replicates (n = 3).

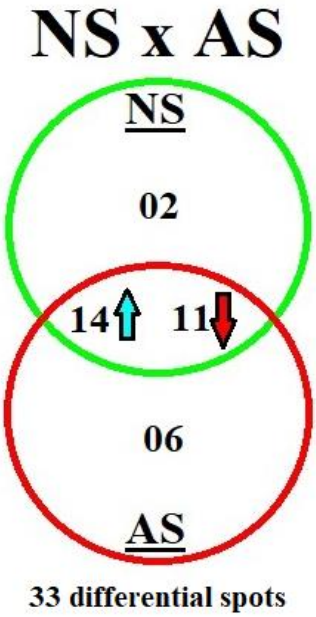

Fig. 9. Venn diagram representation of different subsets of proteins of *Sorghum bicolor* L. Moench cv. CSF 20 leaves grown in nitrate (NS) and ammonium-fed plants under salt stress with 75 mM NaCl (AS). NS was taken as reference.

Table 5 – List of differentially regulated proteins in leaves of salt-stressed sorghum plants as affected by inorganic nitrogen source (nitrate and ammonium). Differential spots were excised from 2DE gels, comparison NS × AS (see Fig. 9), and the proteins were identified by LC–ESI–MS/MS.

Spot	Protein (Species)	Access number (NCBI/SwissProt)	Peptide sequences (MS/MS)	Sequence coverage (%)	Score	Biological process (localization)	pI/MM (kDa) Theoretical	pI/MM (kDa) Experimental
13	Phosphoenolpyruvate carboxylase 3 [*Sorghum bicolor*]	XP_002438521.2	HLLATGFSEISEDAVFTK IEEFLEPLELCYK AIADGSLLDLLR HTDVIDAITTHLGIGSYR RPLLPPDLPMTEEIADVIGAMR	8	351	Carbon Fixation (Cytosol)	6.12 116	5.43 43
18	ATP synthase CF1 beta subunit [*Sorghum bicolor*]	YP_899414.1	TNPTTSRPGVSTIEEK IDQIIGPVLDITFPPGK QINVTCEVQQLLGNNR IFNVLGEPIDNLGPVDTSATFPIHR SAPAFIELDTK TVLIMELINNIAK AHGGVSVFGGVGER VALVYGQMNEPPGAR DVNKQDVLLFIDNIFR QDVLLFIDNIFR FVQAGSEVSALLGR GIYPAVDPLDSTSTMLQPR	35	871	ATP biosynthesis (Chloroplast)	5.31 54	5.60 57
41	Elongation factor Tu [*Sorghum bicolor*]	XP_002452390.1	TKPHVNIGTIGHVDHGK TTLTAALTMVLASVGGSAPK KYDEIDAAPEER GITINTATVEYETETR HYAHVDCPGHADYVK NMITGAAQMDGAILVVSGADGPMPQTK KDMVDDEELLELVELEVR ALEALMANPALK QTDLPFLLAVEDVFSITGR VGDTVDIVGIR TMDDAMAGDNVGLLLR	39	1208	Protein synthesis (Chloroplast)	6.07 51	5.40 47
48	Cytochrome b6-f complex iron-sulfur subunit [*Zea mays*]	ACG28186.1	GDPTYLVVEQDK GPAPLSLALVHADVDDGK VLFVPWVETDFR	18	275	Photosynthesis; electron transporter, transferring electrons within cytochrome b6/f complex of photosystem II activity (Chloroplast)	8.52 24	5.79 14

106	ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR IAQIPVSEAYLGR VINALAKPIDGR LIESPAPGIISR SVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK	19	531	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.47 30
110	Soluble inorganic pyrophosphatase 6 [*Sorghum bicolor*]	XP_002452886.1	AQGQPETLDYR VIEETNESWEK	7	137	Phosphate-containing compound metabolic process; response to salt stress (Chloroplast)	5.78 31	4.79 31
118	Ferredoxin-NADP reductase [*Saccharum* hybrid cultivar GT28]	AFO59573.1	EGQSIGIIADGVDK LYSIASSALGDFGDSK	8	165	Photosynthetic electron transport chain (Chloroplast)	7.53 40	6.47 34
179	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR IAQIPVSEAYLGR LIESPAPGIISR	8	267	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	6.81 51
190	Aldehyde dehydrogenase family 2 member B7, mitochondrial-like isoform X1 [*Sorghum bicolor*]	XP_021304577.1	LLINGNFVDAASGK TGEVIAHVAEGDAEDINR VGPALACGNTLVLK TAEQTPLSALYISK LLHEAGLPEGVVNVVSGFGPTAGAALASH-MDVDK LLHEAGLPEGVVNVVSGFGPTAGAALASH-MDVDKLAFTGSTDTGK SGVDSGANLVTGGDR GYYIQPTIFSDVQDGMK	24	626	Response to stress (Mitochondrion)	7.68 60	6.39 57
198	Polyamine oxidase-like [*Sorghum bicolor*]	XP_021302102.1	VIIVGAGMSGISAGK TFYSDFDSVVGCVYK LFNHQPNGPATPVDMALDYFIYDYEFAEPPR VTSLQNTQPTPTNADFGEDNYFVADQR EYPGANVLLVTVTDDESR GSYSNWPIGVSR	23	529	Polyamine catabolic process (Peroxisome)	5.44 57	5.59 60

201	F0-F1 ATPase alpha subunit, partial [*Sorghum bicolor*]	CAB99314.1	VVSVGDGIAR VVDALGVPIDGK TAIAIDTILNQK	7	190	ATP synthesis coupled proton transport (Mitochondrion)	5.96 48	5.90 65
203	ATP synthase CF1 alpha subunit [*Saccharum* hybrid cultivar NCo 310]	YP_054628.1	VVQVGDGIAR IIGLGEIMSGELVEFAEGTR IAQIPVSEAYLGR VINALAKPIDGR VINALAKPIDGRGEIVASESR LIESPAPGIISR RSVYEPLQTGLIAIDSMIPIGR SVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK QMSLLLR EAYPGDVFYLHSR QSQSNPLPVEEQVATIYTGTR GYLDSLEIEQVK EAIQEQLER	36	1323	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.70 66
227	Elongation factor G-2 [*Sorghum bicolor*]	XP_002448238.1	EDIPVAVTGDIVALAGLK VEVITPEEHLGDVIGDLNSR	4	141	Protein synthesis (Chloroplast)	5.42 85	5.16 80
233	Carbonic anhydrase, chloroplastic-like isoform X1 [*Sorghum bicolor*]	XP_021312698.1	YMVFACSDSR VCPSVTLGLQPGEAFTVR FLTWEPVMDAVDR VCPSVTLGLMPGEAFTVR YTGIGSAIEYAVCALK	16	316	Photosynthesis; response to stress (Chloroplast)	8.95 50	6.97 25
250	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR NVGIVLMGDGLMIQEGSFVK VINALAKPIDGRGEIVASESR LIESPAPGIISR RSVYEPLQTGLIAIDSMIPIGR TAVATDTILNQK HTLIIYDDLSK EAYPGDVFYLHSR	28	996	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	6.92 48

264	NADP (+)-Malate dehydrogenase [*Sorghum bicolor*]	CAA37531.1	QVQDGVATAEAPATR DCFGVFCTTYDLK LASGEVFGQDQPIALK AALLDINGQIFADQGK VLVVGNPCNTNALICLK SSAASTAVSIADAIK	21	458	Carbohydrate metabolic process; response to redox state (Chloroplast)	5.97 47	5.17 46
272	Glyceraldehyde-3-phosphate dehydrogenase A [*Sorghum bicolor*]	XP_021318665.1	GDASPLEVIAINDTGGVK YDSTLGIFDADVKPVGDNAISVDGK VPTPNVSVVDLVVQVSK TLAEEVNQAFR	17	295	Reductive pentose-phosphate cycle (Chloroplast)	7.00 43	6.17 41
275	ATP synthase CF1 alpha subunit [*Zea mays*]	NP_043022.1	VVQVGDGIAR IIGLGEIMSGELVEFAEGTR NVGIVLMGDGLMIQEGSFVK IAQIPVSEAYLGR VINALAKPIDGR LIESPAPGIISR RSVYEPLQTGLIAIDSMIPIGR SVYEPLQTGLIAIDSMIPIGR HTLIIYDDLSK	23	737	ATP synthesis coupled proton transport (Chloroplast)	5.87 55	5.27 29
278	4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl glucoside beta-D-glucosidase 2 [*Sorghum bicolor*]	XP_002443073.2	DYTDFAGLCFER VNNWLTFNEPHTFTCLSYGTGILAPGR GLKDILMITK FGIVYVDR	9	294	Carbohydrate metabolic process (Chloroplast)	6.22 65	5.95 68
312	Cytosolic isocitrate dehydrogenase [NADP]-like [*Sorghum bicolor*]	XP_021312414.1	AFAEASMTTAYEK SHYLNTEEFIDAVATELR	6	172	Response to salt stress (Cytosol)	8.26 52	6.72 44
315	Chlorophyll a-b binding protein [*Zea mays*]	CB22_MAIZE	AKPAAASGSPWYGPDR VLYLGPLSGEPPSYLTGEFPGDYGWDTAGL-SADPETFAK ELEVIHCR GPLENLADHIADPVNNNAWAYATNFVPGK	34	138	Photosynthesis; light harvesting (Chloroplast)	5.29 28	4.71 26

316	ATP synthase subunit alpha [*Zea mays*]	ATPA_MAIZE	VGIENIGR VVQVGDGIAR LIESPAPGIISR	5	125	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.55 34
318	Ricin B-like lectin R40C1 [*Sorghum bicolor*]	XP_002465329.1	ADEGYCLTVR ADDGFSVTVR	5	127	Stomatal Closure; response to stress (Nucleus)	6.27 40	6.69 38
321	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	VVQVGDGIAR IIGLGEIMSGELVEFAEGTR TAVATDTILNQK EAYPGDVFYLHSR	10	296	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.81 42
322	F-ATP synthase subunit alpha [*Saccharum* sp]	Q6L3A1.2	IIGLGEIMSGELVEFAEGTR LIESPAPGIISR TAVATDTILNQK	8	220	ATP synthesis coupled proton transport (Chloroplast)	5.87 56	5.91 46
323	Isocitrate dehydrogenase [NADP] [*Solanum tuberosum*]	IDHC_SOLTU	LIDDMVAYALK GGETSTNSIASIFAWTR LEAACIGAVESGK	9	126	Response to stress (Cytosol)	6.54 47	6.23 50
324	Polyamine oxidase-like [*Sorghum bicolor*]	XP_021302102.1	VIIVGAGMSGISAGK TFYSDFDSVVGCVYK VTSLQNTQPTPTNADFGEDNYFVADQR EYPGANVLLVTVTDDESR GSYSNWPIGVSR	17	389	Polyamine catabolic process (Peroxisome)	5.44 57	5.39 63
329	Pyruvate orthophosphate dikinase [*Saccharum officinarum*]	AAF06668.1	LAEVNPMLGFR VLANADTPEDALAAR	2	179	Photosynthesis (Chloroplast)	5.52 103	4.91 77

333	Phosphoglycerate kinase (Fragment) OS=*Spinacia oleracea*	PGKH_SPIOL	IGVIESLLEK GVSLLLPTDVVIADK	5	73	Reductive pentose phosphate pathway (Chloroplast)	5.83 46	4.30 27
296	Oxygen-evolving enhancer protein 1 [*Sorghum bicolor*]	XP_021305066.1	GTGTANQCPTIDGGVEAFPFK QLVATGKPESFGGPFLVPSYR NAAASTGNITLSVTK	17	209	Photosynthesis; light reaction (Chloroplast)	7.59 34	4.99 31
297	Enoyl-[acyl-carrier-protein] reductase [NADH] 1 [*Oryza sativa* subsp. Japônica]	FABI1_ORYSJ	YAGSSNWTVK VNTISAGPLGSR	5	70	Fatty acid biosynthetic process (Chloroplast)	8.81 39	5.56 36
299	Phosphoribulokinase [*Sorghum bicolor*]	XP_021313843.1	GGNPDSNTLISDTTTVICLDDYHSLDR KEYGVTALDPR DGQAVEKPIYNHVTGLLDPPELITPPK GHSLESIQASIEAR KPDFDAYIDPQK FFNPVYLFDEGSSISWVPCGR FAYGPDTYFGK EVSVLEMDGQFDKLDELIYVESHLSNLSTK HADFPGSNNGTGLFQTIIGLK IRDLYEQIVAER	44	763	Reductive pentose-phosphate cycle (Chloroplast)	6.09 46	5.25 41
311	Oxygen-evolving enhancer protein 1 [*Sorghum bicolor*]	XP_021305066.1	GTGTANQCPTIDGGVEAFPFK LCLEPTSFTVK LTYTLDEIEGPLEVGSDGTLK LTYTLDEIEGPLEVGSDGTLKFEEK FEEKDGIDYAAVTVQLPGGER DGIDYAAVTVQLPGGER QLVATGKPESFGGPFLVPSYR GRGGSTGYDNAVALPAGGR GGSTGYDNAVALPAGGR GDEEELLKENIK NAAASTGNITLSVTK SNPETGEVIGVFESVQPSDTDLGAK	50	875	Photosynthesis; light reaction (Chloroplast)	5.91 35	5.23 30

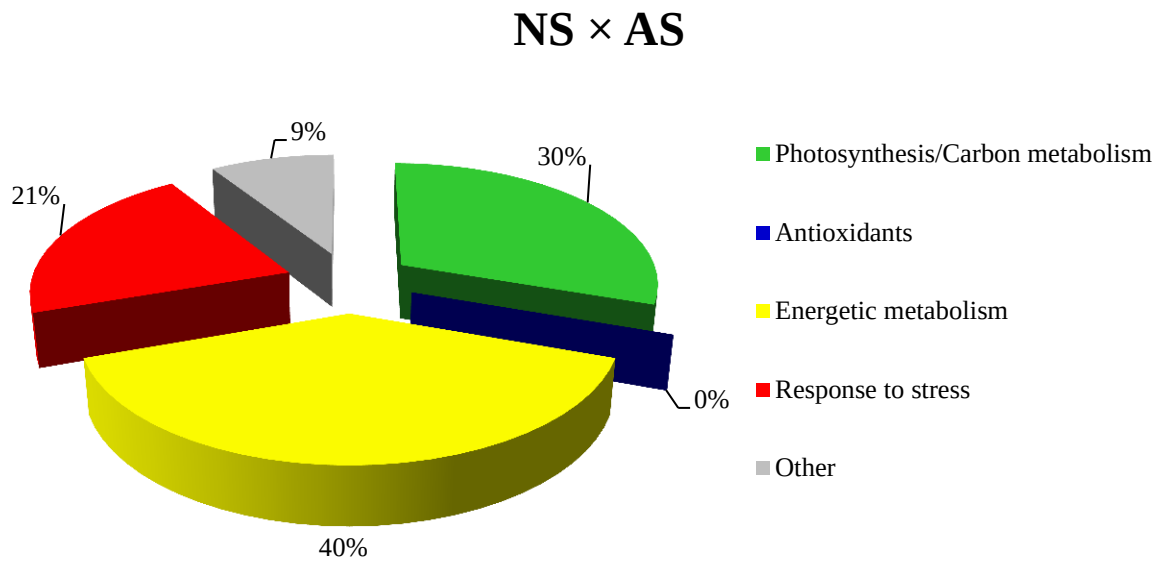

Figure 10. Functional characterization of differentially expressed proteins in leaves of sorghum plants 10 days after salinity as affected by nitrogen source, nitrate (NS) and ammonium (AS).

In the photosynthetic/carbon metabolism term, three proteins were upregulated in ammonium (such as phosphoglycerate kinase) and four downregulated (e.g. phosphoribulokinase), and two oxygen-evolving enhancer proteins were exclusive to nitrate-fed plants (table 3). In energetic metabolism, eight proteins were upregulated in ammonium (four of them ATP synthase alpha subunits), three down regulated ($NADP^+$-malate dehydrogenase), and two F-ATP synthase alpha subunit were exclusive to ammonium-grown plants. Surprinsingly, several stress responsive proteins were more abundant or exclusive from leaves of ammonium-fed stressed plants, as compared to nitrate-fed ones, such as polyamine oxidase-like, ricin B-like lectin R40C1, isocitrate dehydrogenase [NADP] and cytosolic isocitrate dehydrogenase [NADP]-like (Table 3). On the other hand, in other processes, the proteins enoyl-[acyl-carrier-protein] reductase [NADH] and elongation factor Tu were more expressed in the nitrate treatments, whereas the elongation factor G-2 was more abundant in ammonium-grown plants (Table 3).

4.5 Discussion

It has been widely reported that proteomic studies can help find key enzymes and proteins in plants to cope with a variety of stresses, such as salinity. Previously, our research group has shown that NH_4^+ as nitrogen source can mitigate the salt deleterious effects and increase salinity tolerance of forage sorghum plants, in comparison to NO_3^- nutrition. In this

study we did a complete investigation of the proteome of sorghum leaves submitted to salt stress and with distinct nitrogen sources, which prove that ammonium nutrition lead to better physiological results, formerly described by Miranda et al. (2016; 2017).

According to these authors, the positive effects of NH_4^+ supply under salt stress were attributed to: (i) increased accumulation of amino acids that could help to osmotic adjustment in order to maintain a water balance and osmoprotection, as well as ameliorating the salinity-induced K^+ efflux; (ii) a greater efficiency of photosynthetic machinery under salt stress; and primarily (iii) a control on Na^+ accumulation in plant tissues, involving the activation of Na^+/H^+ SOS1 antiporter, enhanced efficiency of proton pumps in roots and low loading of Na^+ into the xylem sap.

4.5.1 Physiological changes in sorghum plants under salt stress

In this study, salinity severely impaired the growth of sorghum plants, irrespective of the nitrogen source. In nitrate treatments the toxic effects of salt excess in plant growth were associated with strong decreases in K^+/Na^+ ratios (Fig. 1A; Supplementary Table S1). Similar results were reported in Yang et al. (2013), who verified that sugar beet plants grown in the Hoagland solution, whose main N source is nitrate, displayed reduced growth and leaves exhibited chlorosis. Recently, Miranda *et al.* (2017) showed that NO_3^--fed plants tend to accumulate Na^+ into vacuole through an increase in Na^+/H^+ exchanger (NHX) activity and gene expression, but the plants are not able to restrict Na^+ transport and accumulation in sorghum shoots. In ammonium-fed plants, although the inhibition in plant growth were also associated with reductions in K^+/Na^+ ratios, it was much less aggravated than those of nitrate-grown plants (Fig. 1B; Supplementary Table 1). Higher SDM, RDM and LA values in ammonium-fed plants are probably related to a more efficient restriction of Na^+ accumulation in shoots. Miranda et al. (2017) also found that ammonium nutrition enhanced plants salt tolerance by increasing expression and activity of Na^+/H^+ antiporters (such as SOS1 and NHX) and V-ATPase, which limited the transport to the leaves via xylem. Fernández-Crespo et al. (2012) also reported that ammonium nutrition increased salt tolerance in citrus plants, especially by elevating proline and polyamines levels.

In the present study, we performed a detailed investigation of the proteomic profile of sorghum plants in order to identify target proteins involved in mechanisms of salt tolerance induced by external nitrogen source. In 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 (Table 1); but failed in activating at least part of the antioxidant system. Although one ascorbate peroxidase 2 was upregulated in response to salinity, one glyoxylase 1 and a cytosolic superoxide dismutase were downregulated (Table 1). It is likely that the losses on defense mechanisms were not able to cope with salt damage, resulting in severe complications in plant metabolism as inhibitions in LA, RDM e SDM . 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 papyfera* and showed inhibition on plant growth and a decrease in activity of SOD. Jakovljević et al. (2017) also reported similar results with the antioxidant enzyme and plant growth while studying early sweet basil plants under salt stress and using Hoagland's nutrient solution.

On the other hand, ammonium-fed plants displayed a specific modulation of proteome that resulted in a more efficient response to salt stress. Firstly, salt-stressed plants synthetized *de novo* some thylakoid structural proteins as well as upregulated diverse enzymes of photosynthetic/carbon metabolism (Table 1). In parallel, ammonium-fed plants activated (exclusively or by upregulation) several proteins involved in energetic metabolism, such as ATP synthase alpha subunits, which in turn provided energy for ammonium assimilation and mechanisms of salt responses, such as H^+-ATPases proton pumps and Na^+/H^+ antiporters. This hypothesis was previously corroborated by Miranda *et al.* (2017), who demonstrated that ammonium decrease Na^+ accumulation in sorghum tissues through Na^+ efflux from cells by efficiently activating SOS1-like Na^+/H^+ antiporters. These authors also showed that the increased SOS1 activity is coupled with a higher P-ATPase H^+-pumping activity. Finally, ammonium-fed plants also upregulated or synthetized *de novo* proteins related to response to stress and other metabolic processes (Table 1), which composed the intricate pathway of plant responses and helped alleviate the salt damage and improve salinity tolerance of sorghum plants (Fig. 1; Supplementary Table 1). For instance, salt-stressed plants displayed a *de novo* synthesis of cytosolic isocitrate dehydrogenase [NADP]-like, which is believed to alleviate oxidative stress in leaves of Arabidopsis (Mhamdi *et al.*, 2010), and cysteine synthase (Table 1), an important component involved in response to biotic and abiotic stresses (Romero *et al.*, 2014). 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 also greater CO_2 assimilation under salt stress in comparison to nitrate nutrition. Such reports are in accordance with results presented here.

4.5.2 Proteins related to photosynthesis and carbon metabolism

Photosynthesis is one of the primary plant processes affected by salt stress. Therefore, the plant ability to survive under salinity is strongly influenced by the activity of photosynthetic enzymes. It is known that salinity disturbs both photochemical and biochemical processes in photosynthesis (Munns and Tester, 2008). Although our results of CO_2 assimilation rate, PSII maximum efficiency (Fv/Fm) and effective quantum yield of PSII (Φ PSII) were not significantly altered by neither salinity nor nitrogen source (Fig. 1A, B; Supplementary Table 1), unlike our proteomic analyses that revealed several proteins related to this process underwent modulation in response to salinity.

The light-harvesting complex (LHC) is composed by chlorophylls *a* and *b* and the chlorophyll *a*-*b* binding protein. LHC is a light receptor that captures and transfers excitation energy to photosystems I and II with which is intimately associated. Under changing light conditions, the reversible phosphorylation of light harvesting chlorophyll *a*/*b* binding proteins (LHCII) represents a system for balancing the excitation energy between the two photosystems (Liu and Shen, 2004). Ammonium nutrition induced *de novo* synthesis in three isoforms of LHC chlorophyll *a*-*b* binding protein (Table 1; spots 287, 280 and 316 in fig. 2D) and one upregulation of these proteins (Table 1; spot 288 in fig. 2C, D), which could have contributed to the mitigation of the deleterious effects of salinity on photosynthesis and growth in these plants. In contrast, only one isoform of this protein was upregulated in nitrate-fed plants (Table 1; spot 18 in fig. 2A, B), which probably contributed to assimilation rate maintenance. Studies show that this protein can be up or downregulated in plants, according to species under salt stress. For instance, Rabey *et al.* (2016) found that salinity caused downregulation of this protein in date palms under salt stress.

The water-splitting reaction in the PSII is catalyzed by the Mn cluster, which is attached to a crucial part of the PSII: the oxygen-evolving enhancer (OEE) protein complex. This complex is composed by three proteins: OEE1, which is bound to the intrinsic subunits of the PSII complex and provides a binding site for OEE2; and OEE3, which is attached to PSII by OEE2 (Momonoki et al., 2009). It is believed that OEE1 might be important to keep the well-functioning of PSII under salt stress (Momonoki et al., 2009). In our studies, this protein was downregulated by salinity in ammonium-fed sorghum plants (Table 1; spots 244 and 245 in figs. 2C, D). Although these proteins were mostly inhibited, that did not seem to

interfere in the photosynthetic process overall. Abreu *et al*. (2014) showed an upregulation of this protein in cowpea leaves under salinity and recovery.

Rubisco catalyzes the fixation of atmospheric CO_2 into ribulose-1,5-bisphosphate, generating two molecules of 3-phosphoglycerate. The holoenzyme is composed by eight large subunits and eight small subunits. In this study, Rubisco large subunit underwent upregulation (Table 1; spots 91 and 119 in figs. 2A; B), downregulation (Table 1; spots 13 and 109 in figs. 2A, B) and repression (Table 1; spots 238, 239 and 240 in fig. 2A). Despite the strong inhibition of this protein, it was not enough to affect CO_2 assimilation. Rabey *et al*. (2016) also reported downregulations of the large subunit of Rubisco in date palms. Our data is also in accordance with Abreu *et al.* (2015), who reported the same result with this protein studying proteomic changes in cowpea leaves submitted to salt stress and recovery.

In the presence of light, rubisco activase replaces inhibitory sugar phosphates such as ribulose-1,5-bisphosphate or carboxyarabinitol 1-phosphate from the catalytical sites of rubisco for CO_2 in order to activate the enzyme. Under nitrate nutrition two isoforms of this enzyme were downregulated in response to salinity (Table 1; spots 55 and 68 in figs. 2A;B), one was upregulated (Table 1; spot 57 in figs. 2A; B) and one underwent *de novo* synthesis (Table 1; spot 209 in fig. 2B). On the other hand, ammonium-fed plants only showed one downregulated isoform (Table 1; spot 228 in figs. 2C;D). Although rubisco activase was mainly downregulated, it seems NaCl could not interfere on CO_2 fixation in the end. Deeba *et al.* (2012) also reported similar results of this enzyme in cotton plants under drought stress; In contrast, two isoforms of rubisco activase underwent upregulation and *de novo* synthesis, respectively.

Chloroplastic 3-phosphoglycerate kinase catalyzes the ATP-dependent phosphorylation of 3-phosphoglycerate to 1,3–bisphosphoglycerate in the Calvin-Benson cycle. Under salt stress, all isoforms of this protein in both nitrogen sources (Table 1; spots 72 and 161 in figs. 2A; B; spots 84, 162 and 313 in figs. 2C;D; spot 333 in figs. 2E;F) underwent upregulations. These upregulations in ammonium nutrition could have contributed to the better results in growth parameters in the presence of NaCl in comparison to nitrate-fed plants. Zhang *et al.* (2014) also reported an upregulation of this enzyme by analyzing proteomic profiles of leaves of two chrysanthemum varieties under heat stress.

Chloroplastic triosephosphate isomerase interconverts glyceraldehydes-3-phosphate and dihydroxyacetone. In both nitrogen sources, this enzyme underwent upregulations (Table 1; spots 153 and 188 in figs. 2A;B; spots 159 and 224 in figs. 2C;D).

These results might have lead to an unchanged CO_2 assimilation in both nitrogen sources. Wang *et al.* (2015) also reported an upregulation of this enzyme in halophyte *Halogeton glomeratus* seedlings exposed to salt stress.

Two C_4 metabolism important proteins were differentially modulated in response to salinity: phosphoenolpyruvate carboxylase (PEPCase) and pyruvate phosphate dikinase. The first step in the C_4 photosynthetic fixation of CO_2 is performed by PEPCase, which catalyzes the carboxylation of phosphoenolpyruvate in the presence of HCO_3^- and Mg^{2+} to produce oxaloacetate and inorganic phosphate. In nitrate-fed plants, three isoforms of PEPCase underwent upregulation (Table 1; spots 15, 140 and 184 in figs. 2A;B). In ammonium-grown plants, two isoforms were upregulated (Table 1; spots 28 and 191 in figs. 2C;D), and another isoform underwent *de novo* synthesis (Table 1; spot 285 in fig. 2D). Comparing both stressed groups, PEPCase was downregulated in ammonium salt stress plants in comparison to nitrate salt stress ones (Table 1; spot 13 in figs. 2E;F). This upregulation might have kept CO_2 assimilation rates nearly unchanged even under salt stress. Jedmowski et al (2014) also reported that sorghum plants under drought stress tend to upregulate this enzyme. Contrary to our findings, Ahmad et al. (2017) reported a downregulation of PEPCase in *Parthenium hysterophorus* leaves submitted to salt stress.

Pyruvate phosphate dikinase, the other C_4 metabolism enzyme differentially expressed, performs the ATP and phosphate-driven conversion of pyruvate to phosphoenolpyruvate, which is the primary CO_2 acceptor in C_4 photosynthesis. In nitrate-fed plants, this enzyme underwent *de novo* synthesis (Table 1; spot 233 in fig. 2B), whereas in ammonium-grown plants it was observed distinct responses to two isoforms: an upregulation (Table 1; spot 153 in figs. 2C;D) and a downregulation (Table 1; spot 161 in figs. 2C;D). Comparing both nitrogen sources under salt stress, this enzyme was exclusive to ammonium-salt plants (Table 1; spot 329 in fig. 2F). These results could have held CO_2 assimilation rates almost unaltered despite the presence of salt stress in both nitrogen sources. In accordance to most of our results, Jedmowski et al. (2014), studying the effects of drought and recovery on the proteic profile of sorghum leaves, detected an upregulation of pyruvate phosphate dikinase. On the other hand, Thagela et al. (2016) observed a downregulation of this enzyme in *Azolla mycrophylla* roots exposed to salinity.

Overall, although nitrate-grown plants increased the abundance of electron transport chain structural proteins and carbon assimilation enzymes (Table 3), these

mechanisms might not be enough to reduce salt damage in photosynthetic performance (Fig. 1A).

4.5.3 Proteins related to energetic metabolism

In nitrate-fed plants, the activation of proteins from energetic metabolism could provide energy for salt responses but these factors might not be efficient and the plants were more sensitive to salinity. On the other hand, ammonium-fed plants activated more proteins of this category, which could have lead to a more efficient alleviation.

Studies have reported that some enzymes involved in sugar and energy metabolism alter their levels in response to salt stress. Chloroplast ATP synthase synthesizes ATP from ADP and inorganic phosphate, utilizing the electrochemical proton gradient formed across these membranes by photosynthetic or respiratory electron transfer reaction (Hisabori *et al.*, 2013). The holoenzyme is composed of a membrane-peripheral component F1, which in turn is formed by five different subunits with an $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ stoichiometry, and a membrane-embedded part Fo, consisted of three different subunits with $a_1b_2c_{10–15}$ stoichiometry (Hisabori *et al.*, 2013). In this study, ATP synthase subunits responded differentially to the nitrogen sources: beta subunit was mainly upregulated by nitrate treatment, with some events of downregulation, whereas alpha subunit was strongly upregulated by ammonium nutrition, being observed some *de novo* synthesis. A high production of ATP synthase is crucial for the mitigation of the deleterious effects of salinity, once the organism will spend more energy to cope with this stress rather than producing biomass (Hu *et al.*, 2012), which might explain better results in growth parameters found in ammonium-fed plants than in nitrate-grown plants. The upregulation of ATP synthase beta subunit is in accordance to Xiong et al. (2017) who assessed proteomic changes in alfalfa shoots in response to salinity. On the other hand, Abreu et al (2014) revealed a downregulation of this enzyme in cowpea leaves submitted to salt stress and recovery. The upregulation of ATP synthase alpha subunit in both nitrogen sources is in accordance with Jia et al (2016) with *Dunaliella salina* under high and low salt stress.

Cytosolic glyceraldehyde 3-phosphate dehydrogenase is an enzyme of the glycolytic pathway involved in the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Two isoforms of this protein underwent downregulation (Table 1; spots 64 in figs. 2A;B, and spot 272 in figs. 2E;F), whereas one was *de novo* synthesized in nitrate-

fed plants (Table 1; spot 190 in fig. 2B). These reductions could mean a lower energy production, required for crucial cellular processes, which could explain the higher inhibition in nitrate-fed plants. Kamal et al (2012), while studying proteomic profiles of wheat chloroplasts under salt stress, also reported downregulation of three isoforms of this enzyme. On the other hand, we also have found a *de novo* synthesis of one isoform of glyceraldehyde 3-phosphate dehydrogenase (Table 1; spot 190 in fig. 2B).

4.5.4 Proteins related to antioxidant system

Reactive oxygen species (ROS) are naturally produced as by-products of several metabolic pathways, such as photosynthesis and respiration. However, ROS can have its levels increased in response to biotic and abiotic stresses, such as salinity. Plants cope with stress-induced ROS accumulation by inducing antioxidant systems, which can be enzymatic like SOD, APX, and CAT, or non-enzymatic, represented by low molecular weight compounds, e.g. glutathione, ascorbate, tocopherol, etc. (Munns and Tester, 2008). However, analyzing protein profiles revealed that both nitrogen sources nutrition did not seem to activate a significant antioxidant system to mitigate the deleterious effects of salinity.

SOD is a metalloenzyme that catalyses the dismutation of two superoxide radicals ($\bullet O_2^-$), reducing one to a hydrogen peroxide molecule (H_2O_2) and oxidizing the latter to molecular oxygen (O_2).Three SOD classes in plants have been identified: copper/zinc SOD (Cu/Zn-SOD), iron SOD (Fe-SOD) and manganese SOD (Mn-SOD). Cu/Zn-SOD is the most abundant SOD family in plant cells, detected in several cell compartments: chloroplasts, mitochondria, cytosol, peroxisomes and in the apoplast as well. Fe-SOD is mainly found as a chloroplastic enzyme. Mn-SOD was already localized in mitochondria and in peroxisomes (Leonowicz et al., 2018). In nitrate-fed plants, cytosolic SOD was downregulated by salinity (Table 1; spot 11 in figs. 2A;B), while in ammonium-fed plants chloroplastic SOD was repressed by the stress (Table 1; spot 233 in fig. 2C). However, despite these results, it seems ammonium nutrition relies on other strategies than antioxidant system to mitigate the deleterious effects of salinity. Saleethong et al. (2016) also reported a downregulation of this enzyme in rice leaves and grains exposed to salt stress pre-treated with exogenous spermidine. Contrary to our findings, Talei et al (2014) observed an upregulation of SOD enzymes in *Andrographis paniculata* roots and shoots in response to salt stress.

Another antioxidant enzyme identified was APX, which was only differentially expressed in nitrate-fed plants, being upregulated in response to salinity (Table 1; spot 31 in figs. 2A;B). APX (EC, 1.11.1.11) is a plant-type heme peroxidase that catalyzes the reduction of H_2O_2 to water and O_2 utilizing ascorbate as an electron donor (Munns and Tester, 2008). Different isoforms are classified into sub-families according to their subcellular localization. Among nine *APX* genes identified in *Arabidopsis*, three were discovered to be encoded in cytosol whereas the other six were located in stroma, thylakoid, and peroxisome. APX activity has also been noticed to increase under several stress conditions. For example, APX is differentially upregulated in response to heavy metal, drought, water, and heat stress (Ozyigit et al., 2016). . This enzyme was differentially upregulated in nitrate-fed plants (Table 1; spot 31 in fig. 2). It appears this enzyme is the main one involved in H_2O_2 scavenging in the presence of NaCl. Our results are in accordance with Xiong et al. (2017), who reported an upregulation of this enzyme in alfalfa plants submitted to salt stress.

4.5.5 Proteins related to response to stress and other cellular processes

Plants have developed several physiological and biochemical responses in order to survive in soils with high salt concentration. The main mechanisms are, among others, ion homeostasis and compartmentalization; ion transport and uptake; biosynthesis of osmoprotectants and compatible solutes; activation of antioxidant enzymes; synthesis of antioxidant compounds; and synthesis of polyamines. Salinity can affect other important cellular processes in plants, such as translation, nitrogen assimilation and amino acid metabolism (Munns and Tester, 2008).

Chaperone protein ClpC1 is a heat shock protein (HSP) of the AAA+ ATPase family (ATPases associated with various cellular activities) and it belongs to the subfamily C of CaseinoLytic Proteases (Trösch *et al.*, 2015). It is known to be required for importing proteins to the chloroplast, as well as preventing the aggregation of misfolded proteins (Bian et al., 2017). In our study, in both nitrogen sources, it was observed events of *de novo* synthesis (Table 1; spot 176 in fig. 2B; spot 312 in fig. 2D) and repression as well (Table 1; spot 237 in fig. 2A; spot 263 in fig. 2C). Since one isoform underwent upregulation, it could play a role in protecting protein structure in response to stress. Our results are in accordance with Bian et al. (2017), who reported an upregulation of this protein in *Brachypodium distachyon* leaves under drought stress. On the other hand, Caprioti et al. (2014) studying

wheat leaves under salt stress, observed a downregulation of this enzyme in response to salinity.

Cysteine synthase (Table 1; spot 293 in fig. 2D) is a heterodimeric complex formed by the enzymes serine acetyltransferase and O-acetylserine (thiol) lyase (Zargochev et al., 2013). The first enzyme performs the generation of o-acetylserine from serine and acetyl-coenzyme A; the latter replaces the acetyl group in o-acetylserine for reduced sulphur in the form of H_2S to form cysteine. This amino acid is a pivotal metabolite, whose role is the donation of sulphate groups to form methionine, some vitamins such as coenzyme A and biotin, glutathione, which is a key water-soluble antioxidant with a central role in ROS scavenging, and other compounds (Zargochev et al., 2013). This complex is also associated with response to stresses, for example in Cd tolerance in transgenic tobacco plants overexpressing cysteine-synthase (Zargochev et al., 2013). In our study, this complex was *de novo* synthesized in ammonium-fed plants, which highlights the importance of this enzyme in response to salinity, producing cysteine as a protective measure against high Na^+ levels (Kamal et al., 2012). Our results are in accordance with Kamal et al. (2012), who studied modulation of the proteome of wheat chloroplasts under salt stress. Shi et al. (2017) also observed an upregulation of this enzyme in *Pyropia haitanensis* responding to high temperature stress.

Poliamine oxidases are FAD-containing enzymes that perform the terminal catabolism of spermidine and spermine to produce 1,3-diaminopropane, H_2O_2, and N-(3-aminopropyl)-4-aminobutanal (spermine catabolism), or 4-aminobutanal (spermidine catabolism). Other polyamine oxidases catalyze the conversion of polyamines (Liu et al., 2015). We observed that two isoforms of polyamine oxidases underwent upregulation and *de novo* synthesis, respectively (Table 1; spots 198 in figs. 2E-F; and 324 in fig. 2F). It is known that polyamines are involved in response to several abiotic stresses, such as salinity and oxidative stress, where they not only mitigate the deleterious effects of the stresses, but also increase synthesis and activity of antioxidant enzymes (Liu et al., 2015). Our results are in accordance with Fatehi et al. (2013) who assessed proteomic changes in *Hordeum spontaneum* submitted to salt stress. Contrary to our findings, Ahmad et al. (2017), while studying ecophysiological and proteomic changes in *Parthenium hysterophorus* in response to salt stress, reported a downregulation of this enzyme.

NADP-isocitrate dehydrogenase catalyzes the oxidative decarboxilation of isocitrate to α-ketoglutarate, reducing $NADP^+$ to NADPH, and it is one of the most important

enzymes able to generate NADPH-reducing power (Leterrier et al., 2012). Here, two isoforms of this enzyme were exclusive to ammonium-fed plants (Table 1; spots 312 and 323 in fig. 2F). Studies have reported that this enzyme is involved in ammonia assimilation, as well as in the promotion of the redox signalling and response to oxidative stress. This might have mitigated the effects of salinity in ammonium-fed plants. Our results are in accordance with Yousuf et al. (2017), who studied the effects of salinity on proteomic profiles of indian mustard leaves. Kamal et al. (2012) also reported an upregulation of this enzyme in wheat leaves under salt stress.

Heat shock 70 kDa proteins (Hsp70 proteins) are a family of chaperones known to interact with developing polypeptides, averting their misfolding and aggregation (Jackson-Constan et al., 2001). This subfamily of HSP has isoforms found in several subcellular compartments, such as cytosol and chloroplasts. It has been related that Hsp70 is one of the cytosolic factors involved in keeping the import competence of the precursor of the light-harvesting chlorophyll a/b-binding protein (Jackson-Constan et al., 2001). Stromal Hsp70 function is yet not elucidated; it is believed that its main role is either part of the protein import complex or to guide precursor proteins with a targeting signal to the thylakoid (Jackson-Constan et al, 2001). In the present study, four isoforms of chloroplast Hsp70 proteins were repressed by salinity, both in nitrate- and in ammonium-fed plants (Table 1; spots 242 and 243 in fig. 2A; spots 274 and 276 in fig. 2C). These results might indicate that salinity can strongly compromise appropriate protein folding and import from the cytosol to the chloroplast. Our results are in accordance with Razavizadeh et al. (2009), who studied proteomic modulations in tobacco leaves in response to salinity and reported a downregulation of this protein. On the other hand, Li et al. (2013), while assessing the proteome responses of alfalfa to heat stress, showed an upregulation of this protein.

Glutamine synthetase is a key enzyme for nitrogen assimilation in plants, once it fixes ammonium into glutamate to generate glutamine. In this study, only the nitrate nutrition displayed differential modulation of this enzyme, with an isoform upregulated and another one downregulated, spots 139 and 164, respectively (Table 1; figs. 2A;B). A strong inhibition of those proteins could represent a serious threat for the organism, once it provides nitrogen groups for the biosynthesis of all nitrogenous compounds in the plant. Xiong et al. (2017) also reported an upregulation of this enzyme in alfalfa roots and shoots under salt stress. On the other hand, Cosentino et al. (2013), while evaluating the proteomic responses of leaves of *Mesembryanthemum crystallinum* to salinity, detected a downregulation of this enzyme.

Peptidyl-prolyl cis-trans isomerases are enzymes that stabilize the interconversion of cis and trans conformations in proline residues involved in peptide bonds, which allows this amino acid to display cis conformation, once the trans configuration is usually the most sterically favored in peptide bonds. The cis/trans isomerization of an amino acid-proline bond results in slow stages in protein folding, which is an important step for folding and a crucial determinant of structure (Kaur et al., 2015). This enzyme was upregulated in nitrate-fed (Table 1; spot 206 in figs. 2A;B) and downregulated in ammonium-fed plants (Table 1; spot 95 in figs. 2C;D). These results could imply that ammonium nutrition does not prevent inhibition of this enzyme efficiently. Vitárnvás et al. (2015), studying the proteome of barley crowns grown under different water deficits, reported two events of upregulation of this enzyme. On the other hand, Qin et al. (2016) revealed a downregulation of this enzyme in wheat leaves transformed with maize *PEPCase* gene in response to drought stress.

Elongation factors are enzymes involved in protein synthesis. Plastid and mitochondrial elongation factor Tu binds GTP and aminoacyl-tRNA, leading it to the codon-dependent placement of this aminoacyl-tRNA at the A site of the ribosome (Fu et al., 2012). We observed that elongation factor Tu underwent a *de novo* synthesis in nitrate-fed plants (Table 1; spot 215 in fig. 2B) and a downregulation in ammonium-fed plants (Table 1; spot 41 in figs. 2E;F). These results might imply that nitrate nutrition induces higher production of elongation factor Tu. Komatsu et al. (2014) also detected an upregulation of this enzyme in wheat plants in response to salinity. Zhang et al. (2016) reported two events of downregulation of this enzyme in petunia seedlings submitted to low temperature stress.

Elongation factor G is a plastid and mitochondrial enzyme that translocates the mRNA one codon further to allow the arrival of the new aminoacyl-tRNA in the A site (Fu et al., 2012). Contrarily, two isoforms of elongation factor G were upregulated in ammonium-grown plants in response to salinity (Table 1; spots 138 in figs. 2C;D; and spot 227 in figs. 2E;F). This could mean that ammonium nutrition enhances elongation factor G production and probably its activity. Our results are in accordance with Wan and Liu (2008), who found an upregulated isoform while studying rice seedling leaves under hydrogen peroxide stress.

5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (also known as cobalamin-independent methionine synthase or MetE) is a B12 vitamin-independent methionine synthase that transfers a methyl group to homocysteine, thus generating methionine (Mordukhova and Pan, 2013). It was observed that two isoforms of this enzyme were upregulated in response to salinity in ammonium-fed plants (Table 1; spots

136 and 176 in figs. 2C;D). One possible explanation for this upregulation is that MetE is involved in S-adenosyl-methionine cycle, which for its turn is related to the Yang cycle that generates ethylene, an important hormone involved in responses to abiotic stresses. Our results are in accordance with Jedmowski et al. (2014), who assessed proteomic changes in sorghum leaves submitted to drought stress and recovery, the authors reported an upregulation of MetE. Liu et al (2014) also described an upregulation of MetE in rice plants in response to salinity.

4.6 Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and by the Instituto Nacional de Ciência e Tecnologia em Salinidade (INCTSal/CNPq).

5 CONCLUSÃO

Em suma, nosso estudo investigative mostrou que a fonte externa de nitrogênio pode alterar as respostas à salinidade em plantas de sorgo ao reprogramar especialmente um vasto número de proteínas envolvidas no metabolismo energético e de resposta ao estresse. Em todos os casos, plantas de sorgo nutridas com nitrato ou amônio regularam o perfil proteômico sob estresse salino; contudo, a modulação em plantas cutltivadas em amônio parece ser mais eficiente em levar a uma melhor aclimatação à salinidade. Particularmente, a indução das enzimas PEPCase, fosfoglicerato-quinase, F-ATP sintase, e a síntese *de novo* de várias proteínas, assim como taxas mais elevadas das razões K^+/Na^+ podem ter contribuído para a melhor tolerância ao estresse salino em plantas nutridas com amônio. Nossos dados apresentam informações importantes para uma melhor compreensão dos mecanismos moleculares de resposta à salinidade em plantas de sorgo mediadas pela fonte de nitrogênio, e fornece novas perspectivas para o desenvolvimento de estratégias biotecnológicas a fim de se produzir safras mais tolerantes ao estresse salino.

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